Wolbachia endosymbiont of *Onchocerca volvulus*: driver of immunopathology and target for therapy

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the University of Liverpool for the degree of Doctor in Philosophy

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

APC	Antigen Presenting Cell
APOC	African Programme for Onchocerciasis Control
A-WOL	Anti-*Wolbachia* Consortium
BmFE	*Brugia malayi* adult Female Extract
BmFEtet	*B. malayi* adult Female Extract from tetracycline treated animals
DALY	Disability-Adjusted Life-Years
DC	Dendritic Cell
DEC	Diethylcarbamazine
ELISA	Enzyme Linked Immunosorbent Assay
FC	Flow Cytometry
GEO	Generalised Onchocerciasis
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HEK	Human Embryonic Kidney 293 cells
HL60	Human Caucasian Promyelocytic Leukemia cells
INFγ	Interferon gamma
IHC	Immunohistochemistry
IL-	Interleukin
ITEM	Immune Transmission Electron Microscopy
IQR	Inter Quartyle Range
IVM	Ivermectin
KO	Knock Out
LF	Lymphatic Filariasis
LPS	Lipopolysaccharaide
Mal	MyD88 Adapter Like protein
MALP-2	*Mycoplasma* Macrophage Activating Lipopeptide 2 kD
MDA	Mass Drug Administration
Mf	Microfilaria
MS	Mass Spectrometry
MyD88	Myeloid Differentiation primary response gene (88)
NEC	Non Endemic Control subjects
NETs	Neutrophil Extracellular Traps
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Programme for the Americas
Ov	*Onchocerca volvulus* adult worm extract
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PI/EN	Putatively Immune/Endemic Normal subjects
PMN	Polymorphonucletaed cells (Neutrophils)
PRR	Pattern Recognition Receptor
REA	Rapid Evidence Assessment
ROS	Peactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SAEs	Severe Adverse Events
TGFβ	Transforming Growth Factor beta
Th-	T helper cell
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor alpha
USG	Ultrasonography
wBmPAL	*Wolbachia* from *B. malayi* Peptydoglycan-Associated Lipoprotein
WB	Western Blot
WT	Wild Type
WoLP	Synthetic diacyl-20-mer of the N-terminus region of wBmPAL

Abbreviations of chemicals and biochemicals are detailed in the Appendix.

ABSTRACT

Onchocerciasis affects an estimated 37 million people in Sub-Saharan Africa and Latin America, causing debilitating skin and eye disease and accounting for a global loss of 1 million Disability-Adjusted Life-Years. The discovery of *Wolbachia* bacterial endosymbionts in filarial nematodes, including *Onchocerca volvulus*, has revolutionised the understanding of the parasite's biology and immunopathogenic mechanisms of disease, and has offered a novel approach to its treatment and control with anti-*Wolbachia* antibiotics. Treatment with doxycycline is effective at sterilising and killing adult *O. volvulus* worms, proving superior to standard microfilaricidal treatment with ivermectin and of great potential as an alternative strategy for the control of onchocerciasis. Although the length of the required treatment has raised concerns about the use of doxycycline in Mass Drug Administration (MDA) strategies, a recent trial in Cameroon demonstrated the feasibility of a six week course of doxycycline MDA delivered with a community-directed approach. In the work presented here we found a significant reduction in microfilaridermia prevalence and loads four years after doxycycline MDA distribution, demonstrating its long-term effectiveness and supporting its implementation in existing control strategies.

Wolbachia peptidoglycan-associated lipoprotein stimulates innate and adaptive immune responses, contributing to disease pathogenesis through the induction of pro-inflammatory cytokines and recruitment of neutrophils. These features together with the cytokine milieu induced by filarial nematodes could support the development of a pro-inflammatory Type-17 immune response. In this work, PBMC from patients with onchocerciasis were found to produce only minimal levels of IL-17 in response to filarial extracts. On the contrary, a rich IL-17^{+} cell infiltrate was found surrounding adult worms in *Wolbachia* positive onchocercomas using immunohistochemistry (IHC), which was depleted from onchocercomas following doxycycline treatment. Although a high percentage of Th17 cells were present in this infiltrate compared to other diseases, the majority of IL-17 producing cells in nodules were neutrophils, within an extracellular trap-like structure. This unexpected result was consistent with the reported IL-17 production by human neutrophils using IHC in the literature, but could not be confirmed at the protein or the transcription level *in vitro* in this work. *Wolbachia* is responsible for an abundant neutrophil infiltration in *Onchocerca*-infected tissues. However, the role of neutrophils in the immune response to filarial parasites and their interaction with *Wolbachia* is poorly known. The work presented here showed that *Wolbachia* lipoprotein activates human neutrophils *in vitro*, supporting previous studies demonstrating the link between *Wolbachia* and neutrophils in the host inflammatory responses to *O. volvulus* infection. These results also indentify *Wolbachia* lipoprotein as a key molecule driving human neutrophil recruitment and activation.

Chapter 1. INTRODUCTION TO ONCHOCERCIASIS

1.1 Parasitological and epidemiological features

Geographical distribution and life cycle of Onchocerca volvulus

Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus*, which affects an estimated 37 million people in 34 countries, mostly in Sub-Saharan Africa and with small foci in Latin America and Yemen (Figure 1.1 B), causing skin and eye pathology, while an estimated 87 million people are at risk of infection [4]. *O. volvulus* is transmitted by *Simulium spp* black flies, vectors that breed along fast flowing rivers, from which comes the common name of the infection, "River Blindness". Adult female worms reside in fibrous nodules (onchocercomas) in subcutaneous and deeper tissues while males migrate between nodules to fertilize them (Figure 1.1 C and D). During their reproductive life span of an average of 10 years, females release 1000-3000 first stage larvae (microfilariae, mf) per day that migrate through the skin and the eye (Figure 1.1 E). Mf are acquired by the black fly vector during a blood meal, develop into infective third stage larvae (L3), and are then transmissible to other people. L3 moult to fourth stage larvae (L4), which moult again to develop into adult worms, with a pre-patency period of ~ 12-18 months. The life cycle of *O. volvulus* is shown in figure 1.1 A.

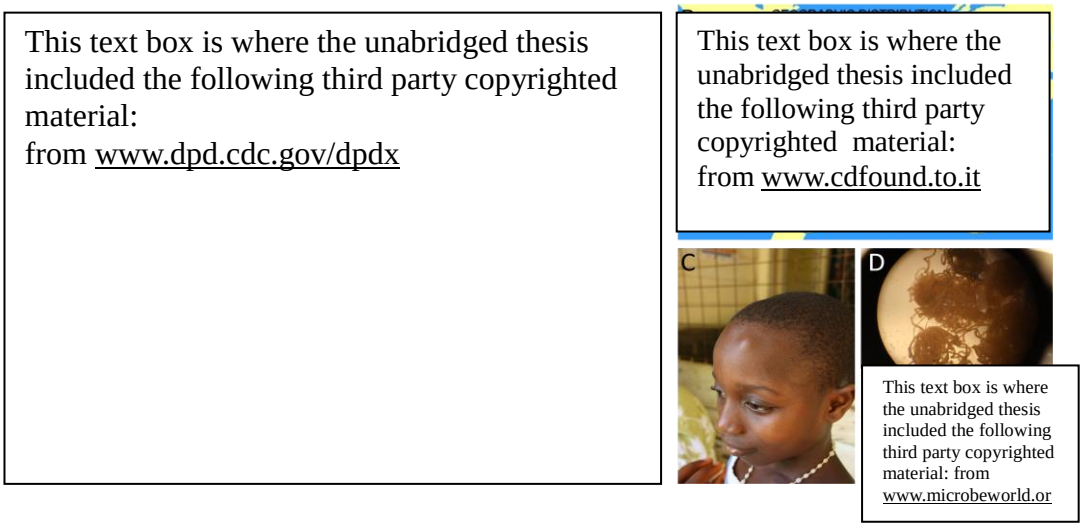

Figure 1-1. Life cycle and geographical distribution of *O. volvulus*. **A**, Life cycle of *O. volvulus* (modified from www.dpd.cdc.gov/dpdx). **B**, Geographical distribution of onchocerciasis (from www.cdfound.to.it). **C**, Subcutaneous nodule (photo F. Tamarozzi, Cameroon, 2011). **D**, Adult *O. volvulus* after nodule collagen digestion, 10x magnification (photo F. Tamarozzi, Ghana, 2009). **E**, Microfilaria in the dermis (from www.microbeworld.org).

Disease burden and socioeconomic impact

Onchocerciasis is characterized by cutaneous and ocular pathology that results from the invasion and death of mf in the skin and in the eye. The socio-economic and public health importance of dermatitis and visual impairment are profound. An estimated 1 million Disability-Adjusted Life-Years (DALYs) are lost globally for onchocerciasis [5] and this disease greatly reduces productivity in endemic areas. These aspects are extensively reviewed in [4, 6].

Cutaneous pathology and troublesome itching are the most common manifestations of onchocerciasis, affecting an estimated 32% and 28% of infected people respectively [6]. Skin manifestations cause social stigma and account for 60% of the DALYs lost globally for onchocerciasis [5-7]. Visual impairment and blindness affect an estimated 500,000 and 270,000 people respectively [8]. Although their incidence has been dramatically reduced in areas where control programmes are implemented [9], they nevertheless account for a well documented excess mortality [10]. Moreover, also disease manifestations of onchocerciasis other than visual loss negatively affect life expectancy. An excess mortality associated with mf load, especially in the younger population, has been recently demonstrated by Walker *et al* [11]. Parasite-induced immunosuppression and infection-associated epilepsy, growth retardation and poor nutrition have been proposed as possible causes [11].

Epidemiological patterns of infection and disease

Prevalence of infection rises with age until about 30 years, after which a strong variation can be observed depending on geographic area and sex [12]. Age- and sex-specific heterogeneous exposure as well as immune-related factors, such as concomitant immunity (i.e. the ability of continually exposed infected patients to mount over time a protective immune response to incoming L3 while maintaining an immune-suppressive state toward already established infections), parasite-induced immune suppression and prenatal exposure, has been suggested as possible explanations [12-15]. In highly endemic areas, skin disease manifestations parallel with prevalence of microfilaridermia, which varies with age and exposure, while prevalence of skin depigmentation and visual impairment increases with age [16, 17]. Some clinical manifestations are associated with geographically distinct endemic areas. For example the hyperreactive form of dermatitis called Sowda is most prevelant in areas of Yemen and Sudan. Most notably, the occurrence of ocular pathology varies between geographical locations, being more common in savannah areas compared with forested endemic areas of West and Central Africa and in Latin America [18]. This has been related to various factors such as area of body

localization of onchocercomas, vector species, mf burden, parasite strain and more recently to a higher *Wolbachia* endosymbiont load in the more virulent *O. volvulus* savannah strain [19].

1.2 *Wolbachia* endosymbionts of filarial nematodes

Most human filarial species, including *O. volvulus* and *Wuchereria bancrofti, Brugia malayi* and *B. timori* (the latter three causative agents of lymphatic filariasis -LF-), harbour intracellular *Rickettsia*-like bacteria of the genus *Wolbachia*, phylogenetically related to the long known *Wolbachia* bacteria found in arthropods [20-22]. Moreover, evidence exists of *Wolbachia*-free filarial species, notably *Loa loa.* Some of these species are likely to have been infected in the past as evidenced by lateral gene transfer, but have subsequently lost their symbionts [23]. In arthropods, *Wolbachia* are both maternally and horizontally transmitted and are mainly considered reproductive parasites, although some features of commensal and mutualistic symbioses have been recognised (reviewed in [24]). *Wolbachia* bacteria in nematodes have been first observed in the 1970s but taxonomically identified only about two decades later [25-28]. They are transmitted vertically and are considered mutualistic endosymbionts on the basis of: i) all individuals and all life stages of infected filarial species harbour *Wolbachia* (although *Wolbachia* has been demonstrated in some but not all populations of *Mansonella perstans* and other filarial parasites of animals), and ii) depletion of *Wolbachia* by tetracycline treatment has detrimental effects on worm development, embryogenesis, fertility and viability [24, 29, 30].

In *O. volvulus* and agents of LF, *Wolbachia* reside in hypodermal cells of the lateral chords and in the female reproductive tract, where bacteria are found in oocytes and developing embryonic stages [22] (Figure 1.2). The biological basis of the *Wolbachia*-filaria symbiosis is still not clear. Studies comparing the genome of *Wolbachia* and *B. malayi* have shown intact *Wolbachia* biochemical pathways, such as the biosynthesis of heme, nucleotides, fatty acids and phospholipids, which are absent or incomplete in worms, suggesting these may contribute to the symbiotic mutualism [31]. Depletion of *Wolbachia* by tetracycline treatment has detriment effects on larval development [32-34] and adult fertility (spermatogenesis, female insemination rate) [35], has long-lasting (possibly permanent) inhibitory effects on embryogenesis (embryostatic effect) resulting in protracted reduction of mf loads [35-38], and ultimately premature adult death after ≥ 12 (LF) to 20 (onchocerciasis) months from treatment (macrofilaricidal effect) [37, 39-41]. These effects have made *Wolbachia* a novel

and promising target for anti-filarial treatment. The mechanism behind these effects is at present not known, but a toxic effect on the worm, either directly from antibiotics or resulting from the release of *Wolbachia*-derived molecules after treatment has been convincingly excluded (ref [22]). Recently Landmann and colleagues have described an increased apoptosis in nematode germline and somatic cells of embyos and larval stages and the disruption of the hypodermal cytoskeleton after *Wolbachia* depletion [43], which explain some of the anti-filarial effects of *Wolbachia* elimination.

Wolbachia has also been suggested to modulate the mammalian host's immunological environment in a manner favourable for nematode survival. For example, the *Wolbachia*-dependent recruitment of neutrophils around adult parasites in onchocercomas has been suggested to facilitate nutrients uptake and mating of the worm or to avert the possibly deleterious recruitment and activation of eosinophils around them [44, 45]. Further evidence of the role of *Wolbachia* in facilitating worm parasitism has been recently provided by the work of Specht *et al* [46]. They showed that the stimulation of mast-cell mediated vascular permeability facilitated the establishment of L3 of *Litomosoides sigmodontis* in the mouse host, and this was dependent upon the presence of *Wolbachia* and TLR2. On the other side, however, the immune response to *Wolbachia* is central in the pathogenesis of filarial diseases and the endosymbiont elicits a T helper 1 (Th1)-type immune response potentially detrimental for the parasite.

A more detailed overview of these aspects is provided in chapter 4.

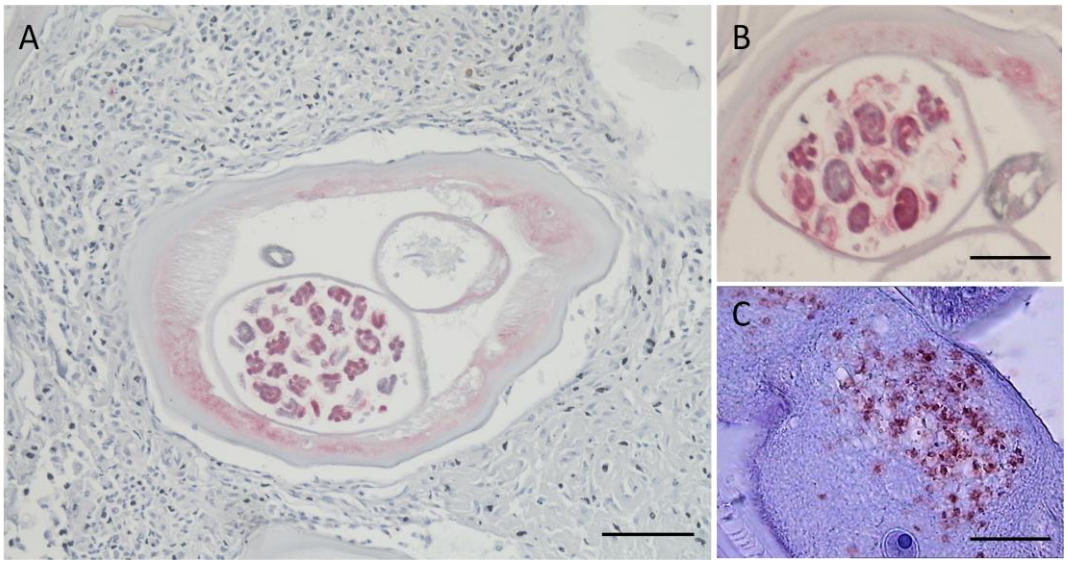

Figure 1-2. *Wolbachia* (in red) in lateral chords and female reproductive organs of filarial nematodes. **A**, Cross-section of adult female *O. volvulus* in a nodule. Original magnification 10x. Scale bar, 50 μm. **B**, Adult female uterine content in cross section showing *Wolbachia* in embryos. Original magnification 10x. Scale bar, 50 μm. A and B, *Wolbachia* is stained with anti-wBmPAL antibody [47] (ref chapter 5). **C**, *Wolbachia* in a lateral chord cell of *Dirofilaria immitis* stained with anti-WSP antibody [48]. Original magnification 60x. Scale bar 5 μm.

1.3 Onchocercal disease in humans

Clinical features

Skin and ocular pathology occur after the death of tissue dwelling microfilariae. The spectrum of cutaneous manifestations is broad (reviewed in [17]). The more common generalized form presents with subclinical or intermittent dermatitis (acute and chronic papular dermatitis) with troublesome itching (Figure 1.3 A). Acute dermatitis, chronic dermatitis, and itching affect 7%, 13% and 32% of the infected population respectively [6]. Acute dermatitis is commonly seen in visitors returning from endemic areas [49]. In endemic areas, generalized dermatitis may progress to skin hyperpigmentation or depigmentation ("leopard skin", figure 1.3 B) and skin atrophy ("hanging groin"). A less common but severe manifestation is a localised hyperreactive form (lichenified onchodermatitis or Sowda) characterized by pruritic hyperpigmented hyperkeratotic plaques associated with local lymphadenopathy. Sowda is mostly seen in certain endemic areas such as Yemen and Sudan.

Ocular pathology is less common than dermatitis, and is generally only observed in residents of endemic areas [49]. The most common eye pathology involves the cornea, but other structures of the anterior and posterior segment

can also be affected [16, 50]. Corneal pathology begins with “fluffy” or “snow-flake” opacities (punctuate keratitis) that later coalesce and may become hyperpigmented (sclerosing keratitis, figure 1.3 C). In the anterior chamber dead mf can cause uveitis with formation of sinechiae, cataract and glaucoma. Posterior segment lesions include atrophy of the retinal-pigment epithelium, choroido-retinal scarring, subretinal fibrosis and post-neuritic optical atrophy. Cutanoeus and ocular as well as systemic symptoms such as fever, tachycardia, hypotension and lymphadenopathy, may result from the death of large mf loads after microfilaricidal treatment with diethylcarbamazine (DEC), called the Mazzotti reaction, or (less severely) with ivermectin (IVM) [51].

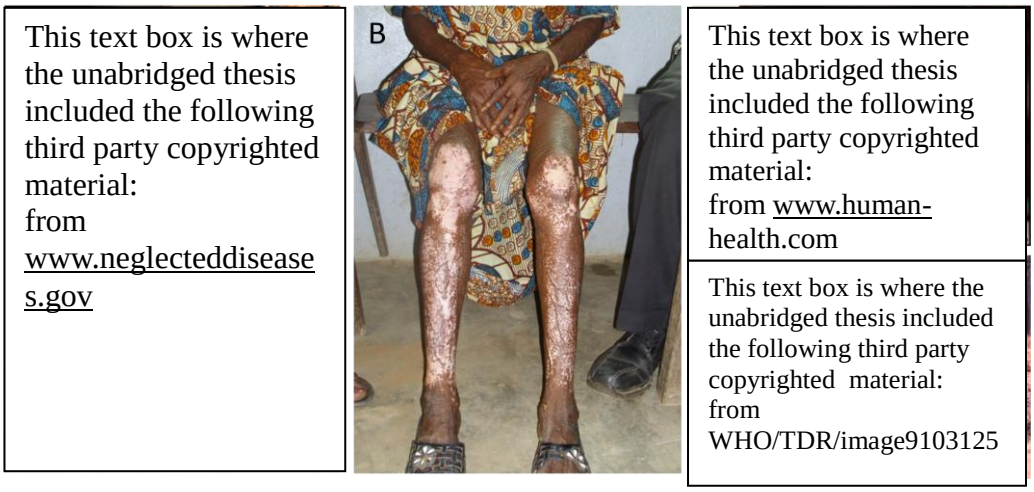

Figure 1-3. Clinical manifestations of onchocerciasis. **A**, Chronic papular dermatitis (from www.neglecteddiseases.gov). **B**, Skin depigmentation (leopard skin) (photo F. Tamarozzi, Cameroon, 2011). **C**, Onchocercal sclerosing keratitis (from www.human-health.com). **D**, A child leading a man blind from onchocerciasis (from WHO/TDR/image9103125).

Immune responses, infection status and pathogenesis of onchocerciasis

The spectrum of *O. volvulus* infection status and pathology has been investigated in relation to immune responses to different parasite life stages and their balance with anti-inflammatory and immunosuppressive mechanisms. Studies on human onchocerciasis tend to classify subjects into their different clinical groups. Patients with generalized onchocerciasis (GEO) represent the vast majority of infected subjects and are characterised by weak or no skin inflammation despite high parasite burden. On the contrary, patients with severe chronic dermatitis (Sowda) present severe symptoms but

low mf and adult burdens. Finally, a small subgroup of people living in endemic areas but apparently able to maintain an infection-free status despite exposure to infective vector bites have been termed “putatively immune” or “endemic normals” (PI/EN). The immune profile of these three polar groups is extensively reviewed in [52, 53] and discussed in detail in chapter 3.

In vitro, Peripheral Blood Mononuclear Cells (PBMC) from GEO show a weak proliferative response to filarial antigens, downregulation of both Th1 and Th2 responses, with a predominance of Th2, and high levels of interleukin (IL)-10. These patients characteristically present with high levels of circulating parasite-specific IgG of all subclasses, but predominantly IgG_4, and IgE [52]. Levels of these parasite-specific antibodies peak in the weeks after microfilaricidal treatment while decrease progressively with multiple treatments over time, although remaining higher than PI/EN subjects also after (presumed) parasitological cure [54, 55]. On the contrary, PBMC production of both Th1 and Th2 signature cytokines interferon (IFN) γ and IL-5 in response to filarial extracts, after peaking shortly after microfilaricidal treatment, remains depressed in persistently exposed (and likely criptically parasitized) subjects, but is heightened after (presumed) loss of adult parasites [54, 56]. The immunological profile of GEO has been interpreted as an immune-suppressed condition favourable for both parasite establishment and survival but with minimal host immunopathology, when compared with that of PI/EN on one side and patients with Sowda on the other [52].

PI/EN show a strong mixed Th1/Th2 cytokine response to filarial extracts while circulating levels of IgG (all subclasses) and IgE are generally very low [52]. The predominance (or at least the presence) of a Th1 response, together with prevailing opsonizing and cytophilic IgG_1 and IgG_3 antibodies, have been suggested to account for the infection-free condition of PI/EN [52]. *In vitro* studies have shown antibody-dependent killing of L3 and leukocyte adherence to L3 in the presence of immune sera from PI/EN and infected individuals [57, 58], and filarial murine models of vaccination are consistent with an antibody-dependent Th2-mediated protection to challenge infections (reviewed in [59, 60] and detailed in chapter 3 and 4). In particular, Th-independent antibodies to carbohydrate-containing molecules appear to be of importance in mediating protection (ref [61]), and are not downregulated in *L. sigmodontis* infected mice, in contrast to Th-dependent antibodies [62]. In humans, IgG_2 have been found to dominate the antibody response to L3 in bancroftian filariasis, suggesting carbohydrates as their target [63], but this isotype has not been analysed in the

work of McDonald and colleagues, that specifically addressed the immune response to L3 in onchocerciasis [14]. In this latter study a positive correlation between anti-L3-specific cytophilic IgG_3 and IgE and levels of IFNγ and IL-5 in response to L3 extract and increasing age, and high levels of anti-L3-specific IgG_1 regardless of age, were observed [14]. However, the strength of these correlations, although statistically significant, was very weak. Moreover, the concept of concomitant immunity in onchocerciasis is still a matter of debate [12, 13], as is the classification of PI/EN as truely infection-free [64]. Therefore, the cause-effect relation of the correlation between immune profile and infection status in onchocerciasis should be interpreted with caution (ref [61] and chapter 3). Human genetic polymorphisms (e.g. of HLA-D and Transforming Growth Factor β) have also been associated with resistance to *O. volvulus* infection and to infection status in LF, but no study so far correlated these traits with parasite-specific immune responses [65-68].

The inflammatory response to dying tissue dwelling mf is responsible for onchocercal pathology. This is clearly shown by the development of skin, eye and systemic symptoms after DEC treatment (that rapidy kills mf) and less so IVM treatment (that induces a slower reduction in mf loads), and the correlation between severity of adverse reactions and mf infection intensity [69-74]. Pro-inflammatory cytokines and granulocytes are the main effectors in these reactions, characterized by marked eosinophilia and neutrophilia, eosinophil and netrophil infiltration in the skin, and high levels of circulating IL-6, TNFα, RANTES, and eosinophil- and neutrophil-derived proteins, which correlate with mf loads and severity of adverse events [69, 70, 73-78].

From a purely pathogenetic point of view, chronic dermatitis and keratitis may be mediated by filarial proteases, both directly by enzymatic attack of the connective tissue and indirectly by inducing autoimmune responses [79-81], and by reactive products realeased by granulocytes [82, 83]. Retinal lesions may result from direct activation of complement by mf or autoimmune processes driven by cross-reaction between retinal and parasite proteins [50, 82, 84].

The majority of studies investigating the relationship between immune responses and pathogenesis of onchocercal dermatitis compared GEO and Sowda patients, correlating the low mf loads and the strong Th2 responses to higher severity of skin pathology, as seen in Sowda [85, 86]. Granulocytes have been reported to be able to kill mf *in vitro*, as detailed in chapter 4. However, with the possible exception of Sowda patients, no evidence exists of their ability to kill live mf in humans [69, 75, 76, 87]. Sowda is characterized by strong Th2

cytokine responses, high levels of parasite-specific IgG of all subclasses, particularily IgG_1 and IgG_3, and IgE, pronounced eosinophilia, mastocytosis, increased circulating levels of eosinophil cationic protein, delayed-type hypersensitivity, and lower expression of the suppressive cytokine Tranforming Growth Factor (TGF)-β in *Onchocerca* infected tissues ([88] and ref [52]). The antibody profile of Sowda patients, similar to what is observed in PI/EN, has been suggested to mediate protection also toward incoming larvae, partially accounting for the low adult worm burden observed in these patients [52]. However, no specific antigen recognition pattern of any IgG subclass has been so far identified in PI/EN and/or Sowda patients compared to GEO [89, 90]. In GEO, the predominant antibodies are IgG_4, IgG_1 and IgE [52]. IgG_4 in sera from LF patients have been found to block IgE-mediated histamine release *in vitro*, suggesting a role in protection against pathology [91]. However, the relevance of this mechanism *in vivo* is unknown, and levels of IgG_4 appear to be a marker of active infection irrespective of pathology in both onchocerciasis and LF [89, 92-94]. GEO encompasses a wide range of skin disease manifestations, as described above. The characteristics of the immune profile observed in Sowda do not lie on a continuum when the spectrum of skin disease severity in GEO is considered, suggesting the mechanisms behind the pathogenesis of GEO and Sowda being different, with this latter rather resulting from a hyperreactive Th2 immune response to mf (discussed in detail in chapter 3). Moreover, Sowda is more common in certain endemic areas and intrinsic human factors such as autoimmune mechanisms and genetic polymorphisms have been suggested to influence its development [66, 81, 95, 96].

The observation that the vast majority of filariasis infected people show chronic infections which persist for many years with high parasite loads but relatively little signs of disease is consistent with the presence of immune evasion and immune modulation mechanisms induced by these parasites (reviewed in [97]). It is evident from animal infections that maximal down-regulation of immune responses coincides with adult parasite maturation at pre-patency and patency [98-102]. However, mf and L3 in the absence of adult parasites were also found to induce immune suppressive mechanisms [62, 103-106]. This is consistent with human studies showing only transient and incomplete reversal of immune suppression after IVM treatment which targets only mf [54, 56, 107, 108], and with the correlation between immune suppression and intensity of exposure [109, 110]. One single report also suggested that vector saliva may have immune suppressive effects [111]. It has also been suggested that increasing immune suppression over time could explain the higher rate of *Onchocerca*

acquisition in adults compared to children [13, 112], a finding that contrasts with the concept of concomitant immunity. Exposure to parasite antigens *in utero* has also been found to account for lowered immune responses toward filarial infections [15, 113-116]. Various mechanisms appear to be involved in immune downregulation. The frequency of total and parasite-specific T and B cells has been reported to be decreased in filariasis infected patients [117, 118]. However, blockage of IL-10 and/or TGFβ was able to restore at least partially the proliferation of PBMC from mf^+ patients, suggesting immune suppression rather than clonal deletion [119-122]. IL-10 has been repeatedly associated with hyporesponsiveness and parasite survival in murine models of filariasis [62, 123-125], and TGFβ, as well as anti-inflammtory prostaglandin E_2 (PGE_2) is highly expressed by immune cells in *Onchocerca* infected tissues from hyporeactive patients [88, 126, 127]. Antigen-specific regulatory T cells (Tr1) are induced in both infection models and human onchocerciasis and seem to play a role in inducing peripheral tolerance by production of IL-10, TGFβ and expression of the down-regulatory molecule CTLA-4 [62, 102, 103, 106, 116, 128, 129]. Alternatively activated macrophages have been studied extensively in mouse models of filariasis (reviewed in [130]) and recently macrophages with an alternative activation phenotype have been described in human onchocerciasis and LF ([126, 131],[97]). Moreover, the functions of antigen-presenting cells have been found to be impaired by exposure to filarial parasites [104, 123, 132-137]. A number of parasite molecules have been identified that neutralise or affect host reactants. These include antioxidant enzymes, protease inhibitors, fatty-acid binding proteins, PGE_2, and cytokine (e.g. TGFβ and Macrophage migration Inhibitory Factor) homologues (reviewed in [52]). Interestingly excretory/secretory products from *O. volvulus* adult females and mf have been shown to directly inhibit proliferation and IFNγ production upon filarial and non-filarial stimuli in PBMC cultures from both infected and unexposed subjects [138]. Subsequently, a conserved filarial phosphorylcholine-containing glycoprotein (ES-62) has been characterised that directly desentitises T and B cells and induces a preferential Type-2 response [139]. Parasite-derived factors and the immune-suppressive cytokine milieu induced by the infection may downregulate not only parasite-specific pro-inflammatory responses, but also immune responses to third-party antigens such as vaccines, allergens or important co-infections like malaria or tuberculosis. Although results from experimental and field studies are still not presenting a conclusive picture, this aspect needs attention to forecast the consequences of filariasis control programmes [15, 97, 140-145].

Wolbachia and the inflammatory response

Wolbachia and *Wolbachia*-derived molecules can come in contact with the mammalian host's immune cells after release from dying parasites or through worm excretory/secretory products [146-150].

Many experimental studies, both *in vitro* and *in vivo*, have explored the interaction between *Wolbachia* and *Wolbachia*-derived molecules and the mammalian immune system (Figure 1.4). *In vitro* studies have demonstrated that exposing innate immune cells to *Wolbachia*-containing filarial extracts or isolated *Wolbachia* bacteria elicit a potent pro-inflammatory response, in contrast to extracts from aposymbiotic species (*Acanthocheilonema viteae, Loa loa*) or extracts from worms depleted from *Wolbachia* by tetracycline treatment. This includes the up-regulation of surface co-stimulatory molecules on dendritic cells (DCs) and macrophages [47, 151, 152], the secretion of pro-inflammatory cytokines (such as IL-1, IL-6, IL-12, IL-23, TNFα, RANTES) by these cells [47, 151-153], and the preferential induction of a Th1 response in co-cultures of DCs and $CD4^+$ T cells, as assessed by the IFNγ/IL-4 ratio [47]. Moreover, *in vitro* incubation of human lymphatic endothelial cells with culture supernatant of monocytes stimulated with *Wolbachia*-containing, but not *Wolbachia*-depleted, filarial extracts induced the upregulation of podoplanin, a marker of lymphatic endothelial cell activation [47]. Neutrophil infiltration in *Onchocerca* infected tissues depends on the presence of *Wolbachia*, which chemoattracts and directly activates these cells resulting in the secretion of CXCL1, CXCL2 and CXCL8 (IL-8), chemotactic for neutrophils, and TNFα [44, 154-156]. The *Wolbachia*-mediated induction of anti-inflammatory cytokines (TGFβ) and the downregulation of pro-inflammatory markers upon multiple stimulations have also been demonstrated [152]. In the study of Turner *et al.* [47] it was also shown that *Wolbachia* can induce a skewed Th1 adaptive immune response *in vivo*, as indicated by the predominant production of IgG_{2c} antibodies by mice inoculated with *Wolbachia*-containing filarial extracts.

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Tamarozzi F, Halliday A, Gentil K, Hoerauf A, Pearlman E, Taylor MJ: **Onchocerciasis: the role of Wolbachia bacterial endosymbionts in parasite biology, disease pathogenesis, and treatment**. *Clinical microbiology reviews* 2011, **24**(3):459-468.

Figure 1-4. *Wolbachia*-induced responses by specific cell type. *Wolbachia*-exposed dendritic cells and macrophages are activated through TLR2/6-MyD88-Mal signalling pathway enhancing the expression of co-stimulatory molecules, producing pro-inflammatory cytokines, and inducing a preferential Th1 response. Macrophages can also be tolerized upon re-stimulation. Neutrophils and corneal stromal cells interact with *Wolbachia* via the same pathway producing CXC chemokines and pro-inflammatory cytokines. Mast cells activated by *Wolbachia* through TLR2 degranulate increasing vascular permeability, which facilitates the establishment of parasite infection. Figure from Tamarozzi *et al* [19]. Copyright © American Society for Microbiology, Clinical Microbiology Review, 2011, 24(3): 459-468, doi: 10.1128/CMR.00057-10.

Early studies indicated an LPS-like activity of *Wolbachia* Surface Protein (WSP), a major component of *Wolbachia* cell membrane [157], as responsible for the activation of immune cells through Toll-like Receptor (TLR)-4 ([158]; an overview of TLRs can be found in chapter 4). However, the results of Turner *et al* [47] suggested that these results were likely due to *E. coli* LPS contamination of recombinant WSP. Indeed, sub-nanogram concentrations of LPS below the threshold to activate macrophages strongly activated them when applied in cultures with *Wolbachia*-containing filarial extracts. Moreover, activation was not elicited through TLR4 upon use of filarial extracts with stringently low

levels of contaminant endotoxins, but through TLR2 [47]. *Wolbachia* (from *B. malayi*) peptidoglycan-associated lipoprotein (wBmPAL [47]) has emerged as the interacting with the host immune system through TLR2/6-Myeloid Differentiation primary response gene 88 adaptor (MyD88)- MyD88 adaptor-like (Mal) signalling pathway, but not TLR4 or TLR9 [47, 151-156] (for an overview of TLRs and PAMPs recognition refer to chapter 4). wBmPAL is a diacylated lipoprotein localised on the bacterial cell wall [47]. They demonstrated that antibodies raised against recombinant wBmPAL could identify *Wolbachia* in filarial tissues and, most importantly, that synthetic wBmPAL (WoLP) elicited nearly identical responses as *Wolbachia*-containing filarial extracts both *in vitro* and *in vivo* [47]. wBmPAL has also been recently identified as an abundant *Wolbachia* protein in the secretome and proteome of *B. malayi* by Bennuru *et al* [146, 157].

Role of Wolbachia in the pathogenesis of onchocercal disease

The most compelling evidence of the role of *Wolbachia* in the inflammatory pathogenesis of onchocerciasis *in vivo* comes from the study of post-microfilaricidal treatment in humans and from murine models of onchocercal keratitis. This aspect has been recently reviewed as part of this PhD project [19].

In onchocerciasis and LF, the occurrence and severity of adverse events after DEC and IVM treatment has been found to correlate with prevalence and levels of *Wolbachia* DNA and *Wolbachia* bacteria in serum, pre-treatment mf loads, and circulating levels of pro-inflammatory cytokines (IL-1, IL-6, IL-12, TNFα) and neutrophil-derived calgranulin and calprotectin. In turn, these laboratory markers correlated with levels of circulating *Wolbachia* DNA [147, 150, 159]. Plasma levels of neutrophil-derived elastase have also been found to correlate with severity of adverse events after treatment with IVM in onchocerciasis [78]. Following depletion of *Wolbachia* in mf by doxycycline treatment, the prevalence and severity of adverse events after DEC has been reported to be significantly lower compared to microfilaraemic patients treated with DEC only [160]. After IVM treatment, a similar trend but without reaching statistical significance was found when *Wolbachia* depletion was only partial, despite significant reduction in mf loads in the doxycycline compared to the IVM only treated group [159]. Finally, in favour of absence of *Wolbachia* rather than just decrease in mf loads after doxycycline treatment being responsible for reduced adverse events after microfilaricidal treatment, is the observation that adverse events after IVM in patients with *Wolbachia*-free *L. loa* occur only with mf loads much higher

compared to infections with *Wolbachia*-positive filarial nematodes (>80,000 mf /10 ml blood or more probably >500,000 mf/10 ml in loiasis compared to <3000 mf/10 ml in LF) [161-163].

The role of *Wolbachia* in the development of onchocercal keratitis has been extensively investigated by Professor Eric Pearlman's group in a mouse model, using intracorneal injections of *Wolbachia* and *Wolbachia*-containing and *Wolbachia*-devoid filarial extracts. In these experiments, corneal inflammation and haze associated with neutrophil recruitment were ablated in the absence of *Wolbachia* [155, 156, 164]. Consistently, these phenomena were dependent on TLR2/MyD88 [154, 156] and were reproduced by the use of WoLP in wildtype but not $TLR2^{-/-}$ and $TLR6^{-/-}$ mice [47].

The role of *Wolbachia* in the pathogenesis of onchocercal disease is exemplified in figure 4.2 and 4.3 of chapter 4, where a more detailed description of the role of neutrophils, eosinophils and adaptive immune responses in onchocercal keratitis is provided.

1.4 Therapy and control of onchocerciasis

Of the three directly filaricidal drugs available for the treatment of onchocerciasis, only IVM is currently used for routine treatment and community-wide control [18, 165]. Of the other drugs, suramin is macrofilaricidal but is highly toxic and requires parenteral administration, while DEC, which is microfilaricidal and only partially effective against adult worms, frequently produces severe adverse reactions especially in heavily infected patients. When used at the standard dose of 150 μg/kg once or twice a year as in onchocerciasis control programmes, IVM is highly effective at reducing microfilarial loads. However, it is only marginally active against adult worms, and mf start to slowly repopulate the hosts' skin a few months after treatment [166-170].

The strategies used for onchocerciasis control programmes include vector control and Mass Drug Administration (MDA) of IVM. The Onchocerciasis Control Programme (OCP, www.who.int/apoc/onchocerciasis/ocp/en) was launched in 1974 and ended in 2002, covering ~ 30 million people in 11 endemic countries of West Africa (Figure 1.5). OCP control strategy relied initially only on larvicidal spraying of *Simulium* breeding sites, introducing yearly IVM MDA in 1989, after Merck & Co., Inc. implemented the generous donation of IVM (Mectizan®) "for as long as necessary to eliminate

onchocerciasis as a public health problem". OCP was highly effective in all covered countries with the exception of Sierra Leone, due to civil war. In 1995 the African Programme for Onchocerciasis Control (APOC, www.who.int/apoc.en) was launched, based on annual IVM MDA only, with the exception of areas of Uganda, Tanzania and Equatorial Guinea where vector control was also implemented until 2005. Twenty three countries are covered by APOC (19 non-OCP and selected areas of 4 former OCP countries, figure 1.5). APOC is planned to end in 2015, with the aim of establishing country-led control systems capable of eliminating onchocerciasis as a public health programme in all endemic countries in Africa [171]. Up to 2010, more than 75.8 million people received regular treatment with IVM, with ~ 1 million DALYs averted and with a reduction in infection prevalence of ~73% compared to pre-APOC levels. In Latin America, 6 endemic countries (Figure 1.5) are under the umbrella of the Onchocerciasis Elimination Programme for the Americas (OEPA, www.cartercenter.org/health/river_blindness/oepa), which was initiated in 1993, using biannual IVM MDA, an approach that has been extremely effective, with at present 10 out of 13 endemic foci having transmission interrupted or suppressed, making the goal of eradicating onchocerciasis from the New World a likely outcome in these foci, although challenges remain for Venezualan and Brazilian Amazonian foci [172].

Figure 1-5. Geographical coverage of onchocerciasis control programmes. From [1]

Limitations of current control programmes in Africa

Although elimination with IVM MDA has been reported in some foci of Senegal and Mali, onchocerciasis is considered not eradicable in other endemic regions of Africa by means of existing strategies used by current control programmes [171-174]. In the absence of a macrofilaricidal drug, even with high treatment coverage, 25 years of annual IVM treatment may not be sufficient for

elimination in areas of high vector density and intensity of human-vector contact [171, 174]. This timeframe is likely to go beyond the realistic length of a sustained control programme [173]. Besides purely cost- and management-related issues, treatment coverage with IVM is also hampered by several factors such as: i) civil unrest, conflict and normal human migration; ii) contraindications of IVM in children under 5 years of age and pregnant women; and iii) the risk of severe adverse events after IVM treatment in areas of *L. loa* co-endemicity, where post-treatment encephalopathy may develop likely as the result of mf-induced cerebral vasculopathy [4, 172, 173]. Another major concern is the potential development of IVM resistance by filarial nematodes [175, 176].

Wolbachia as a target for chemotherapy

Targeting *Wolbachia* bacteria with antibiotics provides an important alternative approach to the treatment and control of onchocerciasis and LF. So far, published *in vitro* screening activities showed that tetracyclines and rifamycins are the most potent classess of antibiotics resulting in *Wolbachia* depletion and detrimental effects (until death) on filarial nematodes [177, 178]. This has also been replicated *in vivo* in infections with *Brugia spp* in rodents, *O. ochengi* in cattle and *L. sigmodontis* in mice [34, 179-181]. An extensive series of human studies conducted in onchocerciasis and LF have shown that ≥ 90% depletion of *Wolbachia* population is required to induce sustained interruption of embryogenesis and adult worm death [36, 159, 182]. At present, the use of doxycycline as anti-wolbachial monotherapy at 200 mg/day for 6 weeks is considered the "gold-standard" macrofilaricidal regime. This leads to a sustained (most likely permanent) block of embryogenesis with consequent clearance of microfilariae, and a macrofilaricidal effect detectable after ≥ 12 months for *W. bancrofti* and ≥ 20 months for *O. volvulus* (up to 70% macrofilaricidal effect demonstrated in this latter infection) [36, 37, 39, 40, 183, 184]. A true measure of the macrofilaricidal effect is compounded by reinfections in subjects living in endemic areas with ongoing transmission and the variable period taken for individual *Wolbachia*-depleted adult parasites to die [185]. Data from field trials and mathematical models suggest a 4 week course of doxycycline at the same dose and possibly a 5 week course at 100 mg/day being equivalent, in the long term, to the 6 week course ([36, 37] and Walker *et al*, unpublished). Most trials have included a single dose of IVM four months after doxycycline treatment. However, both sterilising and macrofilaricidal effects are due to doxycycline alone, with doxycycline as a monotherapy being more effective than IVM alone in reducing long-term mf

prevalence and burden. The combination of doxycycline followed by IVM is superior to doxycycline only for mf reduction, but with no impact of IVM on the macrofilaricidal effects [38, 184, 186]. Current recommendations for individual drug administration, including doxycycline, in onchocerciasis and LF are outlined in [165].

The importance of including macrofilaricidal drugs in current filariasis control strategies is paramount. A very effective macrofilaricidal/permanently sterilising drug would have substantially higher potential to achieve infection elimination than IVM, drastically reducing the timeframe for control programmes ([173, 174] and Basanez *et al*, unpublished). The reduced time and frequency of treatment would also be useful where sustained delivery of IVM is compromised. Moreover, a macrofilaricidal drug could be highly useful in programme endgame situations where a test-and-treat strategy to "mop-up" residual infections could be used to achieve elimination of infection [174].

Using *Wolbachia* as the target for treatment adds the further advantage of: i) overcoming/preventing IVM resistance in filarial nematodes [175, 176]; ii) offering the possibility to safely extend current control programmes in areas previously excluded because of co-infection with *L. loa* and the fear of serious, sometimes fatal, adverse reactions seen in patients having high *L. loa* mf loads when treated with IVM [163, 187], as doxycycline has no microfilaricidal activity against the *Wolbachia*-free *L. loa* [186]; and iii) improve compliance to control programmes by reducing the incidence and severity of post-microfilaricidal adverse reactions [160, 186]. Indeed, doxycycline depletes *Wolbachia* from circulating and tissue dwelling mf, and achieves sustained microfilarial clearance slowly over time by affecting early embryogenesis rather than with a direct microfilaricidal effect. Finally, it has been shown that larval development of *Wolbachia*-depleted mf in the insect vector is impaired, adding a further benefit in terms of interrupting infection transmission [32].

So far, the length of the required treatment has raised some scepticism about the use of doxycycline in MDA strategies because of the perceived risk of poor compliance and potential logistical problems. However, an implementation trial conducted recently in an *O. volvulus* and *L. loa* co-endemic area in Cameroon on ~17,500 subjects has demonstrated that a 6 week regime, after community-directed explanation and organization of the delivery, is feasible, safe, and highly accepted by communities, as shown by the 97% compliance achieved [188]. This notwithstanding, the problem of contraindication of doxycycline in children less than 9 years of age and pregnant and breastfeeding women remains, and a reduction in the length of the required treatment would be desirable. Preliminary studies in humans and using the *L. sigmodontis* mouse

model have shown that treatment with rifampicin could achieve *Wolbachia* depletion, inhibition of larval development, and interruption of embryogenesis [34, 189]. Importantly, rifampicin and doxycycline have synergistic effects, allowing a drastic reduction of treatment length in the *L. sigmodontis* model ([34] and Hoerauf *et al,* unpublished), providing a proof of principle for the use of antibiotic combination therapies to shorten macrofilaricidal treatments. Controlled clinical trials are currently assessing the efficacy of rifampicin alone and in combination with doxycycline for reduced timeframes and doses as part of the regime refinement objective of the Anti-*Wolbachia* Consortium (A-WOL) programme, which also comprises the identification of alternative drugs and drug combinations, which could be used safely in children and pregnant women and effective in shorter timeframes.

1.5 Aim of the work

The discovery of *Wolbachia* endosymbiotic bacteria within filarial nematodes, including *O. volvulus*, has provided pivotal clues for the understanding of parasite's biology, immunomodulation and immunopathogenesis of filarial diseases, and has provided for the first time the target for the delivery of an effective macrofilaricidal treatment. However, many aspects of the relationship between *Wolbachia* and filaria with the host's immune system are still poorly known, especially the role of endosymbionts in inducing host responses favourable for the worm or the host. Moreover, the practical implementation of anti-*Wolbachia* treatment in filariasis endemic areas still requires refinement.
The work presented here explored different novel aspects of *Wolbachia* in human onchocerciasis as a driver of immunopathology and target for treatment.

The work presented in chapter 2 aimed to evaluate the long-term effectiveness of doxycycline MDA distributed using a community-directed delivery strategy four years after implementation and to validate the compliance rate of this implementation trial carried out in 2007 and 2008 in Cameroon [188]. A secondary aim was to evaluate whether the use of ultrasonography to detect motile adult parasites in onchocercomas could be a useful tool to monitor and evaluate the activity of macrofilaricidal drugs. The investigation was carried out by analysing the infection status of subjects who had completed a 6 week course of doxycycline MDA followed by one or two annual rounds of IVM MDA compared to those who only received one or two rounds of annual IVM MDA. The results of this work would be very important to inform the practical aspects of the implementation of macrofilaricidal drugs in current onchocerciasis control

programmes.
Specific objectives were:

- assessing the difference in microfilarial prevalence (primary outcome of the study) and load in subjects who had received the two MDA treatments;
- assessing the difference in palpable nodule prevalence and load in subjects who had received the two MDA treatments; and
- assessing the difference in presence of motile adult worms in onchocercomas of subjects who received the two MDA treatments using USG.

Laboratory work focused on the interaction between human neutrophils and *Wolbachia in vitro* (chapter 4), while chapter 3 and 5 explored the cytokine profile and Type-17 immune responses in onchocerciasis *in vitro* and *ex vivo*.

Wolbachia and *Wolbachia* lipoprotein play a major role in inducing a pro-inflammatory response in onchocerciasis, associated with recruitment and activation of neutrophils [44, 47, 155, 156]. However, the role of neutrophils in the host-parasite interplay remains unclear, and the interaction between *Wolbachia* and neutrophils has not been thoroughly studied. In particular, neutrophils appear to be not detrimental for living intact worms, although abundantly recruited around *Wolbachia*-infected adult worms [44], while they are activated and attack damaged parasites contributing to disease pathogenesis [52, 154-156]. Aim of the work presented in chapter 4 was to investigate the interaction between *Wolbachia* and human neutrophils *in vitro*, evaluating several phenotypes and functions of these cells upon stimulation with *Wolbachia* lipoprotein (WoLP) and filarial extracts containing or depleted from the endosymbiont. This would shed light on the (different?) role of neutrophils in onchocercal infection and disease.
Specific objectives were:

- assessing the activation status of human neutrophils *in vit*ro upon stimulation with WoLP and filarial extracts by analysing the expression of surface adhesion molecules and antibody receptors, the production of reactive oxygen species and cytokines, and the increase in their lifespan; and
- assessing the ability of WoLP and *Wolbachia*-containing worm extracts to recruit human neutrophils in an *in vitro* chemotactic assay.

The cytokine environment promoted by the combination of stimuli provided by the filarial nematode and *Wolbachia* could favour the induction of a pro-

inflammatory Type-17 immune response [47, 121, 152, 190-192]. Moreover, neutrophil recruitment is a signature feature of Type-17 immune responses, and interestingly an abundant neutrophil infilatrate is present in *Wolbachia*-containing onchocercomas and characterises onchocercal keratitis However, most studies investigating the cytokine profile in filariasis have focused on Th1 and Th2 responses while IL-17 and other pro-inflammatoty mediators such as IL-6 and TNFα have received less attention. Also, the individual role of the worm and *Wolbachia* in eliciting these responses has never been explored in human PBMC stimulation experiments so far.

The work presented in chapter 3 aimed to investigate the cytokine profile elicited by stimulation of PBMC from patients infected by *O.volvulus* with WoLP and filarial extracts containing or depleted from *Wolbachia*. Patients were part of a cohort recruited for a randomised, placebo-controlled double-blind clinical trial for the refinement of anti-*Wolbachia* regimes in Ghana.
Specific objectives were:

- assessing the production of Th1 (IFNγ), Th2 (IL-5, IL-13), Th17 (IL-17A) and pro- and anti-inflammatory cytokines (IL-6, TNFα, IL-10) in PBMC cell cultures from patients with onchocerciasis and uninfected controls from the same geographical area;
- assessing the association between these responses and parasite burdens, expressed as mf/mg of skin; and
- assessing the relative contribution of filarial nematodes and bacterial endosymbionts in these responses by comparing the results of *Wolbachia*-containing and *Wolbachia*-depleted filarial extracts in PBMC stimulations.

Although very limited, some evidence exists of the development of Type-17 immune responses in patients with LF [128, 145, 190] and in the *L. sigmodontis* mouse model [193]. However, no published data exist for onchocerciasis, and the role of *Wolbachia* in the induction of IL-17 has not been addreassed so far.
The work presented in chapter 5 aimed to investigate the presence and induction of a Type-17 immune response in onchocerciasis and its relation with the presence of *Wolbachia*.
Specific objectives were:

- confirming the results of preliminary immunohistochemical studies in our lab showing a rich IL-17^{+} cell infiltrate in onchocercomas, dependent on the presence of *Wolbachia*;
- assessing the cellular source(s) of IL-17 in onchocercomas; and

- evaluating the induction of IL-17 by human cells individuated as IL-17 sources in onchocercomas upon stimulation with *Wolbachia* and filarial extracts *in vitro*.

Chapter 2. EVALUATION TRIAL: LONG TERM IMPACT OF COMMUNITY-DIRECTED DELIVERY OF DOXYCYCLINE FOR THE TREATMENT OF ONCHOCERCIASIS

ABSTRACT

Anti-*Wolbachia* treatment with doxycycline has a great potential for the treatment and control of onchocerciasis as it sterilises and eventually kills adult worms. Doxycycline would be of particular use as an alternative approach to ivermectin in areas of *Loa loa* co-endemicity, in areas where sub-optimal efficacy of ivermectin has been reported, and would be a valuable help to existing programmes toward the goal of elimination. Nevertheless, the length of the required treatment has been indicated as a barrier to the use of doxycycline in Mass Drug Administration (MDA) strategies, because of potential logistical problems and risk of poor compliance. In 2007-2008, a feasibility trial of a six week course of doxycycline MDA distributed with a community-directed strategy was carried out in two health districts of Cameroon co-endemic for *Onchocerca* and *L. loa*. The reported 73.8% therapeutic coverage and 97.5% compliance in a population of 17,519 eligible subjects strongly supported the feasibility of a multi-week course of doxycycline delivered with this approach. The study reported here evaluated the effectiveness of this pilot intervention four years after implementation. The study was conducted on a sample of 507 subjects resident in one health district where doxycycline MDA was distributed. Of those, 375 had completed the treatment with doxycycline followed by one or two rounds of ivermectin MDA, distributed after doxycycline as part of the national onchocerciasis control programme, and 132 had only received one or two rounds of ivermectin MDA. We found a significantly lower microfilarial prevalence (p = 0.014) and load (p = 0.012) in people that had received doxycycline followed by ivermectin compared to those who had received ivermection only, while there were no differences in palpable nodule prevalence and burden and in the prevalence of nodules with motile worms, as assessed by ultrasonography. These results demonstrate the effectiveness of doxycycline delivered with a community directed strategy even when evaluated four years after distribution in an area of ongoing transmission, encouraging the introduction of anti-wolbachial therapy in existing control programmes where needed, and providing important data to address the practical aspects of the implementation of macrofilaricidal treatments.

2.1 INTRODUCTION

2.1.1 Rationale

Current control strategies for the control of onchocerciasis rely on Mass Drug Administration (MDA) of ivermectin (IVM) once or twice a year [171]. However, IVM targets only the microfilarial stage of the parasite, effectively reducing dermal microfilarial loads, but has only marginal effects on adult worms [167, 169]. Therefore sustained delivery is required to interrupt transmission: 15-17 years in areas of moderate transmission and up to 20-25 years in foci with very high endemicity [171, 194]. Moreover, IVM can not be safely used in areas where *Loa loa* is co-endemic, because of the severe and occasionally fatal adverse reactions following IVM intake in people with high *L. loa* microfilarial loads [163, 187], raising a barrier to the implementation of current control programmes in these areas.

An alternative treatment is provided by doxycycline, which targets the endosymbiont *Wolbachia*. Clinical trials have demonstrated that doxycyline is highly effective at blocking embryogenesis of adult worms for years, possibly causing permanent sterility, and a six week course of treatment leads to a 60-70% macrofilaricidal rate [35, 37, 186, 195]. Therefore, doxycycline treatment provides an important alternative to IVM in areas of *L. loa* co-endemicity (*L. loa* is naturally devoid of *Wolbachia*, therefore *L. loa* microfilariae will not be affected by doxycycline, therefore will not cause cerebral vasculopathy consequent to their death), in areas where sub-optimal efficacy of IVM has been reported [170, 175, 176], and as a test-and-treat approach in programme endgame situations to achieve elimination, as planned for the North-East focus of onchocerciasis in Venezuela as part of the Onchocercias Elimination Programme for the Americas (OEPA).

Because of its embryostatic and macrofilaricidal effects, it is expected that community-wide distribution of doxycycline will lead to sustained reductions of microfilaridermia, a valuable help to existing programmes [173]. However, the length of the required treatment is perceived as a barrier to the widespread use of doxycycline MDA, due to potential logistical problems and risk of poor compliance.

In 2007 and 2008, Prof. Wanji and his team carried out a feasibility trial of doxycycline MDA in two health districts of the Littoral Region of Cameroon, co-endemic for *L. loa* and therefore naïve to IVM-based onchocerciasis control

[188]. By implementing the distribution of doxycycline with a community-directed strategy in 17,519 eligible people, they achieved a therapeutic coverage of 73.8% per eligible population. More importantly, of the 12,936 people who started the treatment, 97.5% completed the six week course, as assessed by directly observed treatment. These results demonstrated that doxycycline delivery with a community-directed strategy is accepted and feasible in restricted communities of this size [188]. After the distribution of doxycycline MDA, communities received two annual rounds of IVM, as part of the onchocerciasis national control programme that was extended to these areas. During doxycycline treatment, no Severe Adverse Events (SAEs) were reported, while two SAEs were reported during IVM treatment (Prof Wanji personal communication), highlighting the safety of doxycycline.

The evaluation trial presented here aimed to assess the long term effectiveness of this community-directed treatment with doxycycline and to validate its reported compliance rate four years after implementation in the presence of ongoing transmission.

2.1.2 Study design

Overall design

The study was approved by the Research Ethics Committee of the Liverpool School of Tropical Medicine (UK) and the Institutional Review Board of the Medical Research Station of Kumba (Cameroon), and registered in the ISRCTN register (ISRCTN95189962).

The study was designed as a single-blind evaluation trial to be carried out in four health areas of the two health districts (Melong and Mbanga) of the Littoral Region of Cameroon where the feasibility trial of doxycycline MDA was implemented in 2007 and 2008. The study was conducted in May 2011 before the third annual IVM MDA distribution in the area, to assess microfilaridermia prevalence and load at their maximum levels. The study timeframe is shown in figure 2.1.

2007	2008	2009	2010	2011	
				May	June
Doxycycline MDA	**Doxycycline MDA***	**Ivermectin MDA**	**Ivermectin MDA**	**Evaluation study**	Ivermectin MDA
	*For those absent in 2007				

Figure 2-1. Timeframe of the evaluation study.

The study involved the recruitment of participants of both sexes aged 19 years or above who had received and completed the six week course of doxycycline (as assessed using trial registries) followed by one or two rounds of IVM MDA, and participants who only received one or two rounds of IVM MDA.

Exclusion criteria were intake of IVM since June 2010, intake of antibiotics with known anti-wolbachial activity (tetracyclines or rifamycins) for longer than 2 weeks since June 2007, and behavioural, cognitive or psychiatric conditions that in the opinion of the trial clinician may have affected the ability of the subject to understand and cooperate with the study.

Agreement was obtained with local authorities and the community leaders and elders. Participants were informed in detail about the study protocol by the research team in their local language according to Good Clinical Practice using the approved Participant Information Sheet. Participants gave their written informed consent using the approved Informed Consent Form and were enrolled in the study upon meeting the eligibility criteria as assessed by questionnaire. Data were collected using an approved Case Report Form.

Skin biopsies (skin snips) were taken from both iliac crests to assess presence and load of microfilariae (mf) in the skin (microfilaridermia), and body palpation was carried out to assess prevalence and burden of onchocercomas. Participants with at least one palpable nodule were assessed by ultrasound (USG) for the presence of adult worm movements as an index of worm viability.

Objectives and endpoints

Primary objectives were to evaluate the effectiveness of doxycycline MDA followed by IVM MDA and to validate the compliance rate of the implementation trial. The secondary objective was to evaluate whether USG of palpable nodules to detect parasite motility could be used to monitor and evaluate macrofilaricidal activity after doxycycline MDA.

The primary endpoint was prevalence of microfilaridermia as assessed by skin snip. Secondary endpoints were microflaridermia loads (mf/mg of skin),

prevalence and burden of palpable onchocercomas, and prevalence of motile adult worms per patient as assessed by USG.

Sample size calculations and subjects' enrolment

The sample size for the primary outcome was calculated in Win Episcope 2.0 and had 90% power to detect the predicted microfilaridermia prevalence difference between the two groups with a two-tailed $p < 0.005$. A 30% drop-out was accounted for.

The pre-treatment microfilaridermia prevalence was estimated on the basis of the reported Rapid Evidence Assessment (REA) onchocerciasis prevalence in each health area, by multiplying these figures by 1.83 [188, 196]. A drop of 66.7% microfilaridermia prevalence was accounted for in people who had received doxycycline [186]. A drop of 43.4% was accounted for two subsequent rounds of IVM MDA [170]. Details of sample size calculations are shown in table 2.1.

Health District	Health Area	REA prevalence	Pre-treatment mf prevalence	Expected mf prevalence doxy+ivm group	Expected mf prevalence ivm only group	Sample size/ group + 30% drop out
Mbanga	Kotto	48.36%	88.48%	16.68%	50.09%	49
	Matouke	49.72%	90.98%	17.15%	51.49%	47
	Mombo	41.50%	75.94%	14.31%	42.98%	62
Melong	Essekou	22.73%	41.59%	7.83%	23.54%	142

Table 2-1. Sample size calculations for the evaluation study.

Participant enrolment in each health area was planned to start in one randomly selected village in each area. In case the required sample size was not reached from one single village, a second village was to be randomly selected within the same health area. Participants of both groups were to be recruited in all selected villages to prevent confounding from village effect. This process would have been repeated across an increasing number of villages until the required sample size for both groups would have been achieved within each health area.

Team roster and funding

Institution	Name	Role
Liverpool School of Tropical Medicine, UK	Prof. Mark J Taylor	Principal Investigator. Conceived and designed the study and interpreted the results
	Dr. Francesca Tamarozzi	Study Clinician. Designed the study, participated in field activities (USG and data collection), analysed and interpreted the data
	Dr. Brian Faragher	Study Statistician. Participated in the study design and data analysis
Research Foundation for Tropical Diseases and Environment, Buea, Cameroon	Prof. Samuel Wanji	Lead Field Study Investigator. Participated in the study design, organized field activity, participated in the interpretation of data
	Dr. Peter A Enyong Dr. Nicholas Tendongfor Mr. Mathias Esum	Field Study Team. Participated in field activity organization and data collection

Table 2-2. Evaluation study team roster.

The study was funded as part of the A·WOL consortium through a grant awarded to the Liverpool School of Tropical Medicine by the Bill and Melinda Gates Foundation.

2.2 METHODS

2.2.1 Data collection

All investigators involved in the collection of biological data were blind with regard to the treatment group. Each participant received a personal ID code, and the team was divided so that different members were involved in the collection of anamnestic data and in the collection/readout of specimens/tests. The data were de-blinded only after all results were collected.

2.2.2 Evaluation of microfilaridermia and onchocercomas

For the assessment of microfilaridermia prevalence and load, two skin snips were taken from the iliac crests using a sterile corneoscleral punch. Skin snips were incubated in 100 µl normal saline solution in microtitre plates overnight (10-18 hours). The number of mf were counted and the skin wet weight measured to determine the number of mf per mg of skin. Body palpation was carried out by an expert investigator (Prof S Wanji or Dr Peter A Enjong). The

presence, number and location of nodules were recorded on the Case Report Form.

2.2.3 Ultrasonography of onchocercomas

USG examination was performed as described [186, 197] using an M-Turbo® portable ultrasound machine supporting an HFL30x 13-6 MHz linear transducer (SonoSite). Patients were examined in a position that would minimize artifacts due to body movements. Each nodule was measured in the two major dimensions and worm motility monitored for at least 5 minutes. Onchocercomas were identified by a capsule with regular surface, typically lateral shadows with or without posterior artifacts, and a disomogeneous content comprising hypo-, iso-, and hyperechogenic areas. Differential diagnosis was mainly with lipomas (homogeneous, hyperechogenic, absence of lateral shadowing) and lymphnodes (anatomical location, hypoechogenic with a hyperechogenic hilus) [198].

2.2.4 Statistical analysis

Differences in prevalences between groups were assessed by Fisher's Exact test. Differences in continuous variables were analyzed by Mann-Whitney *U* test. A *p*-value ≤ 0.05 was considered significant. All analysis was carried out in SPSS Statistics 17.0 (IBM).
Differences in mf loads and mf prevalence between treatment groups were analysed in: i) the whole sample, ii) only patients with palpable nodules, and iii) only microfilaridermia positive patients. Difference in burden and prevence of palpable nodules between treatment groups was assessed in the whole sample. Finally, the subgroup of patients with palpable nodules that was examined by USG was investigated for differences in nodule structure and adult worm motility between treatment groups.

2.3 RESULTS

2.3.1 Study population

Due to logistical problems in the field, the study was carried out only in the three health areas of Mbanga health district. Also, it was not possible to carry

out the study per separated health areas, therefore the entire Mbanga health district was considered a whole investigated area. A sample size of 500 subjects had 90% power to detect a difference in microfilaridermia prevalence of 16.0% (expected prevalence in the doxycycline plus IVM group when the whole health district was considered) versus 26.8%. This was considered the smallest difference between groups relevant for effectiveness assessment, where the expected prevalence of microfilaridermia after two rounds of IVM MDA was 58.6% when the whole health district was considered. For USG examination, the sample size required to detect the expected difference [186] in the prevalence of subjects having USG detectable motile adult worms within onchocercomas between the two groups would have been 25 per group (two-tailed, 95% confidence, 90% power), which was within the calculated sample size considering the REA prevalence of palpable nodules.

Five-hundred and seven people (260 males and 247 females) were recruited from 17 villages of the three health areas investigated. Of those, 375 had completed the six week course of doxycycline MDA followed by one or two annual rounds of IVM MDA and 132 had received one or two rounds of IVM MDA only. Seventy one people (47 males and 24 females) were investigated by USG. Of those, 50 had completed the six week course of doxycycline MDA followed by one or two rounds of IVM MDA, and 21 received only one or two rounds of IVM MDA. The study population is described in table 2.3.

Treatment group	Males (%)	Females (%)	Total	Mean age (range)	Ivermectin one round (%)
General study population					
Doxycycline + ivermectin	210 (56.0%)	165 (44.0%)	375	43.16 (19-81)	53.1%
Ivermectin only	50 (37.9%)	82 (62.1%)	132	36.86 (19-71)	59.8%
Total	260	247	507		$p = 0.858$
Population examined by USG					
Doxycycline + ivermectin	35 (70.0%)	15 (30.0%)	50	45.60 (19-73)	42.0%
Ivermectin only	12 (57.1%)	9 (42.9%)	21	38.95 (23-69)	76.2%
Total	47	24	71		$p = 0.010$

Table 2-3. Characteristics of the population investigated in the evaluation study.

2.3.2 Microfilaridermia prevalence and load

Microfilaridermia prevalence was found to be much lower than expected (27.0% compared to expected 58.6% in the IVM only group), possibly reflecting the spontaneous fluctuations in this parameter as previously reported [186], or the lower than expected palpable nodule prevalence (20.0% in the IVM only group compared to 46.5% reported REA prevalence). Microfilaridermia prevalence was significantly lower in people who had received doxycycline followed by IVM compared to those who had received IVM alone (p = 0.014; figure 2.2 A), with 83% (±4%) amicrofilaridermic people in the first group compared to 73% (±8%) in the second. A statistically significant difference between groups was also found in mf burden (p = 0.012; figure 2.2 B), with people who had received doxycycline harbouring significantly lower mf/mg of skin. This difference was due to a higher number of mf-negative subjects in the doxycycline plus IVM group, as significance was lost when analysing only mf-positive subjects (p = 0.483), although a trend could still be observed (ref to legend of figure 2.2 B). When only subjects with palpable nodules were considered, these results were replicated, with those who had received doxycycline having significantly lower microfilaridermia prevalence (p = 0.032; figure 2.2 C). This group also had lower microfilarial burden, although not reaching statistical significance (p = 0.091; figure 2.2 D). Results are detailed in table 2.4.

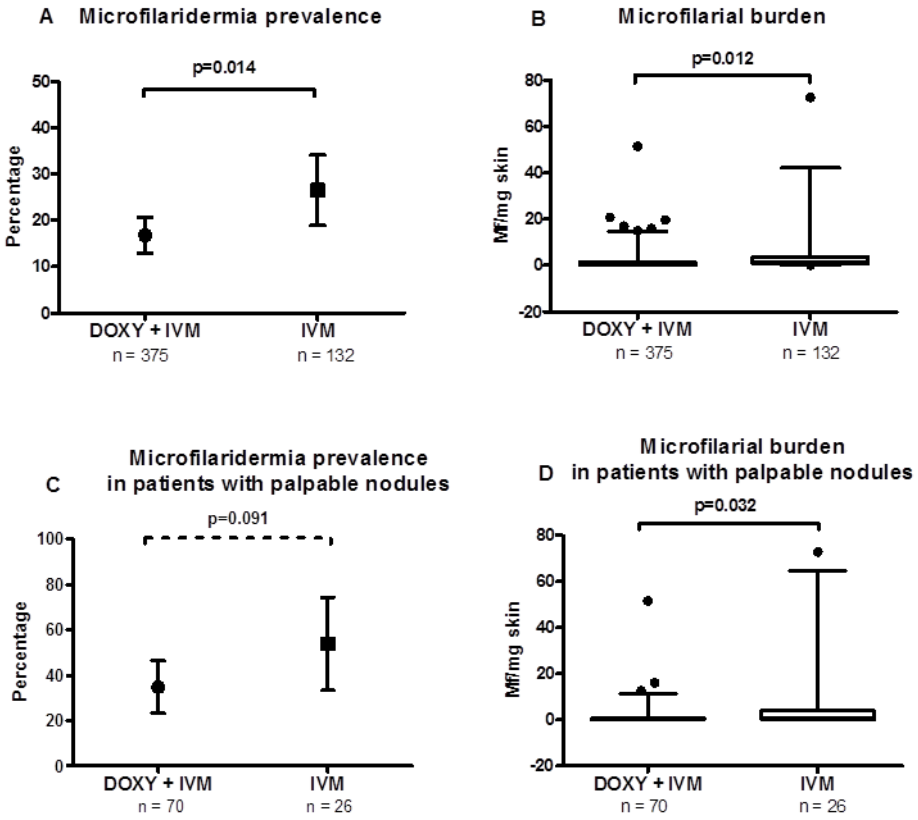

Figure 2-2. Differences in mf prevalence and load between groups. Investigated groups were people who had received doxycycline MDA followed by IVM MDA (DOXY+IVM) and those who had received only IVM MDA (IVM) in the whole population (A and B) and among the subroup with palpable nodules (C and D). **A** and **C**, Difference in mf prevalence between groups; the graphs show mean and 95% CI. **B** and **D**, Difference in mf loads between groups; the graphs show median and 5-95 percentiles. In B, data from mf^+ people are plotted for graphical purposes, with *p*-value referring to the whole population studied. The mf burden of mf^+ patients that had received doxycycline followed by IVM was also lower compared to those who had received only ivermectin, although not statistically significant ($p = 0.483$).

	Doxycycline +ivermectin	**Ivermectin only**	**p-value**
Mf prevalence (95% CI)	17% (13%-21%)	27% (19%-35%)	**0.014**
Mf/mg of skin Median (75-95 centiles)*	0 (0-3.50)	0 (0.37-5.37)	**0.012**
Mf prevalence in nodule$^+$ subjects (95% CI)	34% (23%-46%)	54% (33%-74%)	0.091
Mf/mg of skin in nodule$^+$ subjects Median (75-95 centiles)*	0 (0.71-11.33)	0 (4.00-64.53)	**0.032**
Mf/mg of skin in mf^+ Median (5-95 centiles)	1.5 (0.50-19.4)	2.0 (0.50-60.2)	0.483

Table 2-4. Parasitological results and statistical differences between groups four years after doxycycline MDA implementation. *75-95 percentiles are indicated because median value equals zero.

No difference was found in the number of rounds of IVM MDA between groups in the whole population investigated (p = 0.858). On the contrary, significantly less people received two rounds of IVM in the IVM-only group (27%) compared to the doxycycline plus IVM group (50%) when only subjects with at least one palpable nodule were considered (p = 0.041). We therefore analysed the differences in microfilaridermia prevalence and load in this nodule-positive sub-population analysing separately those who received only one versus two IVM rounds. We found statistically lower microfilaridermia prevalence and load (p = 0.022 and p = 0.006 respectively) in those treated with doxycycline in the group that received only one round of IVM MDA, while no difference was found in the group that received two rounds of IVM MDA. The details of this sub-group analysis are described in table 2.5.

NODULE+ SUBPOPULATION	**Doxycycline +ivermectin**	**Ivermectin only**	**p-value**
1 round ivermectin MDA			
N	36	19	
Mf prevalence (95% CI)	29% (13%-46%)	63% (39%-87%)	**0.022**
Mf/mg of skin Median (75-95 centiles)*	0 (0.35-22.37)	1.75 (9.04-44.20)	**0.006**
2 rounds ivermectin MDA			
N	35	7	
Mf prevalence (95% CI)	40% (23%-57%)	29% (17%-74%)	0.690
Mf/mg of skin Median (75-95 centiles)*	0 (1.00-11.20)	0 (0.00-0.33)	0.529

Table 2-5. Parasitological results and statistical differences between groups in the nodule-positive sub-population analysed separately by rounds of ivermectin MDA. *75-95 percentiles are indicated as median value equals zero.

2.3.3 Palpable nodule prevalence and load

Nodule prevalence in the IVM only group (expected to reflect the pre-treatment REA prevalence) was less than half of the expected value (20% versus expected 46.5%).

No difference in palpable nodule prevalence (p = 0.776; figure 2.3 A) and burden (p = 0.720; figure 2.3 B) was found between groups.

Results are detailed in table 2.6.

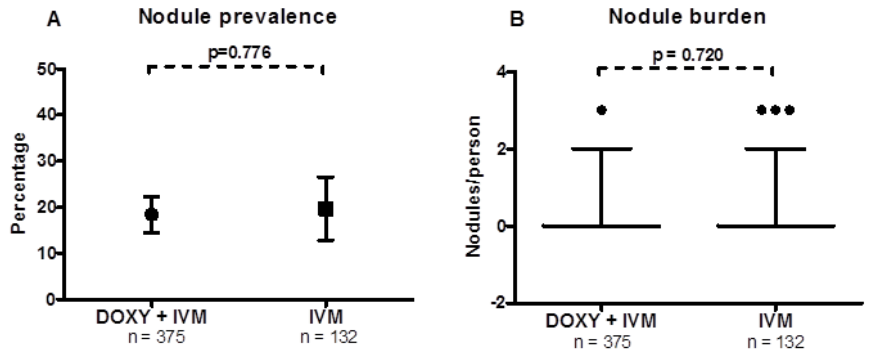

Figure 2-3. Differences in palpable nodule prevalence and load between groups. Investigated groups were people who had received doxycycline MDA followed by IVM MDA (DOXY+IVM) and those who had received only IVM MDA (IVM). **A**, Difference in palpable nodule prevalence between groups; the graphs show mean and 95% CI. **B**, Difference in palpable nodule load between groups; the graphs show median and 5-95 percentiles.

	Doxycycline +ivermectin	**Ivermectin only**	**p-value**
Nodule prevalence (95% CI)	19% (15%-23%)	20% (13%-27%)	0.776
Nodules/person Median (75-95 centiles)*	0 (0-2)	0 (0-2)	0.720

Table 2-6. Differences in nodule prevalence and burden between groups four years after doxycycline MDA treatment. *75-95 percentiles are indicated as median value equals zero.

2.3.4 Results of ultrasonography of palpable nodules

Palpable nodules of 71 patients were investigated by USG. Of these patients, 50 had received doxycycline MDA followed by one or two rounds of IVM MDA, and 21 had received IVM MDA only. In this sub-group, significantly less people received two rounds of IVM in the IVM-only group (24%) compared to the doxycycline plus IVM group (58%) (p = 0.010). On average, every patient had one palpable nodule, with no difference in nodule burden between groups (p = 0.710). Nodules in both groups were particularly small, very few exceeding 1 cm in the maximum diameter. No differences were found in the nodule characteristics (size and calcification) between groups (p = 0.310 and p = 0.408 respectively). Most nodules were ultrasonographically quite homogeneous, iso-hyperechogenic, with a particularly compact appearance (Figure 2.4).

A total of three nodules with motile worms were recorded. Only one nodule per patient had motile worms. Of those, two were found in the doxycycline plus IVM group and one in the IVM only group, a difference that was not statistically

significant (p = 0.884). As expected, motile worms were detectable in nodules with cystic areas, where parasites appear as an acoustic enhancement reflected from tissue moving in hypoechoic areas of the nodule. The results of USG investigation are detailed in table 2.7.

	Doxycycline +ivermectin	**Ivermectin only**	**p-value**
Nodules/person Median (5-95 centiles)	1 (1-4)	1 (1-7.5)	0.710
Prevalence of subjects with nodules containing motile worms	2/50	1/21	0.884
Totally calcified nodules/person Median (75-95 centiles)*	0 (1.00-2.45)	0 (1.00-7.40)	0.408
Nodule size (mean of two major diameters) Median (5-95 centiles)	0.74 cm (0.41-1.09)	0.79 cm (0.40-1.02)	0.310

Table 2-7. Nodule characteristics and differences between groups as assessed by USG. *75-95 percentiles are indicated as median value equals zero.

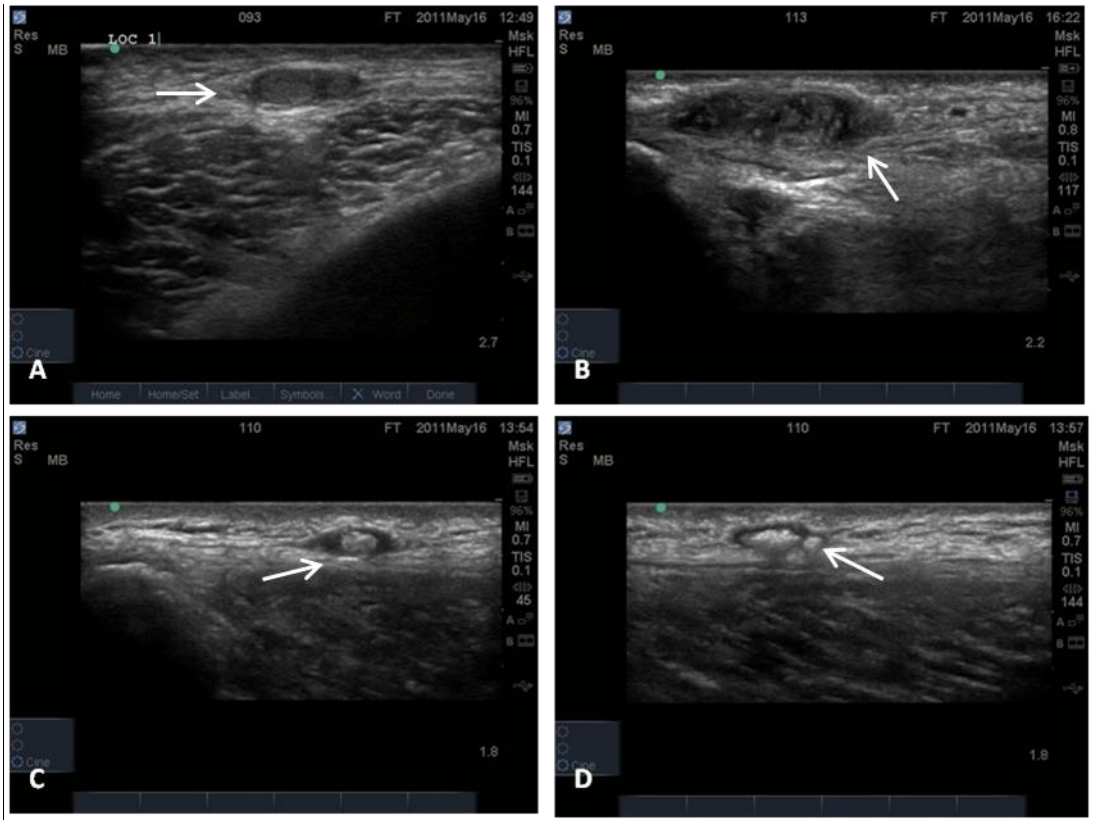

Figure 2-4. Example images of the USG appearance of onchocercomas (white arrows). **A**, Oval nodule with isoechogenic homogeneous appearance. Diameters 0.99 x 0.38 cm, max depth 2.7 cm. **B**, Oval nodule with disomogeneous echogenic content. Anechogenic areas represent cystic areas allowing the visualization of worm movements where motile worms are present. Diameters 4.30 x 9.11 cm, max depth 2.2 cm. **C**, Nodule with calcified core and hypoechogenic periphery. Diameters 1.63 x 0.52 cm, max depth 1.8 cm. **D**, Completely calcified nodule with two small completely calcified satellite nodules; posterior acoustic shadows is visible. Max depth 1.8 cm.

2.4 DISCUSSION

The use of anti-wolbachial macrofilaricidal drugs would be highly beneficial to existing onchocerciasis control programmes in areas of *L. loa* co-endemicity, in areas where sub-optimal efficacy of IVM has been reported, in programme end-game situations to mop-up residual cases, and to shorten the time required to reach the goal of elimination.

Two major barriers to the use of doxycycline as an MDA tool are the contraindications of this drug (not approved in children under 9 years of age and in pregnant and breastfeeding women), and the length of the required treatment, perceived as a potential problem in terms of logistics and compliance. However, concerning this last point, Prof Wanji and co-workers demonstrated that the implementation of a six week course of doxycycline is feasible and accepted by communities when distributed with a community-directed approach [188]. Moreover, this trial highlighted the safety of doxycycline in *L. loa* co-endemic areas, as none of the 12,936 people treated reported any SAEs, in contrast to the two SAEs reported when IVM was distributed.

The study presented here evaluated the long term impact of this community-directed delivery of doxycycline. Our findings demonstrate the effectiveness of this intervention. More importantly, these results were obtained four years after implementation in an area of ongoing transmission and naïve to previous control measures, showing that doxycycline effects are long-lasting. In the whole population, the prevalence of microfilaridermia was 10% lower (p = 0.014) in people who had received doxycycline followed by IVM MDA compared to those who had received only IVM MDA. This result is consistent with the long-lasting sterilization of adult worms and eventually death caused by the six week course of doxycycline, and the production of mf by newly acquired parasites that would have reached adulthood in the time elapsed between doxycycline distribution and evaluation. The absence of difference in the number of rounds of IVM MDA received in the two groups excludes this result being dependent upon this variable.

When analysing the patient subgroup with palpable nodules, significantly fewer people in the IVM only group were found to have received 2 rounds of IVM MDA compared to those in the doxycycline plus IVM group. Therefore, to exclude this being responsible for the differences in mf burden between treatment groups, patients were divided by number of IVM rounds and the analysis carried out

separately. Significantly lower microfilaridermia burden and load were found in those who received doxycycline followed by 1 round of IVM MDA compared to those who received 1 round of IVM MDA only. No statistical difference was found when the sub-groups that received 2 rounds of IVM alone or after doxycycline were analysed, probably due to the small sample size.

The lack of difference in nodule prevalence and burden between groups was not surprising. The rate of nodule re-absorption after the death of adult worms following doxycycline treatment is not known, and palpable nodules can persist as infection scars. Secondly, new incoming parasites are known to colonise existing nodules [185], possibly "rescuing" old nodules from re-absorption.

USG investigation of palpable nodules could not assess a statistical difference in prevalence of motile worms and nodule characteristics between groups, contrary to what reported previously [197]. The statistical difference in the number of rounds of IVM MDA received between the two investigated groups is unlikely to have influenced these results because at the regime used IVM does not affect worm viability [167]. The most likely explanation for this finding resides in the structure of the nodules in this geographical area. Worm motility can be observed in nodules with cystic structure, where loose nodule tissue allows worms to move. In the area of Cameroon where this study was conducted nodules were very small and compact, a structure that greatly loweres the chances of observing worm movements by USG. These findings do not exclude the use of USG to assess macrofilaricidal activity of drugs, but point out that its performance may vary greatly between geographical areas.

To conclude, these results demonstrated that a six week course of doxycycline distributed with a community-direceted approach is not only feasible and safe, but also effective in the long term at reducing microfilaridermia prevalence and burden. This should encourage the introduction of anti-wolbachial drugs in existing onchocerciasis control programmes and their extension in difficult areas such as those co-endemic for *L. loa*. Moreover, as doxycycline is the first macrofilaricidal drug available for onchocerciasis, knowledge of its long-term impact is of particular importance to inform practical aspects and modelling of the implementation of macrofilaricides.

Chapter 3. CYTOKINE RESPONSES IN GHANAIAN MICROFILARIDERMIC ONCHOCERCIASIS PATIENTS AND CONTROLS

ABSTRACT

The cytokine profile in response to parasite stimuli has been linked to infection status in filariasis, most studies focusing on Th1 and Th2 responses. On the contrary, pro-inflammatory mediators linked to the innate immune response and possibly elicited by *Wolbachia* have received little attention. Also, a Type-17 immune response could be elicited in the environment promoted by the *Wolbachia*-filarial nematode combination, but little data exist on its presence and role in filariasis, with no studies carried out in onchocerciasis. In the work presented here, Th1 (IFNγ), Th2 (IL-5, IL-13), Th17 (IL-17A) as well as pro- and anti-inflammatory cytokines (TNFα, IL-6 and IL-10) in response to filarial extracts have been investigated in PBMC from Ghanaian patients with onchocerciasis and control subjects. The results showed a predominant Th2 response in patients compared to controls, while Th1 and Th17 responses where minimal in both groups. Interestingly, it was found a different impact of parasite loads on Th2 compared to pro- and anti-inflammatory responses. In particular, patients with higher mf loads showed depressed Th2 responses but heightened spontaneous production of both pro- and anti-inflammatory cytokines. These results indicate a complex and differentially modulated impact of parasite loads on systemic immune responses, and suggest that filarial parasites are able to promote both pro- and anti-inflammatory responses at the same time. With the use of filarial extracts containing or depleted from *Wolbachia,* it was explored for the first time in human studies of this type the relative contribution of filarial nematodes and bacterial endosymbionts in shaping the cytokine profile in ochocerciasis. This preliminary study did not show any difference in the ability of the two filarial extracts to stimulate PBMC, raising the possibility that *Wolbachia* may not elicit a differential PBMC response. However, further optimization of experimental conditions will be needed in future studies to confirm these results. These should also take advantage of the availability of adulticidal therapy with doxycycline to investigate the relative role of *Wolbachia* and of different parasite life stages in shaping the immune profile in onchocercal infection and disease.

3.1 INTRODUCTION

3.1.1 Cytokine profile in onchocerciasis

The modulation of immune responses by filarial parasites is considered pivotal in determining the course of infection and disease. A wealth of studies investigating the role of immune responses in parasite survival or elimination has been stimulated by i) the existence in both onchocerciasis and lymphatic filariasis (LF) endemic areas of a small proportion of "putatively immune/endemic normal (PI/EN)" individuals (i.e. people living in endemic areas who do not acquire detectable infection nor pathology despite exposure to infective vector bites); ii) field observations reporting a plateau in filarial burdens with age (interpreted as evidence of acquired immunity to newly transmitted parasites, i.e. concomitant immunity) [14, 199-203]; and iii) the availability of animal models where protective immunity to parasites can be observed spontaneously or induced by vaccination (reviewed in [204-206]).

Cytokine profile and the spectrum of infection status

Although studies have reported sometimes inconsistent results, in both onchocerciasis and LF the spectrum of infection status has been generally associated with different patterns of filarial-specific immune responses in PBMC stimulation experiments. Namely, a mixed Th1 (IFNγ, IL-2)/Th2 (IL-4, IL-5, IL-13) response with increased cell proliferation in PI/EN and post-patent subjects (i.e. with evidence of previous but not present infection) and suppressed responses in infected subjects, which show absent or very low Th1 with a predominance of Th2 and IL-10 responses [54, 119, 121, 207-213].

The presence of a strong IFNγ response in PI/EN and subjects with signs of past but not present infection has been interpreted as an association between a Th1 response and immunity to infection. This seems a consistent finding in LF, although only few studies characterized the spectrum of Th1 and Th2 profiles in all categories of infection (i.e. classifying the infection status not only by presence of mf but also by presence of circulating filarial antigens) [207, 209]. In onchocerciasis, however, most studies stringently classifying PI/EN found equal levels of IFNγ but higher levels of IL-5 and proliferation expressed by PBMC in response to adult filarial extracts from PI/EN and microfilaridermic patients, [119, 213, 214].

IL-10 and TGFβ have been linked to immunosuppression and thus to protection of the parasite in infected subjects. These cytokines have been shown *in vitro* to be able to suppress proliferative responses of PBMC stimulated with filarial extracts, as their neutralization restored proliferation in infected subjects [119, 120, 122]. After microfilaricidal therapy and after (presumed) adult death, PBMC production of IL-10 in response to filarial extracts was reported to decrease while levels of IL-5 and IFNγ increased [54, 56]. In accordance with these data, adult female parasites have been found to be the main inducers of an IL-10 dependent environment favourable for the survival of their offspring in the *L. sigmodontis* mouse model [100]. In human studies increased levels of IL-10 and TGFβ have been found in infected (mf positive) patients compared to PI/EN in both LF [122, 211, 215] and onchocerciasis [119, 210, 216]. Although no clear-cut association between infection status and IL-10 levels in response to filarial extracts was observed when the full range of infection conditions was investigated in LF [208, 209], nevertheless several studies in both filarial infections reported increased levels of the spontaneous production of IL-10 by PBMC from infected compared to PI/EN, whereas upon stimulation with filarial extracts levels of IL-10 from infected subjects were reported to further increase compared to PI/EN or be equivalent in the two groups [119, 210, 215-218].
Taken together, this complex picture argues for the balance between immunoreactivity and immunosuppression rather than a shift between the two classical T helper responses to reflect the infection status in filarial infections [53].

Several considerations, however, need to be taken into account when evaluating the immune profile in relation to the infection status. First, in onchocerciasis standard diagnostic tests (nodule palpation and skin snip) lack sensitivity. Therefore it is difficult to assess whether a negative subject is either really not infected, has a non-active infection (e.g. palpable nodule remnants without active infection), or has a cryptic active infection, i.e. with no palpable nodules and/or mf levels below the sensitivity of the diagnostic test.
This issue has been overcome in bancroftian filariasis by the advent of tests detecting adult circulating antigens. Their introduction revolutionized the traditional classification of infection status in LF by showing that many EN or patients with signs of past but not present infections have in fact cryptic infections [219-221]. This appears to be the case also in onchocerciasis, as a high proportion of patients classified as infection-free by nodule palpation and skin snips has been reported to be infected when assessed by more sensitive diagnostic tests, including the Mazzotti reaction after DEC, *Onchocerca* specific

PCR on skin samples or experimental urine antigen test [64, 222]. Moreover, in the absence of long-term follow-up of PI/EN, it is difficult to assess whether these subjects would indeed remain free of infection, although infection status has been reported to be highly stable over time, at least in LF [108, 211]. No consensus exists on the requirements for classifying a subject as PI/EN in onchocerciasis and more or less stringent methods have been used in different studies comparing the immune response of PI/EN and infected patients.

Second, in natural hosts like humans, different parasite life stages and sexes coexist at the same time. The vast majority of studies used mixed sexes adult worm extract in PBMC stimulations. Therefore, stage-specific (and body compartment-specific) responses more linked to protection may have been overlooked. A few studies in humans have used stage-specific filarial extracts, but with contrasting results. Turaga *et al* [222] reported the induction of IFNγ, IL-5 and GM-CSF in response to L3 and adult female but not mf *O. volvulus* extracts, with higher levels of IL-5 induced in PBMC from PI/EN compared to microfilaridermic patients. Moreover, PI/EN could be divided in two subgroups, one expressing only IL-5, and one expressing a mixed Th1/Th2 cytokine profile, suggesting that more than one mechanism may account for protection against incoming *O.volvulus* larvae in humans, or the heterogeneity of the infection status in this group [222]. A mixed Th1 (IFNγ, TNFα)/Th2 (IL-4, IL-5, IL-13) response to L3 and adult worm extracts in EN has also been reported in LF by Babu and co-workers [121]. In this study, EN had higher levels of both Th1 and Th2 cytokines than infected patients in response to L3, while in response to extracts from adult worms only EN produced Th1 cytokines, with levels of Th2 cytokines being equal between groups. In support of the view that a strong Th1 (IFNγ, proliferation) and Th2 (IL-5) response may account for concomitant immunity toward incoming infective larvae, the work of MacDonald *et al* in onchocerciasis [14] found a positive although weak correlation between levels of these cytokines in response to L3 extract and increasing age. Responses to adult female and mf extracts instead decreased with age. On the contrary, no difference was found in IL-5 and IFNγ responses to L3 extract in persons recently (< 10 years) or chronically infected with *O. volvulus* by Cooper and co-workers [120], and no difference in proliferation and Th1/Th2 cytokines to L3 extracts between EN and patients infected with bancroftian filariasis was reported by Steel *et al* [108]. Finally, although filarial extracts containing adult female worms have been consistently found to drive IL-10 in human PBMC cultures, mixed results have been reported for the other stage-specific (L3 and mf) extracts [108, 121, 215].

A number of mouse models have been used to study protective immunity toward different parasite stages, although no mouse strain is fully permissive to filarial parasites used to model human infections [61]. Different parasite species (with different behaviour within the host's body), different infective doses, route of infection, and hosts with different genetic backgrounds and levels of susceptibility have been used. These differences may at least partially account for the discrepancies found between studies. The synopsis of their results, however, argues for a Th2 immune response capable of containing larval (L3 and mf) stages, while both Th1 and Th2 seem necessary to kill adult stages (reviewed in [97, 205]).

Studies investigating protective responses to L3 establishment and vaccination show that vaccination of mice with irradiated *Onchocerca* L3 induce protection to challenge L3 infection with an IL-4 and IL-5 dependent but IFNγ independent mechanism, as assessed using IL-4KO, IFNγKO, or mice treated with anti-IL-5 or anti-IL-4 antibodies, likely implicating eosinophils as the effector cells [223, 224]. The dependency of L3 killing for IL-4 and IL-5 but not IFNγ has also been consistently found in the *L. sigmodontis* model in IL-4KO, IL-5KO, IFNγKO mice, or where IL-5 was neutralized by antibodies [225-228]. The absence of detrimental effects to infective larvae seen when transgenic mice hyperexpressing IL-5 were used [229] are in accordance with the absence of IL-5 requirement for *L. sigmodontis* L3 killing after primary infection [225, 226], and could be explained by the delay in eosinophil recruitment in the skin upon primary infection, regardless of the genetic backround [225]. Less consistent results have been found in the mouse model of *Brugia spp* infection, where the presence of IL-4 was found either to be protective [230, 231] or not having an impact on worm survival [232, 233], and where also IFNγ was shown to have a role in worm containment, as assessed by increased worm recoveries in IFNγKO mice [230]. In this model, however, L3 are implanted in the peritoneal cavity, therefore the different cellular environment encountered by larvae compared to what would have been a natural migration may be at least in part responsible for these contrasting results.

Both Th1 and Th2 responses appear to be able synergistically to contain adult worms, as IL-5/IFNγ double KO mice were reported to have higher adult worm burdens than each of the single KO strain [226]. Killing of adult worms by IL-5 and IFNγ dependent mechanisms has been consistently found in the *L. sigmodontis* mouse model using IL-5KO and IFNγKO mice [226-228], IL-5 transgenic mice [229], and in mice treated with anti-IL-5 antibodies [225, 234]. Moreover, the production of IFNγ, IL-4 and IL-5 in the pleural cavity of *L.*

sigmodontis infected mice has been found higher in resistant C57BL/6 compared to more susceptible BALB/c mice [235]. On the contrary, contrasting results have been found for the other Th2 signature cytokine IL-4, which has been reported to be required for adult worm containment in one study [125] but dispensable in others [228, 233]. Actually, IL-4 elicited by adult females has been suggested to be required for the induction of a worm-protective immunosuppressed environment [104].

From what I have described so far, an apparent discrepancy exists between human and animal studies on the role of Th1 responses in mediating protection toward incoming infections. Thus, a Th2 response mediating protection in mouse vaccination models and a Th1 or more probably mixed Th1/Th2 response being associated with an infection-free status in humans. It is, of course, possible that these contrasting results reflect fundamental differences between mice and human immune responses [236]. However, alternative explanations can be attempted to reconcile the two scenarios. First, the need for a mixed Th1/Th2 response has been found to mediate protection once L3 have reached adulthood in animal models. At present, no human study can dissect whether in uninfected but exposed persons incoming parasites are killed at the L3 stage or later in their development. Second, vaccination studies using purified antigens or fractions of parasite extracts or recombinant *O. volvulus* antigens have reported Th1-, Th2-, or mixed Th1/Th2-mediated protection, showing that a Th1 response may be important at least in some conditions also in animal models [59, 237, 238].
A completely different but not necessarily mutually exclusive hypothesis is the one proposed by Ravindran [61] and also suggested by Rajan and co-workers [239], that a Th1 response, at least in the very early stages of parasite establishment, may actually assist growth and development of filarial larvae. This comes from the observation that: i) T cell proliferation and Th1 cytokines have been consistently found after inoculation of infective larvae in completely susceptible hosts [61, 99], but not in mice where, on the contrary, it appears that the Th1 response to incoming larvae is actively and readily suppressed (reviewed in [206]); ii) the development of *B. malayi* in SCID mice is possible only in the presence of NK cells, which produce Th1 cytokines [239, 240]; and iii) in mouse models using *Brugia spp* or the more permissive *L. sigmodontis*, parasite development is enhanced in conditions where the Th1 response is enhanced [226, 232, 241]. A similar dual role for the same immune response supporting both host protection and parasite development depending on the worm developmental stage has been suggested for IL-5 and eosinophils by the

work of Babayan *et al* [242]. This hypothesis is intriguing in the light of the fact that *Wolbachia* has been found to induce Th1 responses [47]. However, induction of IFNγ during pre-patent period has been reported also after infection with the *Wolbachia*-free *Loa loa* parasite in mandrils (ref [61]), therefore the role of *Wolbachia* in this regard can not be extrapolated with certainty.

Finally, it is at present not clear whether the association between heightened Th1 responses and infection-free status in human filariasis reflects the fact that these subjects are infection-free due to their ability to mount such Th1 responses, or whether the heightened Th1 response reflects the absence of live parasites and therefore of their immunosuppressive mechanisms.

Cytokine profile and disease manifestations

The correlation between immune profile and disease manifestations is less clear. Very few studies investigated the relationship between immune responses and pathogenesis of onchocercal skin [85, 86] and eye [49, 243] disease. In both cases, however, pathology has been linked to prevailing Th2 responses [86, 243]. This is consistent with results from animal models linking IL-5 dependent (but IL-4 independent) responses to mf containment [226, 228, 233, 244-246].

Human studies investigating dermatitis have classified patients into two polar groups. Patients with generalized onchocerciasis (GEO) represent the majority of infected subjects and present a wide spectrum of skin inflammatory manifestations, with generally high skin mf burdens; while patients with severe chronic dermatitis (Sowda) present severe localized skin pathology with generally very low mf and adult loads. Comparing these two groups, a strong Th2 response (IL-5, IL-13, T cell proliferation in PBMC stimulated with *O. volvulus* extract) has been considered responsible for low mf loads and higher severity of skin pathology [86, 247]. However, Sowda is relatively rare compared to GEO, it is geographically localised, and has been correlated with high levels of IgE, eosinophilia and eosinophil reactivity, delayed type hypersensitivity, specific genetic polymorphisms and autoimmune mechanisms ([80, 81, 85, 86, 95, 96, 248, 249] and reviewed in [52]). These characteristics do not lie on a continuum when the spectrum of skin disease severity in GEO is considered, suggesting the mechanisms behind the pathogenesis of GEO and Sowda being different, with the latter rather resulting from a hyperreactive Th2 immune response to mf and being perhaps analogous with Tropical Pulmonary Eosinophilia in LF. GEO are characterized by weak proliferative responses to filarial antigens, low levels of IFNγ (only heightened in acute dermatitis) and a

tendency to show increased levels of IL-5 and IL-13 with increasing severity of pathology and decreasing skin mf loads [86]. However, the correlation between low mf load and severity of pathology is not clear cut [49, 86, 138], and the assessment of mf loads by skin snip may not reflect the real mf burden of patients with severe pathology. These patients may have high burdens of damaged non-motile or dying mf, which may not actively leave the skin sampled in a skin snip, i.e. the real mf load may not be accurately demonstrated by this technique, as pointed out by Ali and colleagues [85]. As for LF, in GEO it is not clear whether increased immune responses in patients with severe pathology and low mf loads are cause or consequence of their status. Indeed, there is no evidence that the partial reversal of down-regulated immune responsiveness after microfilaricidal IVM treatment would predispose to increased pathology due to heightened responses against newly produced mf, although the residual immune suppression due to persistent presence of adult worms may account for that. It is therefore possible that skin pathology would result from the balance between inflammatory responses due to mf death, either spontaneous or immune-mediated, and parasite-mediated immune suppression. Both these pro- and anti-inflammatory conditions are proportional to the burden of adults and mf, in turn a reflection of the burden of newly acquired L3, resulting from intensity of exposure and immune responses [101]. In this context, the heightened IFNγ levels observed in acute dermatitis [86] and in response to mf in mouse models [233, 250-254] may reflect the acute release of mf antigens, possibly from *Wolbachia*, due to the simultaneous death of high mf numbers, rather than being its cause. Indeed Th1 responses are not associated with mf killing in animal models [245, 250]. Alternatively, the heightened Th2 responses observed in chronic skin pathology may reflect the skewing of the immune response toward this arm with chronic exposure to parasite antigens [255].

In studies concerning eye disease, sclerosing keratitis is believed to be caused by the immunological reaction to mf in the cornea [50, 71] and is present in individuals chronically exposed to infection [16, 49, 243]. Only limited data on local immune responses in humans are available, due to obvious limitations on tissue sampling, with a single study reporting the presence of IL-4 mRNA, mast cells, eosinophil major basic protein and $CD4^+$ T cells in the conjunctiva and high levels of IgE in the aqueous humor of patients with onchocercal eye disease [256]. On a systemic level, Plier *et al.* [243] investigated the filarial-induced cytokine production of PBMC from ocular mf^+ individuals with and without pathology, reporting higher expression of IL-4, IL-5 and IL-10 in

patients with inflammation of the anterior segment. The involvement of an adaptive Th2 response in the development of sustained onchocercal keratitis is also supported by animal studies, with involvement of both IL-4 and IL-5 (reviewed in [82]).

3.1.2 Scope of the experimental study

The cytokine profile in response to parasite stimuli has been linked to infection status in filariasis, although it is not clear the cause-effect relationship between these components and the relative role of each parasite stage. Most studies investigating the cytokine profile in filariasis have focused on Th1 and Th2 responses while pro-inflammatory mediators more linked to the innate immune response and possibly elicited by *Wolbachia* (e.g. IL-6 and TNFα) have received little attention [121, 212, 257, 258]. Also, Type-17 immune responses, characterized by the production of the pro-inflammatory cytokine IL-17, could be elicited in the environment promoted by the *Wolbachia*-filarial nematode combination, but little data exist on their presence and role in filariasis [128, 145, 190, 212], with no studies investigating their presence in onchocerciasis (see chapter 5 for a detailed overview of Type-17 immune responses and their investigation in the context of filariasis). Finally, the relative role of the worm and *Wolbachia* in eliciting these responses has never been explored so far in human studies of this type.

In the work presented here, Th1 (IFNγ), Th2 (IL-5, IL-13), Th17 (IL-17A) as well as pro- and anti-inflammatory cytokines (TNFα, IL-6 and IL-10) in response to filarial extracts have been investigated in PBMC from patients with onchocerciasis and control subjects from the same geographic area. Patients were enrolled in a clinical trial for the refinement of anti-*Wolbachia* treatment in Ghana. In particular, we investigated the association between these responses and parasite loads, expressed as mf/mg of skin. More importantly, with the use of filarial extracts containing or depleted from *Wolbachia,* we explored for the first time the relative contribution of filarial nematodes and bacterial endosymbionts in shaping the cytokine profile in onchocerciasis.

3.2 METHODS

3.2.1 Study populations

Patients were part of a population participating in a randomised, placebo-controlled, double blind clinical trial for the refinement of anti-*Wolbachia* regimes. The trial was conducted in 17 villages of the Upper and Lower Denkira Districts, Dunkwa on Offin, Ghana, endemic for onchocerciasis. The trial was approved by the Ethics Committee of the Liverpool School of Tropical Medicine (Liverpool, UK), the Medical Faculty of Rheinische Friedrich-Wilhelm University Bonn (Bonn, Germany), and the School of Medical Sciences of Kwame Nkrumah University of Science and Technology (Kumasi, Ghana). The study was registered in the ISRCTN registry (ISRCTN68861628), where details of the participant selection and inclusion and the study procedures can be found. Before treatment, participants' blood, urine and stool were examined for parasitic co-infections and patients were treated as appropriate. The HIV infection status of patients was not assessed, according to the study protocol. However, a white blood cell count ≥ 4000/ µl was required for enrollment. For the analysis of data described in this chapter, onchocerciasis patients of both sexes (aged 18-55 years with 40-70 kg body weight, without other clinical conditions and not pregnant or breastfeeding) were defined as those positive for microfilaridermia. Non endemic control subjects (NEC) were defined as subjects of the same age range of patients who had been living in Dunkwa town (low risk of *Onchocerca* exposure) for at least 10 years and negative for microfilaridermia and palpable onchocercomas.

3.2.2 Stimuli for cell culture

Soluble extracts from adult *B. malayi* female worms from Mongolian jirds treated (BmFEtet) or not (BmFE) with tetracycline in drinking water as described in [47] were used to obtain filarial soluble extracts as described in Turner *et al,* 2006 [152] and detailed in the Appendix. Extracts from *B. malayi* were used because of unavailability of *Wolbachia*-depleted *O. volvulus* extracts. *O. volvulus* adult worm soluble extract (Ov) was kindly provided by Dr Sabine Specht (Institute for Microbiology, Immunology and Parasitology, University Clinics Bonn, Germany). Mouse anti-human CD3 IgG_{2a} (clone OKT3 –αCD3-)

and mouse anti-human CD28 IgG_{2a} (clone CD28.6 –αCD28-) for T cell stimulation were purchased from eBiosciences.

3.2.3 Isolation and culture of PBMC

Peripheral blood from Ghanaian patients with onchocerciasis and Ghanaian NEC was obtained by venipuncture in EDTA and kept refrigerated until processed. For PBMC isolation, 10 ml blood was transferred onto Ficoll-Paque containing tubes and centrifuged at 550 g (brake off) for 20 minutes at 4°C. PBMC were collected by pipette suction from the white blood cell layer, washed twice in 10 ml sterile PBS, and resuspended in 1 ml RPMI 1640 culture media (PAA labs). Cell counting and viability assessment was carried out by 0.2% trypan blue staining using a haemocytometer. Cells were cultured at 2 x 10^5 PBMC/200µl/well in triplicate in U-shape 96-well tissue culture plates (Cellstar, Greiner bio-one) in complete culture media (RPMI 1640 [PAA labs] supplemented with 2 mM L-glutamine, 10% heat inactivated FCS, 50 µg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin [all antibiotics from PAA labs]). Cells were incubated in the presence or absence of stimuli at 37°C in 5% CO_2. Mouse anti-human CD3 and anti-human CD28 were added together to cultures at a final concentration of 10 µg/ml and 2.5 µg/ml respectively as the positive control for T cell stimulation. Filarial soluble extracts were used at 5 µg/ml. The supernatant was harvested after 72 hours and stored at -80°C. Cultures were performed in the Kumasi Centre for Collaborative Research in Tropical Medicine laboratory at Dunkwa district hospital in collaboration with Dr S Specht and Miss K Arndts (Institute for Microbiology, Immunology and Parasitology, University Clinics Bonn, Germany), and Dr C Martin (Museum National d' Histoire Naturelle, Paris).

3.2.4 Assessment of cytokines in PBMC culture supernatants by ELISA

The assessment of cytokines in supernatants of PBMC cultures was performed using commercial sandwich ELISA kits (DuoSet ELISA Development Reagent Kit, R&D Systems), according to manufacturers' instructions. ELISAs were carried out in Bonn in collaboration with Miss Kathrin Arndts (Institute of Medical Microbiology, Immunology and Parasitology, University Clinics Bonn, Germany). Individual samples were analyzed in triplicate. Culture supernatants from patients and NEC were analysed at the same time on shared plates.

Absorbances were read using a SpectraMax ELISA reader (Molecular Devices) supporting Softmax Pro software. The best-fit curve method was used to calculate the cytokine concentration in the samples.

3.2.5 Assessment of microfilaridermia

For the assessment of microfilaridermia prevalence and load, two skin snips were taken from the iliac crests using a sterile corneoscleral punch. Skin snips were incubated in 100 µl normal saline solution in microtitre plates overnight (10-18 hours). The number of mf were counted and the skin wet weight measured to determine the number of mf per mg of skin (mf/mg).

3.2.6 Statistical analysis

Data were not normally distributed, could not be normalized, and did not fit a Poisson distribution, therefore were analysed by non-parametric tests. Differences of cytokine levels between groups were analysed by Mann-Whitney *U* test for unrelated samples and Wilcoxon signed ranks test for paired samples. Correlation between cytokine levels and mf/mg was analysed by Spearman's rank correlation coefficient. Only correlation coefficients $r \geq |0.5|$ were considered significant. Differences in proportions between groups were analysed by Fisher's exact test. For all tests, a *p*-value ≤ 0.05 was considered significant. When multiple comparisons were performed, the effect size is reported according to Cohen's criteria of 0.1 = small effect, 0.3 = medium effect, and 0.5 = large effect. All analysis was carried out in SPSS Statistics 17.0 (IBM). The analysis was conducted on absolute cytokine levels (pg/ml) as well as net production (i.e. stimulant-induced levels subtracted of spontaneous media-stimulated levels). In consideration of the presence of only 1 male subject in the NEC group, the analysis comparing NEC and patients was only carried out on female subjects. Comparison between baseline and stimulated cytokine levels and between patients with high and low microfilarial burdens were performed on female only as well as the whole (female + male) patient group. For this purpose, patients were further divided in those with high and low microfilarial loads. The cut-off was fixed at 50 mf/mg (16.5% patients) when all patients were examined, and at 20 mf/mg (16.9% patients) when only female patients were analysed.

3.3 RESULTS

3.3.1 Study population

The characteristics of the studied population are detailed in table 3.1.
Three hundred nine subjects, 82 females and 227 males, were available for analysis. Of those, 12 subjects (11 females and 1 male) were NEC, while 297 (71 females and 226 males) were microfilaridermic onchocerciasis patients. Microfilaridermia loads ranged from 0.06 mf/mg to 330.81 mf/mg. Female patients had significantly lower microfilarial loads (median 3.62 mf/mg) compared to males (median 9.30 mf/mg) ($p < 0.001$). This is in accordance with what was previously reported for this age range in West Africa [12]. The distribution of microfilarial loads in patients is shown in figure 3.1.

	N (%)	Age Mean years (range)	Mf/mg Median (5^{th}-95^{th} centile)
All subjects			
Females	82 (26.5%)	40.32 (18-55)	-
Males	227 (73.5%)	36.48 (18-55)	-
Total	309	37.50 (18-55)	-
Non endemic controls			
Females	11 (91.7%)	44.73 (23-55)	0 (0-0)
Males	1 (8.3%)	25	0 (0-0)
Total	12	43.08 (23-55)	0 (0-0)
Patients			
Females	71 (23.9%)	39.63 (18-55)	3.62 (0.12-85.72)
Males	226 (76.1%)	36.54 (18-55)	9.30 (0.19-131.45)
Total	297	37.28 (18-55)	7.16 (0.15-119.08)

Table 3-1. Characteristics of the studied population.

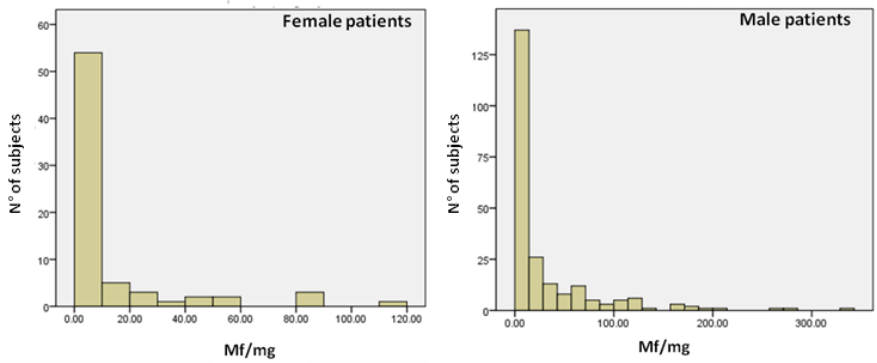

Figure 3-1. Distribution of mf/mg in female and male patients.

No co-infection with blood parasites was found in the studied population. Twenty-four (8.08%) patients (19 males [6.39% of all patients; 8.41% of males] and 5 females [1.68% of all patients; 7.04% of females]) were co-infected with gastrointestinal/urinary parasites. Co-infections were with hookworms (n = 16, 12 males and 4 females), *Strongyloides* (n = 3, 2 males and 1 female), *Schistosoma mansoni* (n = 2, males) and *S. haematobium* (n = 5, males). Of these, only 2 male patients were co-infected with 2 parasites (*S. haematobium* plus *Strongyloides* or plus hookworms). No co-infection was found in NEC. The difference in co-infection prevalence between NEC and female patients was not statistically significant.

Due to low PBMC counts in some patients, not all stimuli were tested on PBMC of all patients, as detailed in table 3.2. On the contrary, all stimuli were tested on PBMC of all NEC.

Stimulus	**Female patients**			**All (male+female) patients**		
	High mf	**Low mf**	**Total**	**High mf**	**Low mf**	**Total**
Media	13	58	71	51	246	297
αCD3/28	12	58	70	49	246	295
Ov	12	58	70	49	246	295
BmFE	8	48	56	24	178	202
BmFEtet	8	50	58	25	185	210

Table 3-2. Number of patients used for PBMC culture for each stimulus. High mf ≥20 mf/mg when female patients are considered and ≥50 mf/mg when all patients are considered.

3.3.2 Type-1 (IFNγ) immune response

A significant IFNγ response, in terms of proportion of responders (Figure 3.2) and cytokine levels, was observed in both NEC and patients only in response to αCD3/28, independently of microfilarial loads in patients (Figure 3.3 A). On the contrary, only low detectable levels of IFNγ were produced, by half of subjects, both spontaneously and in response to filarial extracts, independently of their infection status (Figure 3.3 A and B and figure 3.2). These levels were not significantly higher than spontaneous (media-stimulated) IFNγ production with the exception (though with small effect size) of those induced by BmFE when all (female + male) patients were considered (Figure 3.3 B). No difference in IFNγ

production was observed between NEC and patients and between patients with high and low mf loads, both in terms of absolute values and when respective spontaneous IFNγ levels were subtracted (Figure 3.3 A and B). Also, IFNγ production did not correlate with mf loads. Finally, no differences were observed between IFNγ levels stimulated by BmFE and BmFEtet or Ov (not shown).

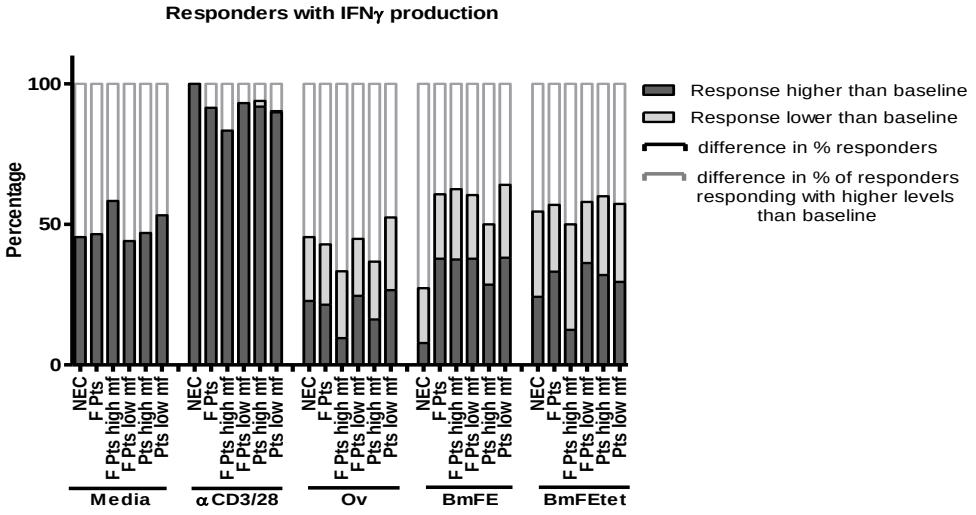

Figure 3-2. Proportion of subjects responding to stimulation with IFNγ production (dark + light grey filled bars) and proportion of responder subjects producing levels of IFNγ higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females+males) with ≥50 mf/mg; Pts low mf = Patients (females+males) with <50 mf/mg. No statistically significant differences between groups were found. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 μg/ml), filarial extracts (all 5 μg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

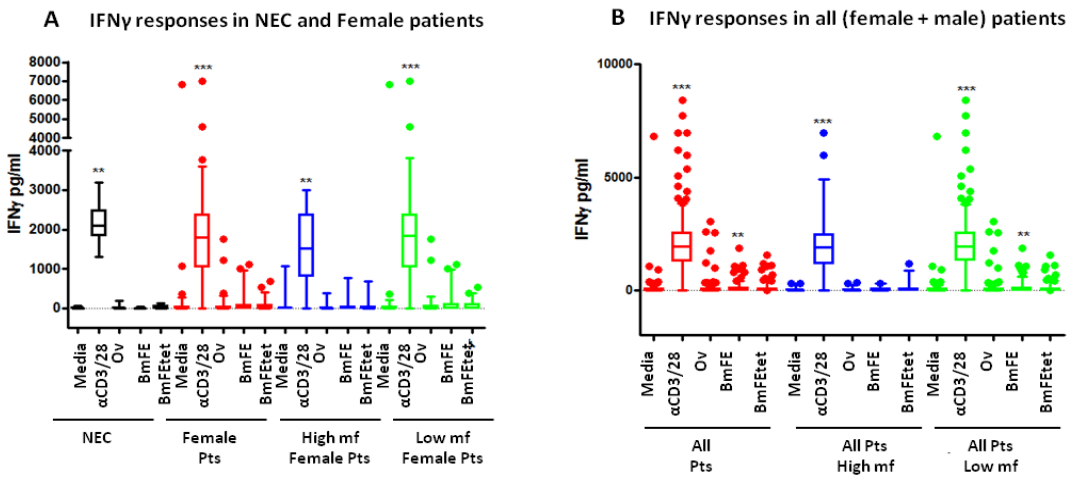

Figure 3-3. Absolute levels of IFNγ produced by PBMC upon stimulation with media alone, αCD3/28 activating antibodies, and filarial extracts in NEC (black), patients (pts) (red), and patients divided in high and low microfilaridermia load. Graphs represent median and 5-95 percentiles. Statistics indicate levels of IFNγ induced upon stimulation compared to spontaneous (media-induced) production. **A**, *** $p \leq 0.001$ (effect size r = 0.59 in both comparisons); ** $p = 0.003$ media vs. αCD3/28 in NEC (r = 0.62), $p = 0.005$ media vs. αCD3/28 in Female (F) patients with high (≥ 20 mf/mg) mf load (r = 0.57). **B**, *** $p \leq 0.001$ (r = 0.58 in all comparisons); ** $p = 0.004$ media vs. BmFE in patients (females + males) as a whole group (r = 0.14), $p = 0.007$ media vs. BmFE in patients (females + males) with low (< 50 mf/mg) mf load (r = 0.14). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

3.3.3 Type-2 (IL-5 and IL-13) immune responses

Type-2 signature cytokine responses in patients, but not in NEC, were generally stronger than Type-1 (IFNγ) and Type-17 (IL-17A) responses. As for IFNγ and IL-17A (see below), filarial extracts elicited the production of lower levels of IL-5 and IL-13 compared to αCD3/28 stimulation. More than 70% of the patients responded to filarial extract stimulation with IL-5 and IL-13 production, a significantly higher proportion compared to NEC (Figure 3.4 and 3.5). Stimulation with all filarial extracts elicited levels of IL-5 significantly higher than media control in patients but not NEC (Figure 3.6 B and C), while this was observed for IL-13 only in response to αCD3/28 and *O. volvulus* extract (Figure 3.7 B). Patients produced higher levels of IL-5 than NEC in response to all stimuli, both when absolute levels and media-adjusted levels were considered (Figure 3.6 A and D), while only absolute levels of IL-13 were higher in patients compared to NEC (Figure 3.7 A). An inverse proportionality between IL-5 levels

and microfilarial burden could be observed, with higher absolute levels (though not statistically significant) and statistically higher increase from basal levels in patients with low microfilarial loads (Figure 3.6 A and D), while this was not observed for IL-13 (Figure 3.7 A). Also, less patients with high mf loads tended to respond with IL-5 and IL-13 production compared to those with low mf loads (Figure 3.4 and 3.5), although this reached statistical significance only when IL-13 produced by patients (females + males) were considered. However, no correlation was observed between IL-5 levels and mf/mg (not shown). Finally, no differences were observed between Type-2 cytokine levels stimulated by BmFE and BmFEtet or Ov (not shown).

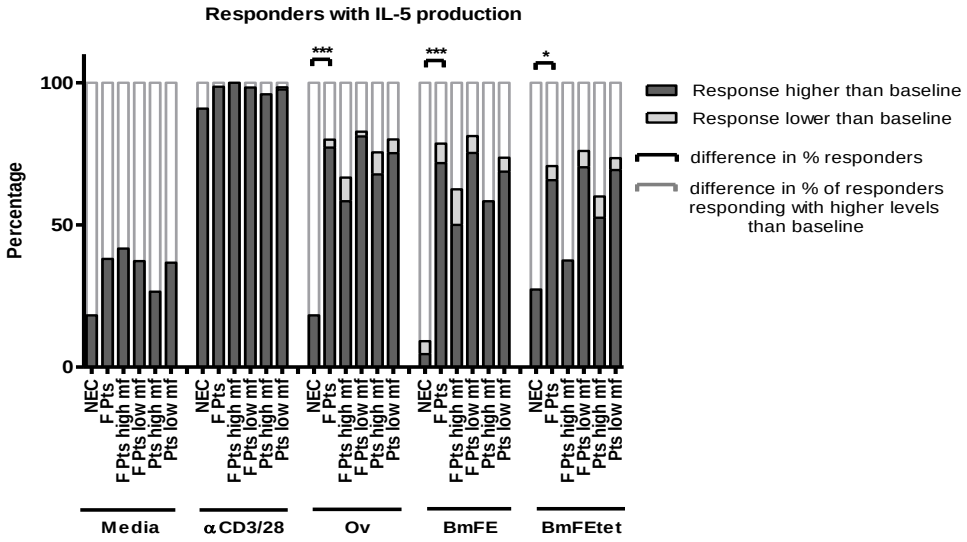

Figure 3-4. Proportion of subjects responding to stimulation with IL-5 production (dark + light grey filled bars) and proportion of responder subjects producing levels of IL-5 higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females+males) with ≥50 mf/mg; Pts low mf = Patients (females+males) with <50 mf/mg. ***$p \leq 0.001$; *$p = 0.013$. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 μg/ml), filarial extracts (all 5 μg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

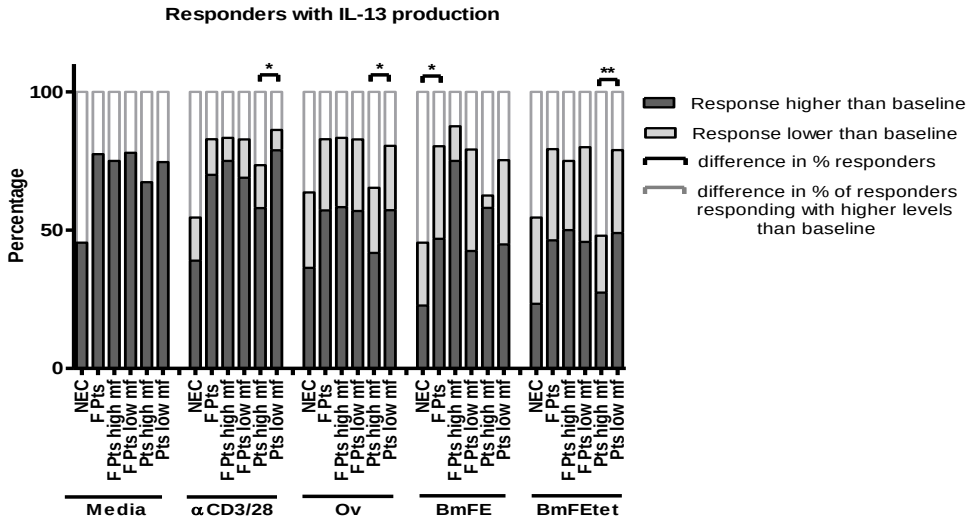

Figure 3-5. Proportion of subjects responding to stimulation with IL-13 production (dark + light grey filled bars) and proportion of responder subjects producing levels of IL-13 higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females+males) with ≥50 mf/mg; Pts low mf = Patients (females+males) with <50 mf/mg. ** p = 0.002 BmFEtet Pts high mf vs Pts low mf; * p = 0.024 BmFE NEC vs F Pts, p = 0.024 Ov Pts high mf vs Pts low mf, p = 0.033 αCD3/28 Pts high mf vs Pts low mf. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

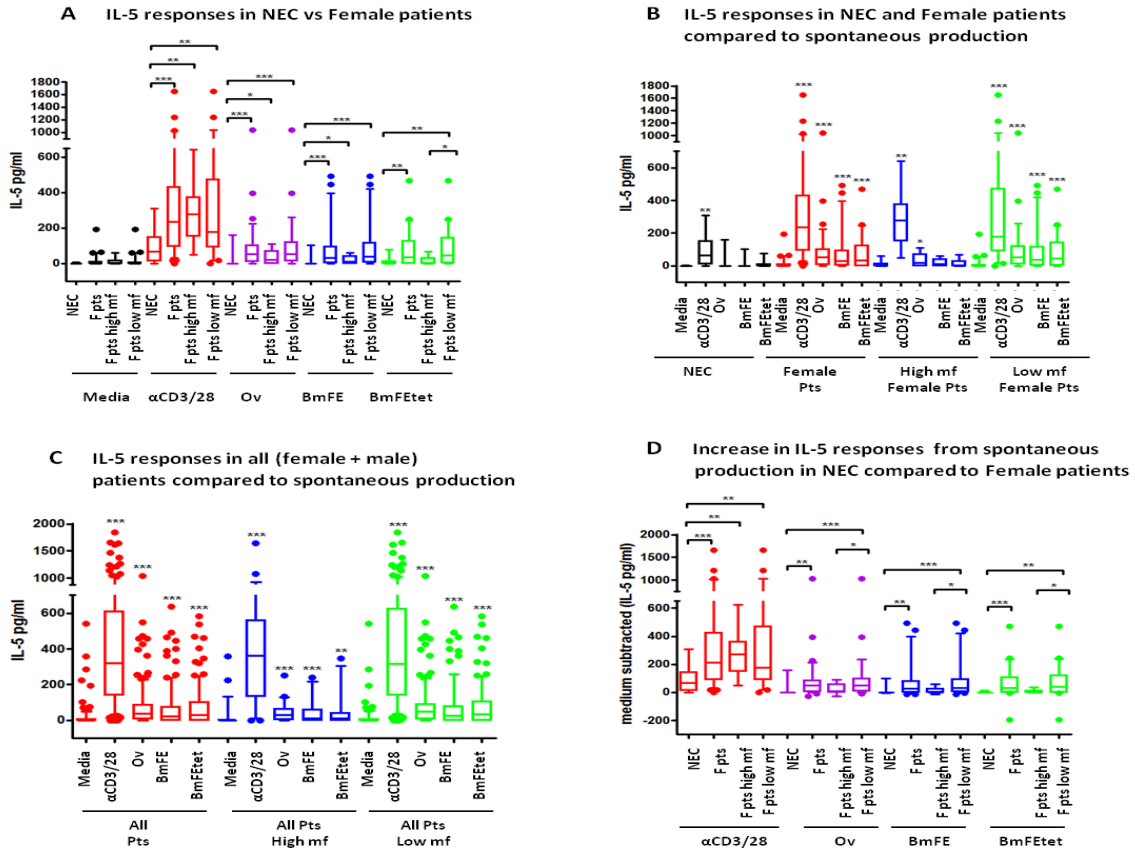

Figure 3-6. IL-5 responses. Graphs show median and 5-95 percentiles. **A**, Differences in IL-5 levels upon stimulation with media (black), αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***$p \leq 0.001$ (effect size r = 0.36 to 0.47); **$p = 0.003$ αCD3/28 NEC vs. Female (F) patients (pts) with high (≥ 20 mf/mg) mf load (r = 0.61), $p = 0.002$ αCD3/28 NEC vs F pts with low mf load (r = 0.37), $p = 0.004$ BmFEtet NEC vs F pts (r = 0.35), $p = 0.002$ BmFEtet NEC vs F pts with low mf load (r = 0.31); *$p = 0.041$ Ov NEC vs F pts with high mf load (r = 0.42), $p = 0.036$ BmFE NEC vs F pts with high mf load (r = 0.48), $p = 0.018$ BmFEtet F pts with high vs low mf loads (r = 0.31). **B**, Differences in levels of IL-5 in NEC (black), F pts (red) and F pts divided by microfilarial load (blue ≥20 mf/mg, green <20 mf/mg). Statistics show comparison with media-stimulated levels. ***$p \leq 0.001$ (r = 0.48 to 0.60); **$p = 0.005$ media vs αCD3/28 in NEC (r = 0.60), $p = 0.002$ media vs αCD3/28 in F pts with high mf loads (r = 0.60); *$p = 0.036$ media vs Ov in F pts with high mf loads (r = 0.43). **C**, Differences in levels of IL-5 in pts (females + males, red) and pts divided by microfilarial load (blue ≥50 mf/mg, green <50 mf/mg). ***$p \leq 0.001$ (r = 0.42 to 0.61); **$p = 0.004$ media vs BmFEtet in pts with high mf load (r = 0.41). **D**, Differences in change of IL-5 levels from baseline upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***$p \leq 0.001$ (r = 0.31 to 0.43); **$p = 0.003$ αCD3/28 NEC vs F pts (r = 0.61), $p = 0.002$ αCD3/28 NEC vs F pts with high mf load (r = 0.36), $p = 0.002$ Ov NEC vs F pts (r = 0.34), $p = 0.002$ BmFE NEC vs F pts (r = 0.37), $p = 0.005$ BmFEtet NEC vs F pts with low mf load (r = 0.36); *$p = 0.047$ Ov F pts with high vs low mf load (r = 0.24), $p = 0.037$ BmFE F pts with high vs low mf load (r = 0.28), $p = 0.017$ BmFEtet F pts with high vs low mf load (r = 0.31). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

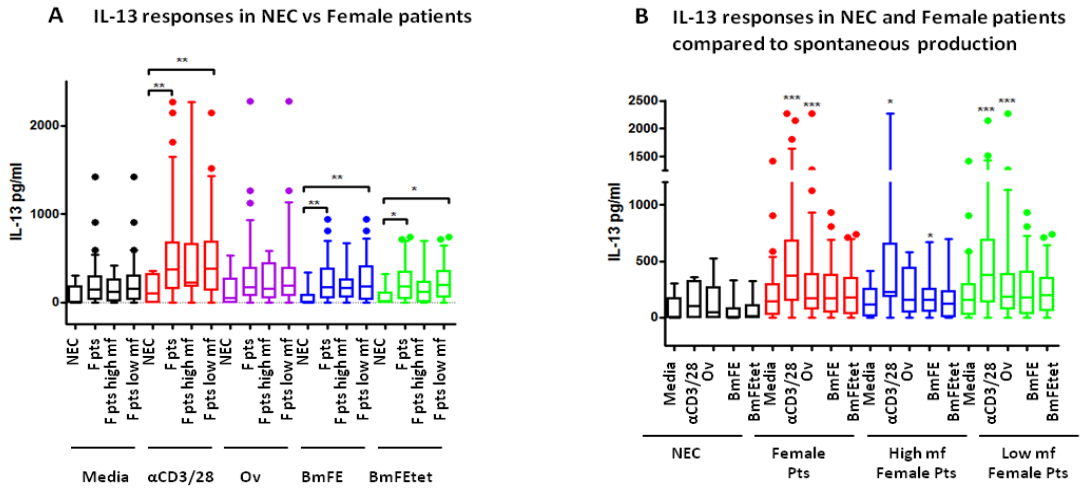

Figure 3-7. IL-13 responses. Graphs show median and 5-95 percentiles. **A**, Differences in absolute levels of IL-13 upon stimulation with media (black), αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***p* = 0.008 αCD3/28 NEC vs Female (F) patients (pts) (effects size r = 0.30), *p* = 0.007 αCD3/28 NEC vs F patients with low (<20 mf/mg) mf load (r = 0.32), *p* = 0.008 BmFE NEC vs F patients (r = 0.32), *p* = 0.008 BmFE NEC vs F patients with low mf load (r = 0.34); **p* = 0.020 BmFetet NEC vs F patients (r = 0.31), *p* = 0.015 BmFEtet NEC vs F patients with low mf load (r = 0.28). **B**, Differences in absolute levels of IL-13 in NEC (black), F patients (red) and F patients divided by microfilarial load (blue ≥20 mf/mg, green <20 mf/mg). Statistics show comparison with media-stimulated levels. ****p* ≤ 0.001 (r = 0.31 to 0.43); **p* = 0.017 αCD3/28 in F patients with high mf load (r = 0.62), *p* = 0.043 BmFE in F patients with high mf load (r = 0.51). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

3.3.4 Type-17 (IL-17A) immune response

The investigation of IL-17A responses gave results similar to those obtained for IFNγ. A significant IL-17A response, in terms of proportion of responders (Figure 3.8) and cytokine levels (Figure 3.9 A and B), was observed in both NEC and patients only in response to αCD3/28, independently of microfilarial loads in patients. On the contrary, very low levels of IL-17A were produced both spontaneously and in response to filarial extracts, by about one half of subjects, with no differences between groups (Figure 3.9 A and B and figure 3.8). However, filarial extracts induced higher levels of IL-17A compared to baseline in patients but not NEC, although this reached statistical significance only when all (female + male) patients were examined (Figure 3.9 B). No

differences were found when high and low microfilaridermic groups were compared, and no significant correlation between mf loads (mf/mg) and IL-17A levels was observed (not shown). Finally, no differences were observed between IL-17A levels stimulated by BmFE compared to BmFEtet or Ov (not shown).

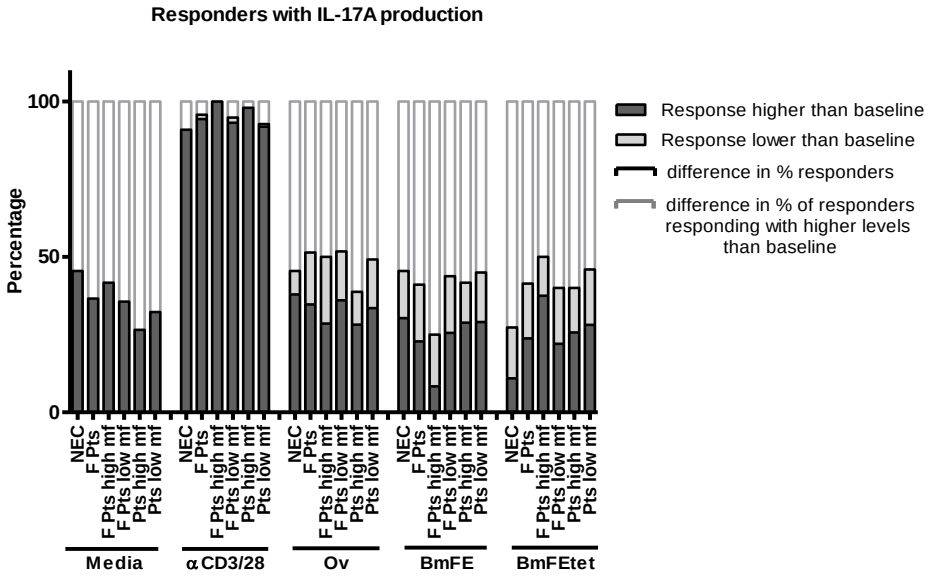

Figure 3-8. Proportion of subjects responding to stimulation with IL-17A production (dark + light grey filled bars) and proportion of responder subjects producing levels of IL-17A higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females + males) with ≥50 mf/mg; Pts low mf = Patients (females + males) with <50 mf/mg. No statistically significant differences between groups were found. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 μg/ml), filarial extracts (all 5 μg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

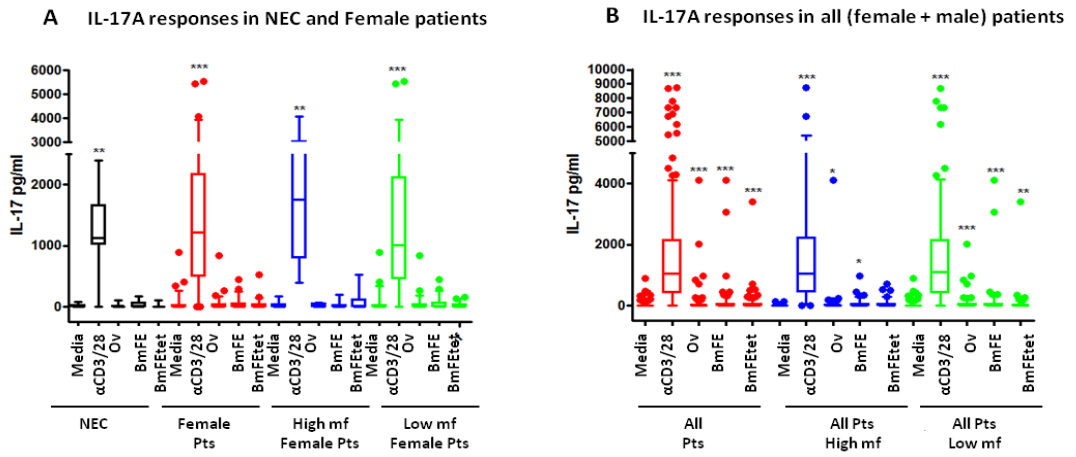

Figure 3-9. Absolute levels of IL-17A produced by PBMC upon stimulation with media alone, αCD3/28 activating antibodies, and filarial extracts in NEC (black), patients (pts) (red), and patients divided in high and low microfilaridermia load. Graphs represent median and 5-95 percentiles. Statistics indicate levels of IL-17A induced upon stimulation compared to spontaneous (media-induced) production. **A**, *** $p \leq 0.001$ (effect size r = 0.60 in both comparisons); ** $p = 0.005$ media vs αCD3/28 in NEC (r = 0.60), $p = 0.002$ media vs αCD3/28 in Female (F) patients with high (≥ 20 mf/mg) mf load (r = 0.62). **B**, *** $p \leq 0.001$ (r = 0.60 for all media vs αCD3/28 comparisons; r = range between 0.15 and 0.20 in all media vs filarial extracts comparisons); * $p = 0.042$ media vs Ov in patients (females + males) with high (≥50 mf/mg) mf load (r = 0.20), $p = 0.019$ media vs BmFE in patients (females + males) with high mf load (r = 0.34). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

3.3.5 Innate pro-inflammatory (IL-6 and TNFα) immune responses

IL-6, and to a lesser extent TNFα were, together with IL-10, the most abundantly produced cytokines in all cultures. The production of innate pro-inflammatory cytokines in response to media or stimuli was different and in some aspects opposite of what was observed for adaptive immunity.

Interestingly, spontaneous (media-stimulated) production of IL-6 and TNFα was significantly lower in patients compared to NEC (Figure 3.12 A and 3.13 A), and a significantly smaller proportion of patients compared to NEC spontaneously produced these cytokines (Figure 3.10 and 3.11). Upon stimulation with αCD3/28 or filarial extracts, patients responded with an increased production of both cytokines (figure 3.12 C and 3.13 C) while NEC maintained levels of IL-6 and TNFα not different or even lower than baseline (Figure 3.12 B, 3.13 B, 3.11 and 3.12). However, absolute levels of both cytokines remained significantly lower, or not different from those produced by NEC (Figure 3.12 A and 3.13 A).

A direct proportionality was observed between microfilarial burdens and levels of IL-6 and TNFα in response to filarial extracts, but not to αCD3/28 (Figure 3.12 A and 3.13 A), even though the increase of these cytokines from baseline levels did not differ between groups with high and low mf loads (Figure 3.12 B and 3.13 B). This also applied when the proportion of patient responders to stimulation in the two groups was considered (Figure 3.10 and 3.11). Also, no correlation was found between mf/mg and pro-inflammatory cytokine levels (not shown). Finally, no differences were observed between IL-6 and TNFα levels stimulated by BmFE compared to BmFEtet or Ov (not shown).

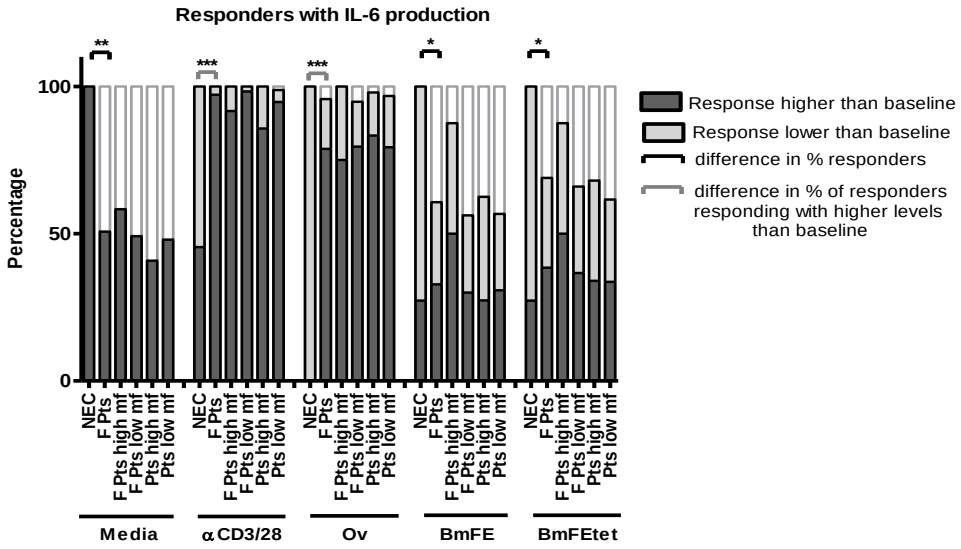

Figure 3-10. Proportion of subjects responding to stimulation with IL-6 production (dark + light grey filled bars) and proportion of responder subjects producing levels of IL-6 higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females + males) with ≥50 mf/mg; Pts low mf = Patients (females + males) with <50 mf/mg. ***$p \leq 0.001$; **$p = 0.002$; *$p = 0.012$ BmFE NEC vs F Pts, $p = 0.050$ BmFEtet NEC vs F Pts. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 μg/ml), filarial extracts (all 5 μg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

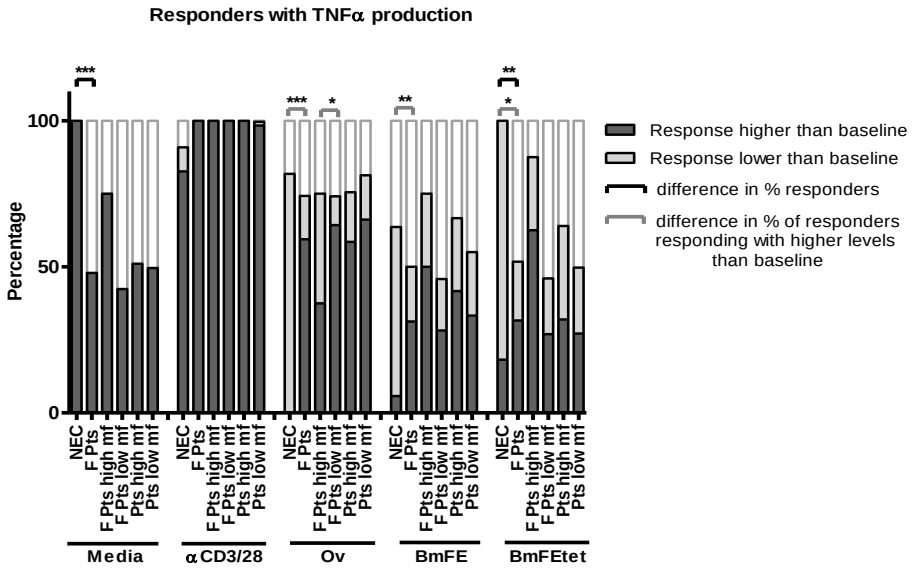

Figure 3-11. Proportion of subjects responding to stimulation with TNFα production (dark + light grey filled bars) and proportion of responder subjects producing levels of TNFα higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (Females + males) with ≥50 mf/mg; Pts low mf = Patients (females + males) with <50 mf/mg. ***$p \leq 0.001$; **$p = 0.002$ BmFEtet NEC vs F Pts, $p = 0.004$ BmFE NEC vs F Pts; *$p = 0.017$ BmFEtet NEC vs F Pts, $p = 0.019$ Ov F Pts high mf vs F Pts low mf. PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

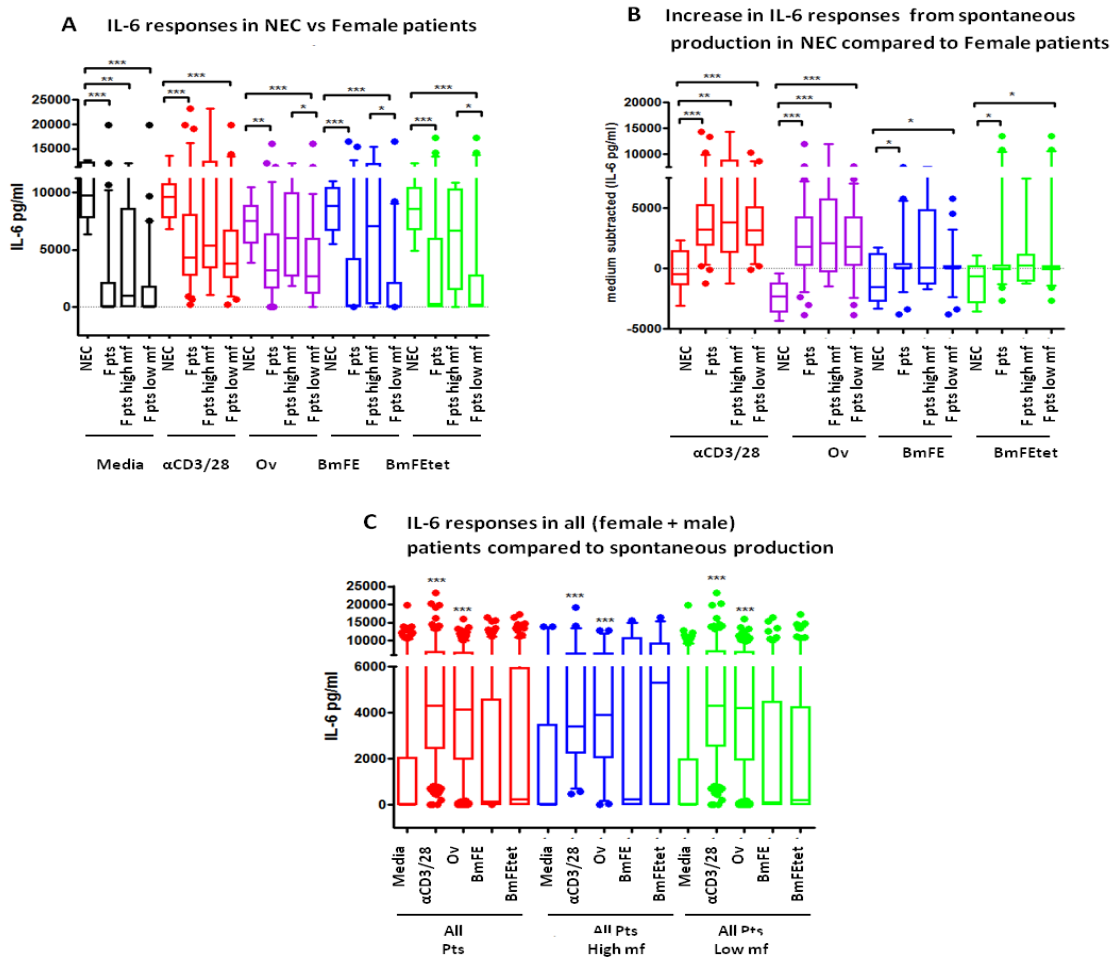

Figure 3-12. IL-6 responses. Graphs show median and 5-95 percentiles. **A**, Differences in absolute levels of IL-6 upon stimulation with media (black), αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green). ***$p \leq 0.001$ (effect size r = 0.50 to 0.61); **$p = 0.004$ media NEC vs F patients with high mf load (r = 0.55), $p = 0.002$ Ov NEC vs F patients (r = 0.35), *$p = 0.012$ Ov F patients with high vs low mf load (r = 0.30), $p = 0.014$ BmFE F patients with high vs low mf load (r = 0.33), $p = 0.022$ BmFEtet F patients with high vs low mf load (r = 0.34). **B**, Differences in change of IL-6 levels from baseline upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***$p \leq 0.001$ (r = 0.48 to 0.58); **$p = 0.002$ αCD3/28 NEV vs F patients with high mf load (r = 0.61); *$p = 0.026$ BmFE NEC vs F patients (r = 0.37), $p = 0.033$ BmFE NEC vs F patients with low mf load (r = 0.28), $p = 0.019$ BmFEtet NEC vs F patients (r = 0.31), $p = 0.020$ NEC vs F patients with low mf load (r = 0.30). **C**, Differences in absolute levels of IL-6 upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green) in patients (females+males as a whole group and divided into high [≥50 mf/mg] or low [<50 mf/mg] mf load). ***$p \leq 0.001$ (r = range between 0.42 and 0.59 in all comparisons). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

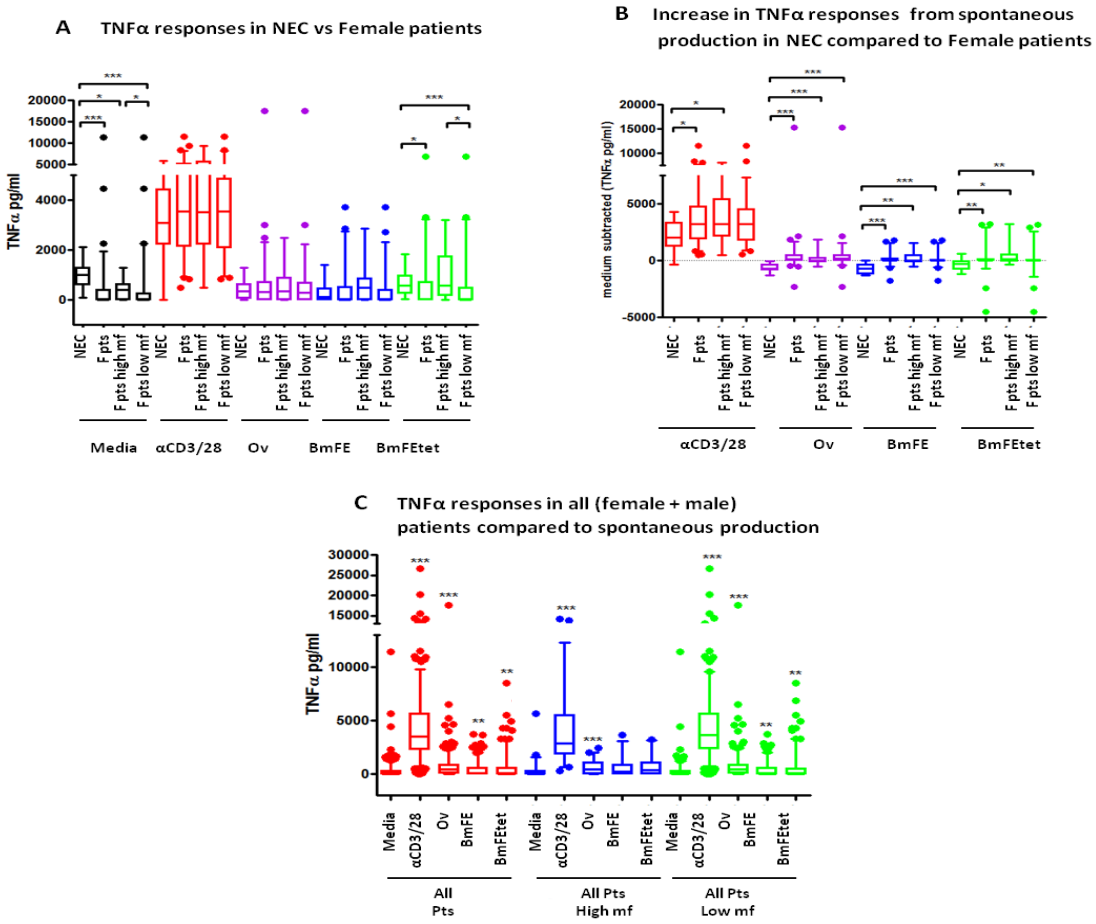

Figure 3-13. TNFα responses. Graphs show median and 5-95 percentiles. **A**, Differences in absolute levels of TNFα upon stimulation with media (black), αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green). ***$p \leq 0.001$ (effect size r = 0.38 to 0.50); *$p = 0.012$ media NEC vs F patients with high (≥20 mf/mg) mf load (r = 0.53), $p = 0.029$ media F patients with high vs low mf load (r = 0.26), $p = 0.016$ BmFEtet NEC vs F patients (r = 0.29), $p = 0.032$ BmFEtet F patients with high vs low mf load (r = 0.36). **B**, Differences in change of TNFα levels from baseline upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***$p \leq 0.001$ (r = 0.53 to 0.58); **$p = 0.002$ BmFE NEC vs F patients with high mf load (r = 0.70), $p = 0.005$ BmFEtet NEC vs F patients (r = 0.33), $p = 0.008$ BmFEtet NEC vs F patients with low mf load (r = 0.34); *$p = 0.034$ αCD3/28 NEC vs F patients (r = 0.23), $p = 0.044$ αCD3/28 NEC vs F patients with low mf load (r = 0.40), $p = 0.021$ BmFEtet NEC vs F patients with high mf load (r = 0.53). **C**, Differences in absolute levels of TNFα upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green) in patients (females + males as a whole group and divided in high [≥50 mf/mg] and low [<50 mf/mg] mf load). ***$p \leq 0.001$ (r = 0.40 for media vs Ov and 0.61 for media vs αCD3/28); **$p = 0.002$ media vs BmFE in patients (r = 0.16), $p = 0.004$ media vs BmFEtet in patients with low mf load (r = 0.16); *$p = 0.012$ media vs BmFE in patients (r = 0.12), $p = 0.029$ media vs BmFEtet in patients with low mf load (r = 0.11). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

3.3.6 Anti-inflammatory (IL-10) immune response

IL-10 responses overall mirrored those observed for innate pro-inflammatory cytokines. Spontaneous (media-stimulated) production of IL-10 was significantly lower in patients compared to NEC (Figure 3.15 A) and a significantly lower proportion of patients compared to NEC spontaneously produced IL-10 (Figure 3.14) Upon stimulation with αCD3/28 or filarial extracts, patients responded with an increased production of IL-10 while NEC maintained levels of IL-10 not different or even lower than baseline (Figure 3.15 B and C and figure 3.14). However, absolute levels of IL-10 remained significantly lower, or not different from those produced by NEC (Figure 3.15 A). A direct proportionality was observed between microfilarial burdens and levels of IL-10 in response to all stimuli (Figure 3.15 A), although the increase from baseline levels did not differ between groups with high and low mf loads (Figure 3.15 B), as did not differ the proportion of patients responders to stimulation between the two groups (Figure 3.14). Also, no correlation was found between mf/mg and IL-10 levels (not shown). Finally, no differences were observed between IL-10 levels stimulated by BmFE compared to BmFEtet or Ov (not shown).

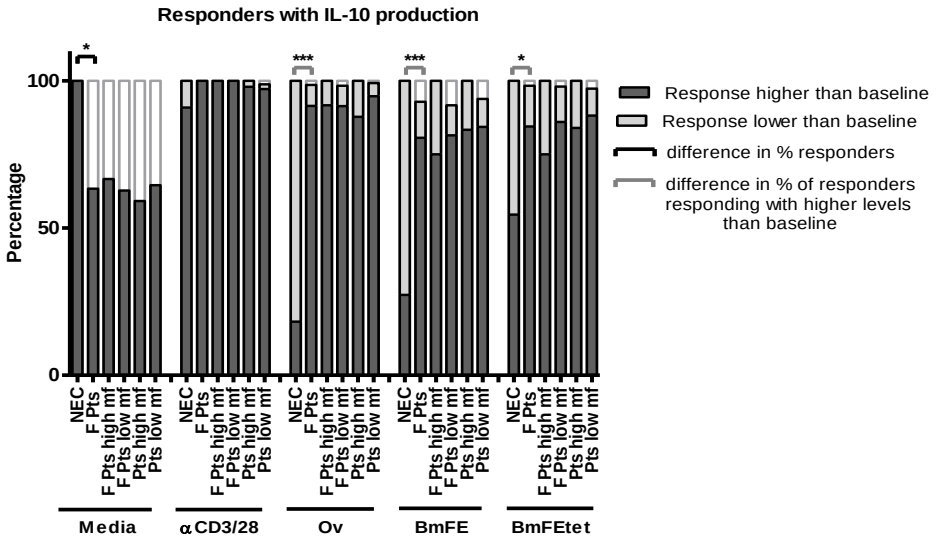

Figure 3-14. Proportion of subjects responding to stimulation with IL-10 production (dark + light gray filled bars) and proportion of responders producing levels of IL-10 higher than baseline (dark filled bars). NEC = Non Endemic Controls; F Pts = Female patients; F Pts high mf = Female patients with ≥20 mf/mg; F Pts low mf = Female patients with <20 mf/mg; Pts high mf = Patients (females+males) with ≥50 mf/mg; Pts low mf = Patients (females+males) with <50 mf/mg. ***$p \leq 0.001$; *$p = 0.014$ Media NEC vs F Pts, $p = 0.029$ BmFEtet NEC vs F Pts.

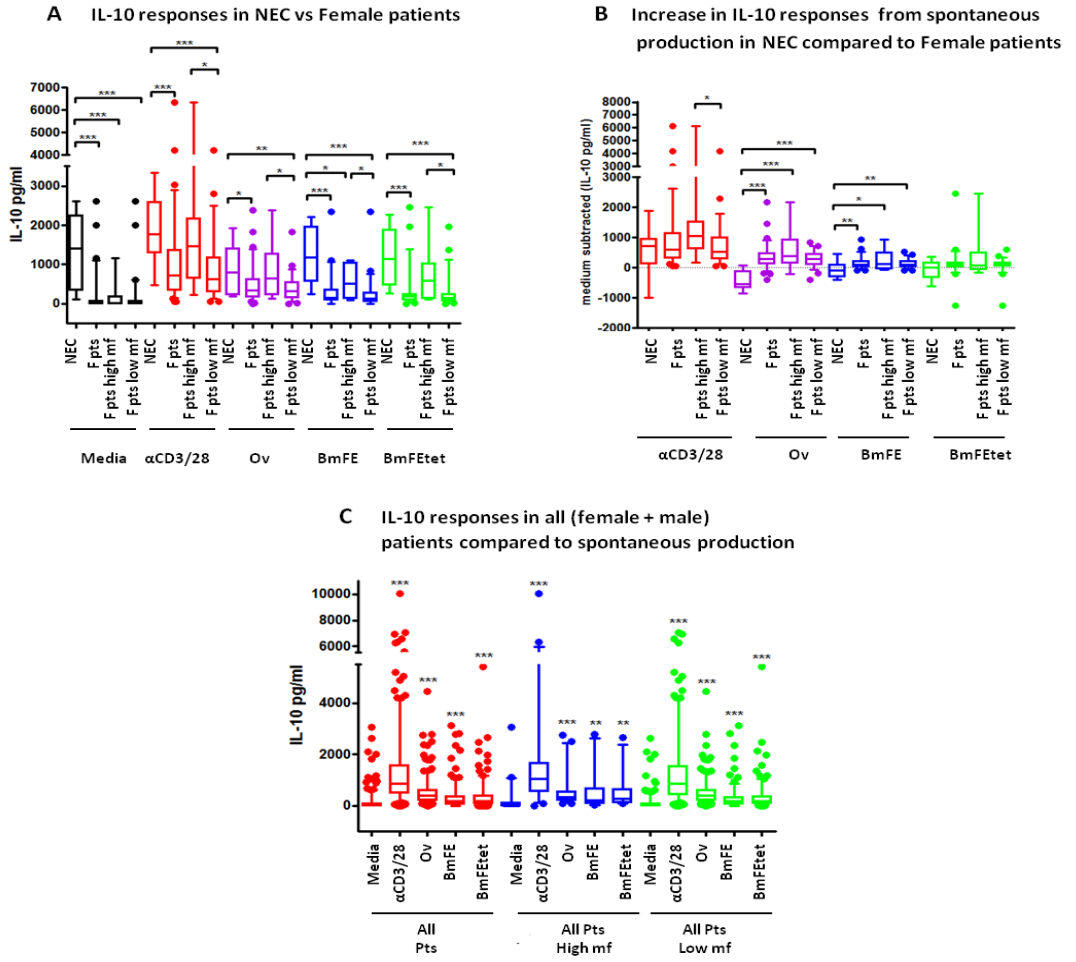

Figure 3-15. IL-10 responses. Graphs show median and 5-95 percentiles. **A**, Differences in absolute levels of IL-10 upon stimulation with media (black), αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green). ***$p \leq 0.001$ (effect size r = 0.37 to 0.58); **$p = 0.009$ Ov NEC vs F patients with low (<20 mf/mg) mf load (r = 0.31); *$p = 0.019$ αCD3/28 F patients with high vs low mf load (r = 0.28), *$p = 0.023$ Ov NEC vs F patients (r = 0.25), $p = 0.027$ Ov F patients with high vs low mf load (es 0.26), $p = 0.032$ BmFE NEC vs F patients with high mf load (r = 0.49), $p = 0.044$ BmFE F patients with high vs low mf load (r = 0.26), $p = 0.030$ BmFEtet F patients with high vs low mf load (r = 0.28). **B**, Differences in change of IL-10 levels from baseline upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue) and BmFEtet (green). ***$p \leq 0.001$ (r = 0.55 to 0.59); **$p = 0.002$ BmFE NEC vs F patients (r = 0.38), $p = 0.003$ BmFE NEC vs F patients with low mf load (r = 0.39); *$p = 0.024$ αCD3/28 F patients with high vs low mf load (r = 0.27), $p = 0.026$ BmFEtet NEC vs F patients with high mf load (r = 0.51) **C**, Differences in absolute levels of IL-10 upon stimulation with αCD3/28 (red), Ov (purple), BmFE (blue), and BmFEtet (green) in patients (females+males as a whole group and divided in high [≥50 mf/mg] and low [<50 mf/mg] mf load). ***$p \leq 0.001$ (r = 0.49 to 0.61); **$p = 0.004$ BmFE in patients with high mf load (r = 0.42), $p = 0.007$ BmFEtet in patients with high mf load (r = 0.50). PBMC were cultured in triplicate for 3 days at 1 x 10^6 cells/ml with αCD3/αCD28 antibodies (10/2.5 µg/ml), filarial extracts (all 5 µg/ml) and media. ELISA were performed in triplicate for each biological sample. N patients per group is indicated in table 3-2.

3.3.7 Synopsis of results

	NEC	Patients	High mf	Low mf
IFNγ*				
Percentage responders	27.3-54.5%	36.7-64.1%	36.7-60.0%	52.4-64.1%
Increase after stimulation	-	-	-	-
IL-5*				
Percentage responders	18.2-27.3%	58.3-80.1%	58.3-75.5%	73.5-80.1%
Increase after stimulation	-	↑	↑	↑↑
IL-13*				
Percentage responders	45.5-63.6%	48.0-80.5%	48.0-63.3%	75.3-80.5%
Increase after stimulation	-	↑↑	↑	↑↑
IL-17A*				
Percentage responders	27.3-45.5%	38.8-51.4%	38.8-41.7%	44.9-51.4%
Increase after stimulation	-	↑	↑	↑
IL-6§				
Percentage responders	100%	56.7-97.9%	62.5-97.9%	56.7-96.7%
Increase after stimulation	-/↓	↑	↑↑	↑
Spontaneous production	+++	+	++	+
TNFα§				
Percentage responders	63.6-100%	49.7-81.3%	64.0-75.5%	49.7-81.3%
Increase after stimulation	-/↓	↑	↑↑	↑
Spontaneous production	+++	+	++	+
IL-10§				
Percentage responders	100%	93.8-100%	100%	93.8-99.2%
Increase after stimulation	-/↓	↑	↑↑	↑
Spontaneous production	+++	+	+	+

Table 3-3. Synopsis of results of cytokine production from PBMC of patients (males + females) with onchocerciasis and non endemic controls (NEC) stimulated at 1 x 10^6 cells/ ml for 3 days with filarial extracts or media alone (spontaneous production). High mf ≥50 mf/mg; low mf <50 mf/mg. The table provides an overview of the general behaviour of cytokine production upon filarial extracts stimulation without distinguished between *O. volvulus, B. malayi* and *B. malayi* tetracycline-treated extract as no significant differences were observed with the use of either of these stimuli, which all induced similar responses. - = no increase from spontaneous production levels; ↓, ↑, ↑↑, ↑↑ ↑ = illustrative scale of cytokine decrease or increase relative to baseline upon stimulation; +, ++, +++ = illustrative scale of spontaneous cytokine production. For detailed statistical analysis refer to specific graphs for each cytokine. * Spontaneous production not detailed as not different between groups; § upon stimulation absolute levels remain lower than NEC.

3.4 DISCUSSION

The study presented here investigated Th1 (IFNγ), Th2 (IL-5, IL-13), Th17 (IL-17A) as well as pro- and anti-inflammatory cytokines (TNFα, IL-6 and IL-10) in response to filarial extracts in PBMC from patients with onchocerciasis and control subjects from the same geographic area. In particular, we investigated

the association between these responses and parasite loads, expressed as mf/mg of skin. More importantly, with the use of filarial extracts containing or depleted from *Wolbachia,* we explored the relative contribution of filarial nematodes and bacterial endosymbionts in shaping the cytokine profile in patients with onchocerciasis.

The predominance of filarial-specific Th2 over Th1 responses found in onchocerciasis patients is in accordance with the literature [119, 210]. Moreover, negligible levels of IL-17A were found in this study, comparably to what reported in a recent investigation in bancroftian filariasis [212]. In previously published work, these response profiles were obtained with PBMC stimulations ranging from two to five days, therefore it is unlikely that the results obtained here may be due to a non optimal PBMC stimulation period. A heightened filarial-specific Type-17 immune response has been reported in correlation with filarial pathology in LF [190], while this and others work suggest that filarial infection would down-regulate filarial-specific and third party antigen-specific Type-17 responses [145, 212, 259], mirroring the dynamic of Th1 and Th2 responses in filariasis.
No direct comparisons can be made between this and other published studies regarding the different responses of patients and controls. Indeed, NEC investigated in this work did not strictly belong to either of the control categories generally used in this type of investigations. The low but present response to filarial extracts in some of these subjects indicates their likely exposure to *Onchocerca* infection (no filarial-induced cytokine response is observed in control subjects never exposed to the parasite [54, 214, 260]), but they can not be classified as PI/EN based on their exposure history and recruitment criteria. Net filarial induced cytokine responses (i.e. increase from media-stimulated baseline levels in response to filarial extracts) were higher in patients compared to NEC. This might reflect the low exposure of the NEC included in this study, possibly not enough to boost an ongoing immune response.

Of particular interest are the results concerning pro- and anti-inflammatory cytokines. Higher spontaneous production of IL-10 has been reported before in onchocerciasis in infected patients compared to PI/EN [119, 217, 218]. This was linked to the presence of active infection, as levels of spontaneous IL-10 have been found to be no more detectable after repeated ivermectin treatment and supposed adult death [54]. Our results showing a direct proportionality between parasite load and absolute production of these cytokines are

consistent with this picture. Interestingly, in this investigation higher levels of pro- and anti-inflammatory cytokines TNFα, IL-6 and IL-10 were spontaneously produced by NEC compared to onchocerciasis patients. These results may reflect a generalized state of suppression of the innate arm of the immune response in patients with active onchocerciasis compared to subjects only occasionally exposed to infection. Indeed, filarial parasites have been shown to mediate immune suppression also through inhibition of the functions of antigen-presenting cells [133, 135-137]. The source of these cytokines has not been specifically investigated in this study. When antigen-specific net production of these cytokines was considered, no difference was observed between patients with high and low mf loads, suggesting that filarial-stimulated production may be induced by paracrine activation from cytokines less modulated by the parasite load.

No correlation was found between mf/mg of skin and levels of any investigated cytokine. However an impact of filarial load on cytokine responses in onchocerciasis patients was found, with the exception of Th1 and Th17 responses. Absolute and net levels of IL-5 were lower in patients with high compared to low mf burdens, consistent with previous reports [14, 213]. On the contrary, a direct proportionality between absolute levels of TNFα, IL-6 and IL-10 and levels of microfilaridermia was observed. This was in contrast to what was found by Arndts *et al*, who reported lower levels of these cytokines in mf^+ compared to infected mf^- patients with LF [212]. Taken together, the presence of an impact of filarial loads on cytokine responses in the absence of a correlation with mf loads suggests that adult parasite load, rather than mf, may be related to the profile shown in this study. Moreover, these results suggest that filarial parasites are able to promote both pro- and anti-inflammatory responses at the same time. A similar result has been found recently in schistosomiasis, where infected patients were reported to express higher level of both TNFα and IL-10 in response to parasite extract [261].

Wolbachia-containing and *Wolbachia*-depleted filarial extracts did not elicit a different cytokine profile upon stimulation of PBMC. This was an unexpected result, as a clear difference in the ability to stimulate macrophages and DCs and to induce an adaptive Th1 response by *Wolbachia*-containing compared to *Wolbachia*-devoid parasites has been reported before [47, 153]. From a technical point of view, several possible explanations could be put forward. First, parasite extracts were used at 5 μg/ml. Although this concentration is in the range used in most published work investigating the cytokine responses of

PBMC stimulated with filarial extracts, nevertheless it is much lower (5 μg/ml vs 200 μg/ml) than what was used to demonstrate *Wolbachia*-related differences in macrophage stimulations [47]. Thus, levels of *Wolbachia*-derived molecules in 5 μg/ml BmFE may not have been enough to stimulate strong differential responses compared to BmFEtet. Second, the *Wolbachia*-depleted *B. malayi* extract used in this study was not tested for effective depletion of *Wolbachia* due to time constraint and no residual extract available at the end of the field work. Therefore, it can not be completely excluded that the lack of difference upon use of BmFE and BmFEtet may be due to the residual presence of *Wolbachia* in the BmFEtet extract. Third, for the same reasons stated above, *B. malayi* extracts could not be tested for the presence of contaminating LPS and an LPS-blocking reagent such as polymyxin B was not used in these cultures. Therefore it is equally possible that low levels of LPS contaminating both extracts may have induced the equal responses observed. Besides technical-related reasons, it is also possible that none of the investigated cytokines would be specifically induced by *Wolbachia*. However, this is unlikely as both IL-6 and TNFα have been reported to be induced in DCs and macrophages by *Wolbachia*-containing but not *Wolbachia*-devoid filarial extracts [47, 153].

To conclude, the results presented here added novel information about cytokine responses in *Onchocerca* infected patients. However, further studies should investigate the role of *Wolbachia* in this respect. In particular, preliminary titration experiments should be performed and the presence of possible contaminants in filarial extracts carefully ruled out. It would also be important to link this investigation with the spectrum of clinical manifestations, to shed light on the role of *Wolbachia*, if any, not just in acute but also in chronic skin pathology. Furthermore, stringent criteria should be applied in the classification of mf$^-$ patients. In this study, these subjects were excluded from the analysis because of the uncertainty in classifying their true infection status, but it would be important to investigate this patient category to better understand the role of mf versus adult parasites in shaping the immune profile in onchocerciasis. Finally, of particular importance would be to use stage-specific parasite extracts with and without *Wolbachia* in PBMC stimulations, and to investigate cytokine production changes after macrofilaricidal treatment. This in particular would shed light on the role of each parasite stage, and *Wolbachia* within them, in shaping the systemic cytokine response, for example in response to newly acquired L3 after adult clearance or in the presence of adults.

Chapter 4. THE INTERACTION BETWEEN NEUTROPHILS AND *WOLBACHIA*

"To view immunology as the host's participation in the competition between genomes [the host's genome and genomes other than that encoded in the germline] helps explain what makes the neutrophil as fascinating as it is indispensable. Surprisingly, some immunologists seem not to share this view. Say neutrophil, and they move on, thinking: inflammation, not immunity. They disrespect the cell's 'nonspecificity' and consider its best-studied behaviours – crawling, eating, and disgorging prepacked enzymes and partially reduced molecules of oxygen – as rudimentary. Finally, scientists who are interested in anti-inflammatory therapeutics are discouraged from targeting neutrophils because it seems futile to try to suppress neutrophil-dependent tissue damage without serious side effect of increasing the host's risk from infection. [...] It is time to set aside the view that neutrophils are destructive cells that arrive too early, lash out too blindly and live too briefly to be of interest to immunologists."

Carl Nathan
Neutrophils and immunity: challenges and opportunities

Nature Reviews Immunology, 2006 [262]

ABSTRACT

The host inflammatory response to *Onchocerca volvulus* microfilariae and their endosymbiont *Wolbachia* is at the basis of onchocercal pathology. A major role is played by *Wolbachia* peptidoglycan-associated lipoprotein (wBmPAL) as the trigger of this response via TLR2/6 activation and by neutrophils as the effector cells. Neutrophil infiltration into *O. volvulus* infected tissues depends on the presence of *Wolbachia,* but their role in the host-parasite interplay remains poorly understood, and the interaction between *Wolbachia* and these cells has not been thoroughly studied.

The work presented here investigates the effect of a synthetic diacylated lipopeptide of wBmPAL (WoLP) on purified human neutrophils *in vitro*. It was found that WoLP modulated the surface expression of adhesion molecules involved in rolling and adhesion and of Fcγ Receptors, and exerted a chemotactic effect on neutrophils. Neutrophils produced IL-8 upon exposure to WoLP, which also induced and primed for the production of reactive oxygen species. Finally, WoLP enhanced neutrophil survival by delaying cell apoptosis. These results show that WoLP activates human neutrophils *in vitro*, supporting previous studies demonstrating the link between *Wolbachia* and neutrophils in the host inflammatory responses to *O. volvulus* infection and disease pathogenesis. These results also identify *Wolbachia* peptidoglycan-associated lipoprotein as a key molecule driving human neutrophil recruitment and activation.

4.1 INTRODUCTION

4.1.1 Overview of neutrophil biology

Neutrophilic polymorphonuclear leukocytes (neutrophils) are the most abundant population of circulating white blood cells and major effectors of innate immunity [263]. They are classically regarded as mediators of the earliest phases of acute inflammation, with a primary role in resistance against extracellular pathogens through phagocytosis and killing of phagocytosed pathogens with lytic enzymes such as lysozyme, bactericidal molecules such as defensins, and reactive oxygen species (ROS) [264-266]. In the past 20 years, however, the view of neutrophils being rather "basic" and "dead end" effector cells has been completely revolutionized. Indeed, neutrophils have emerged as a vital component of the effector and regulatory networks of both innate and adaptive immunity, orchestrating the response to pathogens from the early "non-specific" phase, to the regulation of adaptive immunity, resolution and tissue repair phase [263]. They have also been recently recognized as functional antigen presenting cells (APCs) [267]. Moreover, increasing evidence exists of their involvement in chronic inflammation, autoimmunity and cancer. These aspects have been recently extensively reviewed in [262, 263].
The interaction of neutrophils with pathogens and their cross-talk with other immune and non-immune cells in inflamed tissues and lymph nodes leads to the modulation of their main physiological activities, which can be summarized as adherence and migration, phagocytosis, degranulation, production of pro- and anti-inflammatory mediators, and death. A brief overview of some of these functions is provided below, in light of the functional assays carried out in the experimental work presented here.

Neutrophils are recruited from the blood stream into tissues, crossing the blood vessel wall. This is a complex multi-step process consisting of rolling, adhesion and transendothelial migration (reviewed in [264, 265]). It occurs mostly in post-capillary venules where endothelial cells, activated by pro-inflammatory stimuli generated in the inflamed/infected tissue, up-regulate adhesion molecules and present surface chemoattractants for neutrophils such as IL-8 on their luminal surface. Up-regulated adhesion molecules on endothelial cells bind corresponding ligands constitutively expressed on neutrophils in a sequence of attachments and detachments that progressively reduces the speed of neutrophil rolling on the endothelium.
L-Selectin is the most important neutrophil adhesion molecule involved in the

rolling process. Constitutively expressed, it transiently increases its binding capacity upon cell activation, and is then shed from the cell surface [264, 268]. After rolling, firm adhesion to the endothelium is mediated by β_2 integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1, CR3). Integrins are constitutively expressed at low levels on circulating neutrophils. Upon cell activation they are up-regulated due to the mobilization of preformed receptors from granules and their ligand binding capacity increases. After adhesion, β_2 integrins also mediate the transendothelial migration of neutrophils [264, 268]. CD11b/CD18 is the major adhesion glycoprotein of neutrophils. It is also involved in the binding of complement C3bi-coated particles and fibrinogen, and its engagement activates phagocytosis, respiratory burst, degranulation, adhesion, chemotaxis and aggregation [265]. The modulation of surface adhesion molecules is a pivotal step in the activation of neutrophils, and shedding of L-Selectin and up-regulation of CD11b have been used extensively as markers of neutrophil activation *in vitro*.

Upon activation, the generation of ROS and the lytic digestion of phagocytosed material can occur both separately [269, 270] or be part of the same process [266]. NADPH oxidase activity, the core of ROS production, is required for optimal activity of neutral proteases contained in the granules and myeloperoxidase contained in azurophil (primary) granules potentiates the bactericidal activity of the ROS H_2O_2 by producing halogenate intermediates [266]. ROS production, named “respiratory burst”, is a multi-step process. NADPH oxidase is a complex of cytosolic and membrane-bound components, which are disassembled in resting neutrophils. Upon stimulation, the components of the complex assemble in an electron transport chain, and generate ROS, mainly superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Most stimuli induce low-level and delayed production of ROS. The time gap between respiratory burst triggering and actual production of reactants is the time required for the assembly of the oxidase complex. The quantity and speed of ROS production are increased when pre-activated (primed) neutrophils are stimulated. Priming is induced by sub-stimulatory concentrations of activating molecules and leads to up-regulation of structures and functions, in this case oxidase components. Upon stimulation after priming, the neutrophil response is then faster and quantitatively increased. The details of activity and regulation of NADPH oxidase are reviewed in [271].

Neutrophils possess biosynthetic capacity and can be induced to express genes encoding for inflammatory mediators such as Fc receptors, cytokines,

chemokines and MHC II molecules [272, 273]. Whereas some are synthesized upon stimulation, others are pre-stored in granules and quickly released after activation [274]. The range of cytokines and chemokines produced by neutrophils is wide, but there are discrepancies between cytokines produced by mouse and human neutrophils, and no consensus exists, so far, on the production of IL-6, IL-10, IL-17 and IFNγ by human neutrophils [263]. IL-8 is a potent neutrophil chemoattractant and activator [275-277] and is the cytokine most abundantly secreted by neutrophils themselves[278]. Production of IL-8 upon stimulation is a widely used indicator of neutrophil activation in experimental studies.

Resting neutrophils are short lived cells, with a half life of ~8-12 hours. Nevertheless, their life span is considerably increased upon stimulation by microbial products and cytokines, hypoxia, transmigration, and adhesion [279-281]. Apoptosis followed by ingestion by macrophages is the major mechanism of neutrophil clearance and is involved in active suppression of the production of inflammatory mediators [282]. Macrophages recognise apoptotic neutrophils via the exposure of phosphatidylserine that translocates from the inner to the outer cell membrane sheet during apoptosis. Besides necrosis and apoptosis, a novel type of cell death has been described for neutrophils and other cells such as mast-cells, eosinophils and macrophages (reviewed in [283, 284]), which leads to the formation of neutrophil extracellular traps (NETs), in a process referred to as NETosis [285]. NETs are composed of nuclear components (DNA and histones) and molecules from the granules, constituting a net that traps microorganisms, promoting their contact with anti-microbial proteins [286] and antibody-like molecules that facilitate their uptake via Fcγ receptors and opsonisation [287, 288].

4.1.2 TLRs, lipoproteins and neutrophils: a literature review

Toll-like receptors

The innate immune system interacts with pathogens through a number of receptors named Pathogen Recognition Receptors (PRRs), which recognise microbial conserved structures called Pathogen-Associated Molecular Patterns (PAMPs) [289, 290]. Recognition of PAMPs rapidly activates intracellular signalling pathways leading to the induction of inflammatory responses. The first recognised and best studied PRRs are Toll-like receptors (TLRs), so called because of their homology with the Toll protein of *Drosophila*, responsible,

among other functions, for fly development and anti-fungal responses [291, 292]. Structurally, TLRs are transmembrane glycoproteins characterized by a ligand-binding domain, a transmembrane region, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain required for downstream signalling [293]. Dimerization of TLRs upon ligand binding, and in some cases aggregation with specific co-receptors (such as MD-2, CD14 and LBP for TLR4 or CD14, CD36 and LBP for TLR2/6), induces the recruitment of adaptor molecules activating intracellular signalling pathways which lead to the expression of inflammatory cytokines and type I interferon [294] (Figure 4.1). To date, 10 TLRs have been identified in humans, but only TLR1-9 are well characterized. Table 4.1 summarises their cellular location, major ligands, and signalling pathway(s). TLR2 constitutes an exception among TLRs, as it forms heterodimers with either TLR1 or TLR6 (and possibly TLR10) [295], whereas other TLRs form homodimers. TLR2/1 binds triacylated lipopeptides from GRAM- bacteria, while TLR2/6 is activated by diacylated lipopeptides mainly from GRAM+ bacteria and *Mycoplasma*. So far, TLR2 activation by human helminths, besides *Wolbachia*-containing parasites, has only been reported with *Schistosoma* and *Ascaris* (ref [295]).

TLR2 and TLR4 have been reported as the most promiscuous receptors, with binding capacity of extremely diversified structures [296, 297]. Nevertheless, extensive debate exists on their effective ligand range, as the activity of some putative ligands may be attributable to low-level contamination of other TLRs agonists [47, 295, 297, 298]. Endogenous ligands for TLRs have also been proposed as a possible link between TLR activation and the induction of chronic inflammation and autoimmune diseases [294]. However, emerging evidence indicates that these molecules should be regarded as PAMP-binding molecules or PAMP-sensitizing molecules rather than true ligands, increasing TLRs sensitivity to “classic” non-self PAMPs [299].

TLR expression has been described in most cells of the innate immunity, including dendritic cells, monocytes/macrophages, Natural Killer cells, mast cells and granulocytes. Each of these cells express a specific range of TLRs, which can be simultaneously activated by multiple structures of a same pathogen, providing articulated immunological responses, in a complex interplay of additivity, synergy and tolerization [300-303]. Non-immune cells such as epithelial cells, endothelial cells, smooth muscle cells, and fibroblasts have been shown to express TLRs as well. Activation of TLRs on these cells leads to the expression of a pro-inflammatory phenotype with the production of

cytokines, chemokines, antimicrobial peptides and up-regulation of surface molecules involved in leukocyte migration [304]. Finally, it has been shown that TLRs provide an important link between innate and adaptive immunity, influencing the quality of T and B cell responses [304, 305]. This happens via activation of APCs, but there is increasing evidence of TLR expression on B and T cells and direct activation of these cells by TLR ligands, both in mice and humans [133, 134, 306-309].

Localization	**TLRs**	**Ligands***	**Main signalling pathway(s)**
Cell surface	TLR2/1	Triacyl lipopeptides	MyD88, TIRAP, NF-κB
	TLR2/6	Diacyl lipopeptides	MyD88, TIRAP, NF-κB
	TLR4	Lipopolysaccharide	MyD88, TIRAP, TRAM, TRIF, NF-κB, IRF3/7
	TLR5	Flagellin	MyD88, NF-κB
Intracellular vesicles	TLR3	ss and ds viral RNA	TRIF, NF-κB, IRF3/7
	TLR7	ss RNA from viruses, bacteria and fungi	MyD88, NF-κB, IRF7
	TLR8	ss viral RNA	MyD88, NF-κB, IRF7
	TLR9	DNA with unmethylated CpG motifs	MyD88, NF-κB, IRF7

Table 4-1. Description of human TLRs. *Major and un-equivocally accepted ligands.

Figure 4-1. TLR trafficking and signalling. MP=macrophages, cDC=conventional dendritic cells, pDC=plasmacytoid dendritic cells, LPDC=lamina propria dendritic cells, iMO=inflammatory monocytes, LRO=lysosome related organelle. Reproduced with permission (License Number: 2854240358967) from [294].

TLR activation in human neutrophils

Human neutrophils express all TLRs with the exception of TLR3 [310], and major attention has been directed to TLR2 and TLR4 activation in these cells [311]. Stimulation of human PMN with Pam_3CSK_4 (TLR2/1 ligand), MALP-2 (TLR2/6 ligand) and LPS (TLR4 ligand) induces neutrophil activation, as assessed by acquisition of an activated cell shape, shedding of surface L-Selectin and up-regulation of CD11b, induction and priming for fMLP-stimulated respiratory burst, degranulation, IL-8 secretion, and increased phagocytosis [310, 312-316]. These functions appear to be directly mediated by TLR2 and TLR4 ligands as they occur upon stimulation of monocyte-free neutrophil cultures [313, 316], but are amplified in the presence of monocytes, at least where TLR4 stimulation is concerned [317].

TLR2 and TLR4 agonists potentially regulate neutrophil migration into tissues. Pam_3CSK_4, MALP-2 and LPS have been reported to increase random migration of neutrophils [316, 318] and to partially inhibit their recruitment by IL-8 and fMLP [310, 319]. This is possibly achieved via down-regulation of chemokine receptors on neutrophils [310, 320]. The fact that this anti-chemotactic effect is induced when neutrophils are pre-treated with TLR agonists and subsequently exposed to a concentration-gradient of the chemoattractant has been suggested to be a mechanism of control of neutrophil recruitment into tissues [310].

Modulation of neutrophil apoptosis by TLR2 and TLR4 ligands has received much attention. Sabroe and co-workers [313] found that in highly pure neutrophil cultures, short-term (4 hours) apoptosis was delayed by stimulation with LPS but not by Pam_3CSK_4, while apoptosis delay at 22 hours was achieved only by LPS when neutrophil cultures were supplemented with 5% PBMC. They suggested that neutrophil survival over a prolonged time course could be due to the LPS-induced monocyte synthesis of cytokines with pro-survival activity, such as IL-1β, IL-8, GM-CSF or TNFα. These cytokines are indeed secreted by PBMC stimulated with LPS and Pam_3CSK_4 [313]. Subsequent studies from the same group found that IL-1β did not exert direct effects on highly pure neutrophils, but did enhance cell survival in 5% PBMC co-cultures, suggesting that the IL-1β-dependent effect could be mediated by bystander cells [321]. Parallel studies showed TNFα to have both a direct and a more potent PBMC-mediated indirect effect on neutrophil life span, possibly by inducing an unidentified pro-survival factor in monocytes [322]. Contrasting results on the effect of TLR2 ligands on neutrophil survival have been provided by other groups. Francois *et al* [323] found that stimulation with Pam_3CSK_4 and MALP-2 induced long-term apoptosis delay in highly purified neutrophils, and this effect was increased when neutrophils were stimulated in whole blood cultures. Long-

term delayed apoptosis in gradient-purified (i.e. non-highly pure, < 5% PBMC contaminated) neutrophil cultures upon stimulation with comparable concentrations of Pam_3CSK_4 was also reported by Power *et al* [324]. Finally, in the work of Wilde and colleagues [316], MALP-2 was found to have direct effects on short-term but not long-term apoptosis delay in highly pure neutrophil cultures, whereas MALP-2 induced long-term apoptosis delay on neutrophils transmigrated through endothelial cells.

4.1.3 The role of neutrophils in helminth infections

The role of granulocytes in helminth infections has been recently reviewed [325, 326]. The vast majority of studies have investigated the role of eosinophils in host defences and pathogenesis of helminth infections. Although there is a lack of a complete consensus, the overall picture indicates a role for eosinophils in protection against the invasive larval stages (but less so adult worms) of most tissue-dwelling heminths or helminths whose life cycle include tissue migration but dispensable in the case of strictly gastrointestinal parasites (reviewed in [327, 328]).

On the contrary, neutrophils have received less attention. *In vitro*, neutrophils were found to mediate killing of larval nematodes and cestodes in the presence of immune sera [329, 330]. Moreover, heminth-derived factors were reported to be able to both promote [331-333] but also inhibit [334-343] neutrophil recruitment and functions. In animal models, neutrophils have been reported to be recruited around the infection site of larvae of *Schistosoma mansoni* [344, 345], *Taenia crassiceps* [346] and *Heligmosomoides polygyrus* [347], however, their active role in mediating protection has been only shown in murine infection with *Strongyloides stercoralis* [348-350].

The results obtained on the role of granulocytes in mediating protection against helminths may be highly influenced also by the host-parasite system. This has been clearly pointed out for eosinophils [328]. Indeed, in natural infection systems non-specific inflammation is minimal whereas it is significant in surrogate hosts. For example, primary infection with *S. ratti* induces minimal eosinophil recruitment in the natural rat host, while a strong and protective early recruitment of eosinophils and granulocytes is elicited in the surrogate mouse host [351, 352]. Similarly, eosinophil depletion obtained by interference with IL-5 had no impact on primary infection of *H. polygyrus* and *Trichuris muris* in natural hosts [353-355] whereas it greatly reduced protection to *Strongyloides spp, O. lienalis* and *Angiostrongylus cantonensis* in non-

permissive hosts [245, 356-360]. On the other hand, eosinophil-mediated parasite killing may be more involved during the adaptive phase of immunity in natural hosts, as observed in cestode infections in sheep and pigs [361-363]. These considerations, together with the knowledge that neutrophil and eosinophil phenotype of mice and humans differ in several aspects [236], highlight the need to interpret and extrapolate results with caution.

4.1.4 Neutrophils-*Wolbachia* interaction and their role in onchocerciasis

Neutrophils are a major component of the inflammatory infiltrate around filarial nematodes, but their role in protection against the different life stages of the parasite is not completely clear. On the other hand, strong evidence supports their involvement in onchocercal pathology and their presence depends on that of *Wolbachia* (Figure 4.2 and 4.3)

The interaction of neutrophils with different filarial life stages

In vitro experiments showed that neutrophils are able to kill *O. volvulus* L3 in the presence of immune serum [57], and this does not differ when sera from mf^+ of mf^- subjects are compared. Neutrophils are also recruited upon primary infection of mice with *L. sigmodontis* and *B. pahangi* L3 [364-366], suggesting that presence of acquired immunity is not required. Nevertheless, protection upon larval challenge after vaccination with irradiated L3 has been reported to be mediated mainly by eosinophils in an antibody-dependent manner [59, 225, 234, 364, 367]. The role of *Wolbachia* in the early recruitment of neutrophils by infective larvae and its impact on protection upon challenge infection has not been investigated so far.

In contrast to eosinophils that appear to be involved in the early phase of infection, neutrophils appear to have a role in later stages. In mouse models of infection with *L. sigmodontis*, neutrophil containing granulomas form around adult worms, and mouse strains with defective neutrophil recruitment and functions have impaired ability to kill adult worms at late stages of infection [226-228, 234, 368]. Interestingly, both IFNγ and IL-5 (possibly *via* eosinophils and macrophages) were found to be responsible for neutrophil recruitment in this model, linking both Th1 and Th2 responses to protection through granulocytes [226-228, 234]. Moreover, the fact that IL-10, strongly induced during filarial infections, is able to inhibit neutrophil recruitment [369] may be

consistent with a role for neutrophils in protection toward adult filarial stages. In contrast to these observation, however, in the natural host no obvious detrimental effect of neutrophils for adult *Onchocerca* has been found [44, 370], although defensin and calgranulins have been found on the surface of adult worms, suggesting neutrophil degranulation [81, 371, 372], and calgranulin C has been reported to inhibit worm motility and have a filaricidal effect on adult *B. malayi in vitro* [372]. However, as part of the more abundant inflammatory infiltrate around worms, neutrophils may contribute to the lower parasite load in nodules from patients with Sowda [87]. In *Onchocerca* infections, but also around *L. sigmodontis* worms in the mouse [234], neutrophils are found surrounding and attached to adult worms in onchocercomas, often forming cyst-like structures around males and the anterior end of females, and their presence depends upon the presence of *Wolbachia* [44] (Figure 4.2). Neutrophil infiltration in nodules from patients treated with doxycycline, containing *Wolbachia*-depleted worms, was dramatically reduced compared to placebo controls [44]. Furthermore, neutrophils were shown to accumulate only around adult *Wolbachia*-containing *Onchocerca spp* parasitizing animals, and not around worms of species naturally devoid of the endosymbiont [44, 373]. Although treatment with doxycycline renders adult females sterile, the absence of neutrophils in doxycycline-treated nodules did not appear to be due to lack of microfilariae (mf), as neutrophils were found to accumulate also around nulliparous females and males [44, 374, 375]. Also, the absence of neutrophil recruitment in *Wolbachia*-depleted nodules was not a consequence of the presence of degenerated or dead worms after treatment with doxycycline [44]. Indeed, this reduced number of neutrophils in onchocercomas from doxycycline-treated patients occurred around parasites still alive, suggesting that excretory/secretory products of viable worms, likely *Wolbachia*-derived products, may be the source of neutrophil chemoattractants. Further evidence of the association between the presence of *Wolbachia* and recruitment of neutrophils comes from cattle onchocerciasis, where neutrophil depletion in *O. ochengi* nodules occurred after *Wolbachia* depletion by oxytetracycline treatment, and neutrophil infiltration reappeared upon recovery of *Wolbachia* loads in worms [45].

Complementary *in vitro* experiments also demonstrated that *Wolbachia*-containing but not *Wolbachia*-depleted *O. volvulus* extracts exerted a chemotactic effect on isolated human neutrophils [44, 375]. Moreover, only extracts from *Wolbachia*-containing *O. volvulus* parasites were able to induce IL-8 secretion by these cells, in contrast to *O. volvulus* extracts from doxycycline-treated parasites or extracts from the *Wolbachia*-devoid

Acanthocheilonema viteae [44].
In contrast to neutrophils, infiltration of eosinophils in nodules is scanty, and associated with patency [52, 376], with the exception of Sowda patients, where they constitute an abundant infiltrate around adults and mf [87, 377].

In studies on microfilariae, eosinophils rather than neutrophils have been indicated as responsible for mf clearance in animal models of filariasis [244, 245, 378]. However, live mf have been shown to attract and be attacked *in vitro* by both neutrophils and eosinophils in the presence of immune serum and complement [379-383], with serum-mediated eosinophil cytoadherence to mf being stronger in patients with severe compared to mild onchodermatitis and with an inverse correlation with mf load [85]. In lymphatic filariasis, a recent study has reported that human neutrophils are activated *in vitro* (degranulation and cytokine production) preferentially by immune complexes from infected compared to uninfected subjects [384]. However live mf are not seen to be attacked *in vivo* in histological studies of skin biopsies [69, 75, 76, 385], possibly because of motility of mf, which impedes adherence of inflammatory cells to worms [386]. Greene and colleagues [379] also suggested that this may be due to the fact that intact mf in the skin are located in the interstitium, soaked in the antibody- and complement-poor interstitial fluid, an environment overall not favourable for antibody and complement mediated cellular activation. In contrast, Folkard *et al* [387] showed that eosinophil but not neutrophil depletion by anti-IL-5 and anti-NIMP-R14 antibodies respectively, impaired the clearance of both primary and challenge infections of *O. lienalis* mf in the mouse, suggesting that the presence of an adaptive immune response would not be required for the eosinophil-mediated clearance of mf in this model, as also shown by Hogarth *et al* [245] in the same mouse model. The absence of a role for neutrophils in mediating mf killing has also been reported by Simons *et al* [124] in mouse model of *B. malayi* infection. Neutrophils, together with eosinophils, have been shown to attack damaged mf after DEC treatment and in patients with Sowda [75, 87, 377] and strong evidence exists of their role in the pathogenesis of onchocercal keratitis and dermatitis.

The role of Wolbachia and neutrophils in onchocercal dermatitis and keratitis

Experimental models and observations in patients show that *Wolbachia*-induced recruitment and activation of neutrophils have a key role in the pathogenesis of onchocercal keratitis and dermatitis, as illustrated in figure 4.2 and 4.3.

The death of mf in eye tissues and the subsequent inflammatory reaction have long been known to be at the basis of onchocercal ocular pathology [50, 71, 388, 389]. Mouse models of onchocercal keratitis have been widely used to study this process. Neutrophils surround mf in the cornea and are recruited after injection of filarial extracts within 24 hours in both primary infection and after vaccination, while eosinophils peak after 72 hours [154-156, 390, 391]. Neutrophils were also found to mediate corneal opacity and to replace eosinophils as late cell infiltrate in eosinophil-lacking IL-$5^{-/-}$ mice [390], while their depletion by antigranulocyte antibodies ablated corneal opacification in IL-$5^{-/-}$ mice (ref [391]). Granulocyte recruitment to the stroma leads to corneal opacity and haze that resolves within a few days in the case of primary injection but is exacerbated and protracted in the presence of adaptive immunity, with both T cells, B cells and antibodies being required [154, 155, 391-395]. It has been demonstrated that *Wolbachia* is responsible for the recruitment and activation of neutrophils with subsequent induction of corneal opacity and haze [154-156, 164]. After injection of mf in the cornea, *Wolbachia* was found in neutrophil phagosomes fusing with granules [155]. Moreover, neutrophil recruitment, development of corneal opacity, and production of chemokines in the corneal stroma occurred upon injection of isolated *Wolbachia* bacteria and *Wolbachia*-containing filarial extracts, but not of *Wolbachia*-negative extracts from nematodes treated with tetracycline or from *A. viteae*, [155, 164]. Neutrophil recruitment and corneal haze are TLR2 and MyD88 dependent [151, 154, 156]. As shown in figure 4.3, TLR2-induced production of chemokines by resident cells likely mediates the initial recruitment of neutrophils into the corneal stroma [154, 396]. In turn, recruited neutrophils are also activated by *Wolbachia* via TLR2-MyD88 and produce chemokines that promote further recruitment of cells into the cornea [154-156]. When an adaptive immune response is present, TLR2-dependent *Wolbachia* induction of IFNγ enhances the recruitment of neutrophils to the cornea [151, 397] in conjunction with specific antibodies present in the corneal stroma [392, 395].

As described by Pearlman and colleagues in a mouse model of onchocercal dermatitis [398], the temporal recruitment of granulocytes in the skin appears to mirror that occurring in the cornea, with neutrophils constituting an early infiltrate and eosinophils being recruited later. In infected patients, an inflammatory infiltrate composed mainly of neutrophils appears within the first 24 hours from topical application of DEC, surrounding only damaged but not intact mf (Figure 4.2) [75]. The link between presence of *Wolbachia* and recruitment of neutrophils is further strengthened by the observation of Brattig

[52], who reported the recruitment of eosinophils, but not neutrophils, in the skin after DEC administration to doxycycline-treated patients.

Neutrophil activation was also observed during adverse reactions following treatment with DEC and ivermectin (Figure 4.2). The occurrence and severity of adverse reactions after microfilaricidal treatment correlated with microfilarial loads and the presence of *Wolbachia* DNA, whole bacterial cells, neutrophilia and levels of pro-inflammatory cytokines and neutrophil derived molecules (calprotectin, calgranulin, elastase) in the blood [72, 78, 147, 150]. Also, circulating levels of the neutrophil chemoattractant IL-8 were reported to peak at 12 hours post-ivermectin treatment [73].

The Wolbachia-neutrophil partnership in onchocerciasis

Knowledge of the biological basis of *Wolbachia* – filariae symbiosis is limited. Comparative genomics of *Wolbachia* and filarial hosts suggested that several biochemical pathways are provided by the symbiont to the worm [24], in addition, a role for *Wolbachia* in the modulation of the inflammatory and immune response to the worm has also been proposed [44-46, 97].

The observation that *Wolbachia*-dependent encapsulation by neutrophils has no apparent detrimental effect on *O. volvulus* adults, which live more than 15 years in an immunocompetent host, has raised the question of whether neutrophils may be considered an effective host defence mechanism against the parasite, or may play a role in parasite survival [44]. In this context, it has been proposed that neutrophil-filled cysts around the anterior end of females could facilitate nutrient uptake and mating with males [44]. Neutrophils have also been suggested to protect adult worms from the potentially lethal attack of eosinophils [45, 370]. Following *Wolbachia* clearance by oxytetracycline, neutrophils were replaced after a short time by degranulating eosinophils in nodules of *O. ochengi* in cattle, and neutrophil infiltration resumed when *Wolbachia* loads in worms recovered [45]. Further work by the same group showed that eosinophil recruitment was not a non-specific consequence to the presence of moribund or dead worms, as degranulating eosinophils were associated with intact, fertile worms [370]. Moreover, significant influx of eosinophils was not observed after treatment of animals with melarsomine that kills adult parasites but has no effect on *Wolbachia* [370]. Unfortunately, investigation of human nodules at equivalent time points has not been carried out so far. Findings in the *L. sigmodontis* model gave inconclusive results. Depleting neutrophils by anti-GM-CSF antibodies, Al-Qaoud *et al* [234] showed that neutrophils rather than eosinophils are required for adult worm killing.

Moreover, Saeftel *et al* [227] reported increased worm loads in IFNγKO mice, which had fewer neutrophils but comparable eosinophil infiltrates around adult worms than wild type controls. However, work conducted with IFNγKO and IL5KO mice by the same group showed a parallel dynamics of the two granulocyte populations, making it difficult to dissect the relative role of each cell type [226, 228].

Neutrophils clearly mediate the inflammatory response to damaged mf, but the only study specifically addressing their possible role in the containment of mf burden *in vivo* suggested that these cells, although recruited in higher numbers in parallel with mf clearance, were not involved in this process [124]. In the *L. sigmodontis* mouse model, increased neutrophil recruitment around adults in B-cell deficient mice was associated with decreased production of mf by adult females [364], but the causative involvement of neutrophils in this phenotype was not investigated.

Finally, although a role for both Th1 (IFNγ) and Th2 (IL-5) responses in neutrophil recruitment have been found in mice [226-228, 234], the contrary, i.e. the possible immunomodulatory role of neutrophils on T cell differentiation, has yet to be investigated in filarial infections. A role of neutrophils in directing the Th response toward a Th1- or Th2-type has been shown, for example, in mouse models of *Leishmania major* [399] and *Candida albicans* [400, 401] infection, with the type of immune skewing depending on the strain of pathogen used. Thus, it is possible to speculate that *Wolbachia* may influence the type of cellular response toward different filarial parasite stages also via interaction with neutrophils.

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Tamarozzi F, Halliday A, Gentil K, Hoerauf A, Pearlman E, Taylor MJ: **Onchocerciasis: the role of Wolbachia bacterial endosymbionts in parasite biology, disease pathogenesis, and treatment**. *Clinical microbiology reviews* 2011, **24**(3):459-468.

Figure 4-2. Neutrophils are major effector cells in onchocerciasis, and their infiltration in *O. volvulus* infected tissues depends on the presence of *Wolbachia*. *Wolbachia* released after Mf death in the cornea activate resident cells in the corneal stroma via TLR2/6-MyD88 activation. These cells recruit neutrophils through secretion of CXC chemokines, a process perpetuated by inflammatory cells. After microfilaricidal treatment, large loads of *Wolbachia* are released, leading to cutaneous and systemic side effects. In the skin, neutrophils are the first cells to be recruited and activated, and at a systemic level, adverse events are associated with neutrophilia and circulating levels of neutrophil-derived antibacterial proteins. Neutrophils constitute an abundant cell infiltrate around *Wolbachia*-containing worms in onchocercomas, but are virtually absent in nodules containing *Wolbachia*-free or *Wolbachia*-depleted worms. Figure from Tamarozzi *et al* [19]. Copyright © American Society for Microbiology, Clinical Microbiology Review, 2011, 24(3): 459-468, doi: 10.1128/CMR.00057-10.

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Figure 4-3. Neutrophils are major effector cells in the mouse model of onchocercal keratitis. *Wolbachia* released from dying Mf in the corneal stroma (1) activates resident cells (fibroblasts and bone marrow-derived cells) via TLR2/6 (2). This activation induces these corneal resident cells to produce chemokines (3), which in turn induce the recruitment of neutrophils in the corneal stroma (4). Here, neutrophils are activated by *Wolbachia* via TLR2/6 and produce chemokines inducing further neutrophil recruitment (5). Activated neutrophils degrade the corneal matrix causing haze and visual impairment (6). Figure from Tamarozzi *et al* [19]. Copyright © American Society for Microbiology, Clinical Microbiology Review, 2011, 24(3): 459-468, doi: 10.1128/CMR.00057-10.

4.1.5 Scope of the experimental study

The host inflammatory response to *O. volvulus* mf and their endosymbiont *Wolbachia* is at the basis of onchocercal pathology. Neutrophils are pivotal effector cells in the pathogenesis of onchocercal diseases and their presence in *O. volvulus* infected tissues depends on that of *Wolbachia*. Nevertheless, their role in the broader *O. volvulus* parasitism is not clear.

So far, very few studies assessed the response of neutrophils to filarial stimuli and *Wolbachia*, with only few functions being investigated. Mouse neutrophils have been found to up-regulate CD11b, shed L-Selectin and to produce TNFα and chemokines upon stimulation *in vitro* with isolated *Wolbachia* and *Wolbachia*-containing filarial extracts but not with extracts from parasites depleted of *Wolbachia* by tetracycline treatment [154-156]. Moreover, *Wolbachia*-containing but not *Wolbachia*-depleted filarial extracts have been reported to be chemotactic for human neutrophils *in vitro,* and to induce IL-8

production from these cells [44, 375].

Wolbachia peptidoglycan-associated lipoprotein (wBmPAL) is highly expressed on the surface of *Wolbachia* bacteria [157]. The synthetic diacylated lipopeptide of wBmPAL (WoLP) has been shown to exert the same TLR2/6-mediated effects of *Wolbachia*-containing filarial extracts on macrophages and DCs *in vitro*, and to induce TLR2-dependent neutrophil mediated keratitis in mice [47]. Nevertheless, its effects on the major functions of human neutrophils have not been explored.

Knowledge of how the filariae-*Wolbachia* system and their derived molecules shape neutrophil functions is a first important step to understand the role of these cells in the broader infection and disease pathogenesis. This work specifically investigated the effects of WoLP and filarial extracts containing or depleted of *Wolbachia* on the recruitment and activation of human neutrophils *in vitro*. The use of synthetic lipopeptides offers the advantage of excluding possible co-stimulatory effects of bacterial-derived TLR ligands, in particular lipoproteins and LPS, that can contaminate stimulating molecules expressed in *E. coli* [47].

4.2 METHODS

4.2.1 Stimuli for neutrophil functional assays

Soluble extract from *B. malayi* adult females containing *Wolbachia* (BmFE) and *Wolbachia*-depleted by tetracycline treatment (BmFEtet) was prepared as destribed in the Appendix. Synthetic WoLP (20-mers of the N-terminal region of wBmPAL, di-palmitoylated at the N-terminal cysteine residue [47]) was from EMC Microcollections, as well as synthetic triacylated lipoprotein (Pam_3Cys-SK_4) and synthetic diacylated lipoprotein (Pam_2Cys-SK_4). CpG oligonucleotide (ODN1826) was from Pfizer/Coley, recombinant human TNFα from Calbiochem, recombinant human GM-CSF from Roche, and N-formyl-methionine-leucine-phenylalanine (fMLP), Phorbol 12-myristate 13-acetate (PMA), Ionomycin, and ultra-pure LPS from Sigma Aldrich.

4.2.2 Evaluation of filarial extracts and WoLP reactivity

To evaluate successful depletion of *Wolbachia* of *B. malayi* worms by tetracycline treatment and activation of TLR2/6 by both *Wolbachia*-containing extract and WoLP, reactivity of BmFE, BmFEtet and WoLP was tested using mouse macrophages selected by plastic adherence from peritoneal inflammatory cells obtained from Wild Type (WT) C57BL/6, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ mice, all on a C57BL/6 background. Mice were obtained from Prof S. Akira, Osaka University, Japan, and maintained in the Biomedical Services Unit, University of Liverpool. Animal genotyping was carried out by Miss A. Halliday, (Molecular and Biochemical parasitology, LSTM) according to the protocol detailed in the Appendix. Cells from 3 mice per genotype were obtained by intraperitoneal injection of thioglycollate and kindly provided by Dr J Turner (Molecular and Biochemical Parasitology, LSTM) and cultured as described in the Appendix. Cells were cultured in triplicate at 2 x 10^5 cells/well in flat bottomed 96-well tissue culture plates (Nunc) at 37°C with 5% CO_2 for 24 hours in the presence of WoLP (0.1 μg/ml), BmFE (200 μg/ml), and BmFEtet (200 μg/ml). CpG (25 μg/ml), Ultra-pure LPS (0.1 μg/ml), Pam_3Cys-SK4 (1 μg/ml), Pam_2Cys-SK4 (0.1 μg/ml), and medium alone were used as control stimuli for TLR9, TLR4, TLR2/1 and TLR2/6 respectively. Levels of secreted TNFα were measured by ELISA (Invitrogen), according to manufacturer's instructions.

4.2.3 Isolation and culture of human neutrophils

The use of white blood cells from adult healthy volunteers for this study was approved by the Research Ethics Committee of the University of Liverpool, UK.
Peripheral blood was collected by venipuncture in lithium-heparin and neutrophils (PMN) were isolated by Polymorphprep following manufacturer's instructions. Briefly, 10 ml blood was layered onto 10 ml Polymorphprep, centrifuged at 500 g (brake off) for 35 minutes at RT, and the PBMC collected by pipette suction from the more superficial cell layer. Polymorphonucleated cells were collected from the lower cell layer and washed once with RPMI 1640 culture media (Gibco) by centrifugation at 700 g for 5 minutes at RT. Contaminating red blood cells were lysed by incubation with 30 ml ammonium chloride lysis buffer (13.4 mM $KHCO_3$ + 155 mM NH_4Cl + 96.7 μM EDTA) in culture media (9:1 lysis buffer: culture media) for 3 minutes followed by 3 minutes centrifugation at 400 g at RT. Cells were resuspended in RPMI 1640

culture media and counted using a Beckman Coulter cell counter supporting Multinizer 3 software (Beckman Coulter). Cell viability was assessed by 0.2% trypan blue staining using a haemocytometer. The purity of isolated cell populations was assessed by cytospin and rapid Romanowsky stain. Briefly, 1 x 10^5 cells in 200 µl 1mM EDTA in PBS were loaded into cytospin chambers and centrifuged at 500 rpm for 5 minutes using Cytospin3 (Shandon). The cell monolayer was stained by rapid Romanowsky stain as per manufacturer's instructions. Differential count was performed on ≥ 700 cells by optical microscopy.

All cultures were performed at 37°C in a humidified incubator. Cell culture media was RPMI 1640 (+ 25mM HEPES + 2 mM L-glutamine), from Gibco. For incubations < 8 hours, PMN were resuspended at 5 x 10^6 cells/ml and cultured in 1.5 ml screw-top tubes (Eppendorf) with gentle rotation. For incubations ≥ 8 hours, PMN were resuspended in media supplemented with 10% heat inactivated human AB serum at 1 x 10^6 cells/ml in 24-well culture plates (Costar), and incubated in the presence of 5% CO_2. WoLP was used in a concentration range of 1 ng/ml to 5 µg/ml. DMSO was added to cultures in the same volume of WoLP stock and used as vehicle control for WoLP. BmFE and BmFEtet extracts were used at a concentration range of 50 µg/ml to 200 µg/ml. Ultra-pure LPS was used at 0.1 µg/ml, GM-CSF at 5 ng/ml and TNFα at 10 ng/ml.

4.2.4 Morphological assessment of neutrophil activation

PMN were cultured for 1.5 hours with WoLP (1 µg/ml), BmFE (200 µg/ml), BmFEtet (200 µg/ml), fMLP (0.01 µM), DMSO (vehicle control) and media alone. The morphology of PMN was visualised using a Zeiss Axiovert S100TV microscope (Carl Zeiss) supporting a Hamamatsu multiformat CCD camera (Hamamatsu Corporation) with AQM Advance 6 software (Kinetic Imaging).

4.2.5 Chemotaxis and chemokinesis assays

Chemotaxis and chemokines assays were performed using a transwell system (Millicell 24-wells Cell Culture Hanging Inserts, Millipore) in 24-well tissue culture plates pre-coated with sterile Poly-Hema to avoid strong adhesion of PMN to the plate surface. Briefly, 12 mg/ml Poly-Hema was dissolved in constant vigorous agitation in ethanol at 37°C. The Poly-Hema solution (400 µl/well) was added to wells. Plates were incubated for ≥ 12 hours at 37°C until

complete evaporation of ethanol, and stored at RT in sterile conditions.
For the chemotaxis assay, 800 µl of the chemoattractant to be tested (dissolved in RPMI 1640 culture media) was added in the well and the culture hanging insert placed above, allowing the bottom membrane to soak for at least 10 minutes. PMN (1 x 10^6 in 200 µl cell suspension) were placed in the upper chamber. To differentiate between chemotaxis (migration toward a chemoattractant gradient) and chemokinesis (increased random movements upon exposure to a stimulus in the absence of a gradient), the assay was carried out with equal concentrations of stimuli in both the upper and the lower chamber. The chambers were incubated for 1.5 hours at 37°C with 5% CO_2 and the cells migrated transwell resuspended and counted using a Beckman Coulter cell counter, supporting Multinizer 3 software (Beckman Coulter). To avoid counting of cell debris, only particles between 8 and 12 µm in diameter were counted.
WoLP (0.5-5 µg/ml) and BmFE (50-200 µg/ml) were tested for chemotaxis. WoLP (1 µg/ml), BmFE (200 µg/ml) and BmFEtet (200 µg/ml) were used to assess chemokinesis vs chemotaxis effect. DMSO was added to media in the same volume of WoLP stock (vehicle control) and fMLP (0.01 µM) was used as a positive control for chemotaxis.

4.2.6 Assessment of surface adhesion molecules and Fcγ Receptors expression

After 1 hour culture in the presence of 0.1 µg/ml WoLP, equal volumes of DMSO, or 200 µg/ml BmFE and BmFEtet, the surface expression of CD11b, CD18, L-Selectin, CD16 (FcγRIII) and CD32 (FcγRII) by PMN was assessed by flow cytometry (FC). GM-CSF (5 ng/ml) was used as positive control.
PMN (5 x 10^4 in 10 µl 0.2% BSA in PBS FC buffer) were stained with FITC-conjugated rat anti-human CD11b IgG_{2b} (Miltenyi Biotec, 5 µl antibody in 10 µl FC buffer), mouse anti-human CD18 IgG_1 (R&D Systems, 5 µl antibody in 10 µl FC buffer), mouse anti-human L-Selectin IgG_1 (R&D Systems, 5 µl antibody in 10 µl FC buffer), mouse anti-human CD16 IgG_1 (BD Pharmigen, 1 µl antibody in 10 µl FC buffer), mouse anti-human CD32 IgG_{2b} (BD Pharmigen, 1 µl antibody in 10 µl FC buffer) and mouse IgG_1 isotype control (Santa Cruz Biotechnology, 5 µl antibody in 10 µl FC buffer) for 30 minutes on ice in the dark. Previous work in the lab showed that mouse IgG_1 isotype control was equivalent to rat and mouse IgG_{2b}.
After fixation with 200 µl 2% paraformaldehyde in FC buffer for 15 minutes at

RT, cells were resuspended in 200 µl FC buffer (2 x 10^5/ml final concentration) and analysed with Guava EasyCyte Plus (Millipore) supporting CytoSoft 5.3 software

4.2.7 Assessment of neutrophil apoptosis

PMN were cultured with WoLP (1 ng/ml-5 µg/ml), BmFE (50-200 µg/ml), BmFEtet (200 µg/ml) and control stimuli GM-CSF (5 ng/ml, positive control for apoptosis delay), DMSO and media alone for 15 and 20 hours.
Cells (2.5 x 10^4 in 25 µl cell suspension) were transferred to a 96-well tissue culture plates (Costar) and apoptotic cells labelled with 25 µl Annexin-V-FITC 1:50 in HBSS (1:100 final dilution for cell staining) by incubation in the dark for 15 minutes at RT. Annexin-V is a phospholipid-binding protein with high affinity for phosphatidylserine, found on the outer surface of early apoptotic cells. A marker of apoptosis is the redistribution of phosphatidylserine from the inner to the outer surface of the cell membrane, where it can be labelled with FITC-conjugated Annexin-V. Propidium Iodide (PI) (200 µl, 1 µg/ml final concentration in HBSS) was added to wells to label late apoptotic and necrotic cells. Non-stained cells in HBSS were included as a control for background fluorescence. Cells (1 x 10^5/ml final concentration) were analyzed using Guava EasyCyte Plus (Millipore) supporting CytoSoft 5.3 software.

4.2.8 Respiratory burst chemiluminescence assay

For the assessment of total reactive oxygen species production, a luminol-enhanced chemiluminescence assay that measures both intra- and extra-cellular oxidants was performed.
PMN were primed for 30 minutes with WoLP (1 ng/ml-5 µg/ml), BmFE (50-200 µg/ml), WoLP 0.5 µg/ml + BmFE 50 µg/ml, and control stimuli TNFα (10 ng/ml), DMSO and media alone. Cells (2 x 10^5 in 40 µl cell suspension) were added in duplicate to white low-adhesion 96-well microplates and stimulated with fMLP (1 µM), PMA (100 ng/ml) or DMSO (unstimulated cells) in the presence of Luminol (10 µM) in HBSS in a final volume of 200 µl/well. Chemiluminescence was read every 30 seconds for 60 readings in a Wallac VICTOR™ Light 1420 Luminescence counter (Perkin Elmer) at 37°C. Background chemiluminescence was assessed by inclusion of one well without cells per each stimulus. Total chemiluminescence was calculated using the

area under the curve method [402]. Cell viability at the time of peak ROS production was assessed by 0.2% trypan blue staining.

4.2.9 Measurement of cytokines by ELISA

Levels of IL-1β, IL-12p70, IL-8, GM-CSF and TNFα in cell culture supernatants were measured in duplicate using DuoSet ELISA Development kits as per manufacturer's instructions. Absorbance was read in a FLUOstar Omega plate reader supporting MARS data analysis software 1.20 (BMG Labtech). The best-fit curve method was used to calculate the cytokine concentration in the samples.

4.2.10 Statistical analysis

Unless specified otherwise, samples from ≥ 3 different donors were used for the functional assays. Means were compared using independent-samples *t* test. For PMN surface molecules expression, mean percentage changes in mean fluorescence intensity compared to control were analysed using one-sample *t* test. A *p*-value ≤ 0.05 was considered significant. Computations were done in SPSS Statistics 17.0 (IBM).

4.3 RESULTS

4.3.1 Reactivity of filarial soluble extracts and WoLP

To assess i) absence of *Wolbachia* in BmFEtet and its presence in BmFE worm extracts, ii) WoLP activation of TLR2/6 comparably to BmFE, and iii) absence of LPS contamination of stimuli, it was examined the TNFα production of peritoneal macrophages from WT and TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR6$^{-/-}$ mice upon BmFE, BmFEtet and WoLP stimulation. WoLP selectively activated TLR2/6 as demonstrated by absence of activation of macrophages to produce TNFα from TLR2$^{-/-}$ and TLR6$^{-/-}$ mice. The same activation pattern was found for the *Wolbachia*-containing BmFE, while no activation was found when the *Wolbachia*-depleted BmFEtet extract was used.

These results (Figure 4.4) confirm *Wolbachia*, and particularly *Wolbachia*

diacylated lipoprotein, being the main macrophage activator stimulus in filarial extracts [47]. They also confirm *Wolbachia*-depletion of BmFEtet, and exclude LPS contamination of filarial extracts and WoLP stocks.
Parallel experiments with mouse neutrophils were not carried out due to the difficulty in purifying these cells.

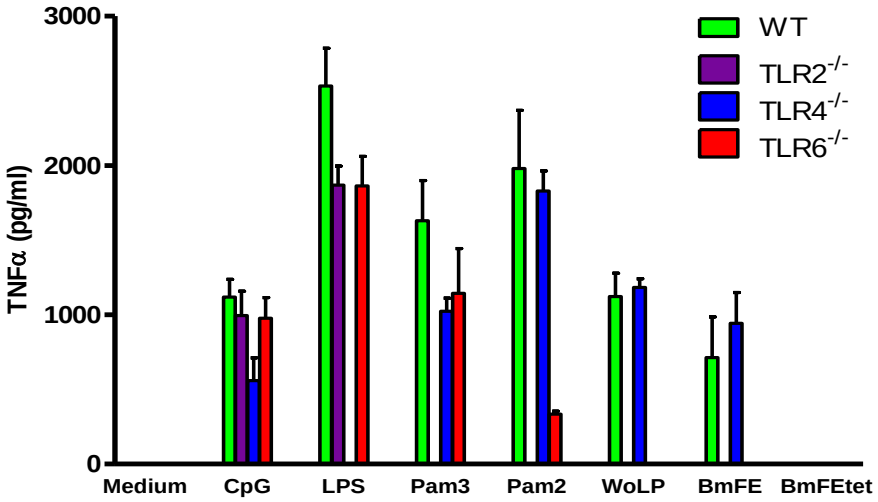

Figure 4-4. TNFα levels in the supernatant of wild type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR6$^{-/-}$ murine macrophages. Macrophages were selected by adherence to plastic from peritoneal inflammatory cells and stimulated for 24 hours at 1 x 10^6 cells/ml with WoLP (0.1 μg/ml), BmFE and BmFEtet (200 μg/ml) and control stimuli: medium, CpG (25 μg/ml, TLR9 agonist), Ultra-pure LPS (0.1 μg/ml, TLR4 agonist), Pam_3CSK_4 (1 μg/ml, TLR2/1 agonist), and Pam_2CSK_4 (0.1 μg/ml, TLR2/6 agonist). Bar graph represents pg/ml (mean ± SD) of 3 mice per genotype, each assessed in triplicate.

4.3.2 Neutrophil purity and viability after isolation

After isolation with Polymorphprep, PMN purity was always ≥ 97% and viability ≥ 98%. The monocyte:PMN ratio was constantly ≤ 1:700 (0.14%). This is in line with published work using gradient isolation [317].

4.3.3 Neutrophils acquire activated cell morphology upon exposure to WoLP

Activation of PMN results in a change in cell morphology. Typically, resting PMN are round in shape, with a smooth cell surface, while after activation cells appear elongated. After 1.5 hours exposure to WoLP (1 μg/ml), PMN showed an

evident activated cell shape, similar to that obtained with exposure to fMLP (0.01 μM). Exposure to BmFE (200 μg/ml) and BmFEtet (200 μg/ml), on the contrary, did not result in an evident change in cell morphology. PMN had resting cell morphology upon exposure to DMSO and media controls (Figure 4.5).

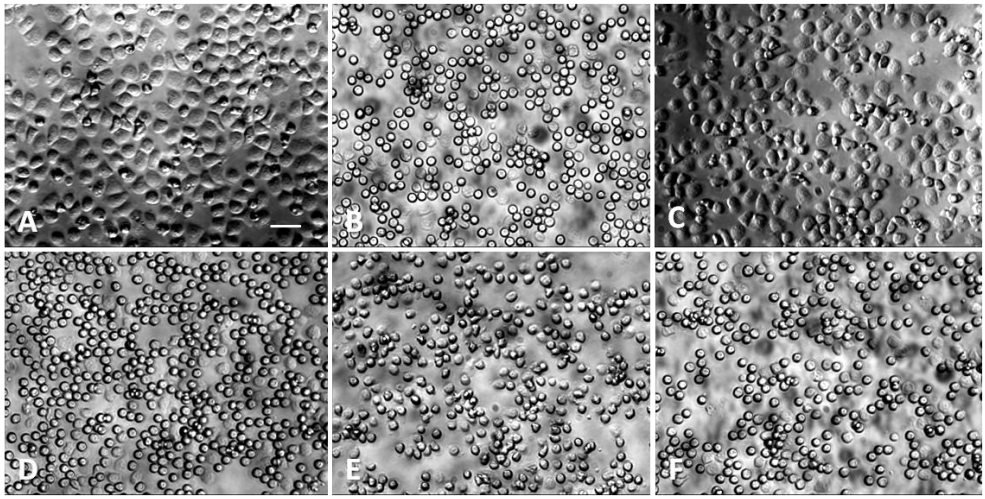

Figure 4-5. PMN acquired activated cell morphology upon exposure for 1.5 hours to WoLP (0.1 μg/ml) (**A**), similar to that acquired after exposure to fMLP (0.01 μM) (**C**). In contrast, PMN exposure to DMSO (same volume as WoLP) (**B**), media alone (**D**) and filarial extracts BmFE (200 μg/ml) (**E**) and BmFEtet (200 μg/ml) (**F**) did not result in an evident change in cell morphology. Original magnification 32x. Scale bar, 50 μm.

4.3.4 WoLP exerts a chemotactic effect on neutrophils

Migration of PMN in response to WoLP and filarial extracts was analysed in a transwell system using fMLP as a positive control.

When stimuli were present only in the lower chamber (i.e. in the presence of a concentration-gradient), WoLP and BmFE induced the migration of PMN in higher numbers compared to control stimuli at all concentrations used (Figure 4.6).

It was then assessed if the observed enhanced migration of PMN into the lower chamber was due to chemotaxis toward a concentration-gradient of stimuli or to increased random movements (chemokinesis). As shown in figure 4.7, the WoLP-induced migration of PMN into the lower chamber was significantly impaired when the stimulus was present in both chambers ($p < 0.001$), indicating that WoLP exerts a chemotactic effect on PMN. In contrast, BmFE and BmFEtet induced PMN migration also in the absence of a concentration-gradient, indicating that these stimuli are chemokinetic rather than

chemotactic. Also, no difference was found between BmFe and BmFEtet in inducing PMN transwell migration.

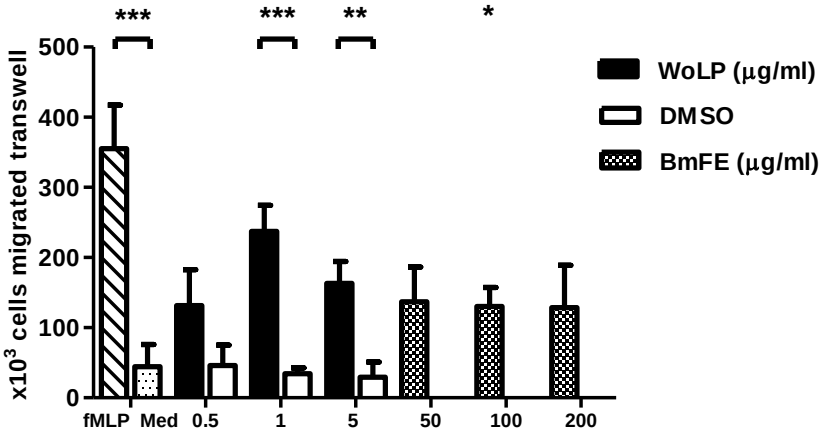

Figure 4-6. Migration of PMN after 1.5 hours in the presence of a concentration-gradient (chemotaxis) of WoLP (0.5-5 μg/ml as indicated), BmFE (50-200 μg/ml as indicated), fMLP (0.01 μM) and control DMSO (same volume as WoLP) and media (Med). Placed in the lower well of the transwell system. PMN (2 x 10^5 cells in 200 μl media) were placed in the upper well of the transwell system. Bar graph represents n x 10^3 cells migrated transwell (mean ± SD of 3 donors each tested in duplicate). * p = 0.023 BmFE 100 μg/ml vs media; ** p = 0.004; *** p = 0.001.

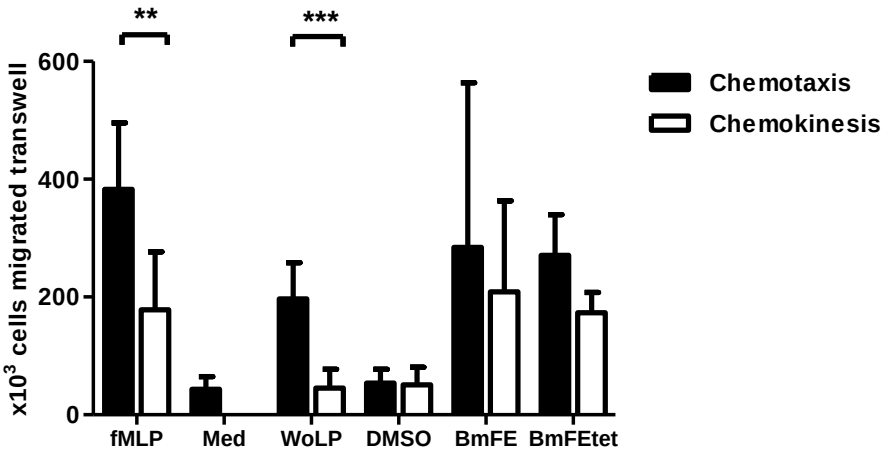

Figure 4-7. Migration of PMN after 1.5 hours in the presence and in the absence of a concentration-gradient of stimuli (chemotaxis and chemokinesis, respectively). For chemotaxis, stimuli were placed only in the lower chamber of the transwell system; for chemokinesis stimuli were placed at equal concentration in both chambers of the transwell system. Stimuli were: WoLP (1 μg/ml), BmFE and BmFEtet (both 200 μg/ml), fMLP (0.01 μM) and control DMSO (same volume as WoLP) and media (Med). PMN (2 x 10^5 cells in 200 μl media) were placed in the upper well of the transwell system.Bar graph represents n x 10^3 cells migrated transwell (mean ± SD) n = 6 donors for fMLP, WoLP and DMSO; n = 3 donors for BmFE and BmFEtet, each tested in duplicate. ** p = 0.007; *** p < 0.001.

4.3.5 WoLP modulates the surface expression of the β_2-integrin CD11b/CD18 and of L-Selectin in neutrophils

The recruitment of PMN requires physical interaction with endothelial cells of blood capillaries. L-Selectin is the major selectin expressed on PMN, contributing to leukocyte rolling and is rapidly shed from the cell surface upon activation. CD11b/CD18 is one of the major neutrophil integrins, involved in strong binding of activated neutrophils to the endothelium. Upon activation, surface expression of CD11b/CD18 is up-regulated via recruitment from granule stores.

One hour incubation with WoLP (0.1 μg/ml) led to significant down-regulation of surface L-Selectin expression ($p < 0.001$) and up-regulation of CD11b and CD18 ($p = 0.001$ and $p = 0.025$ respectively) compared to incubation with DMSO control. A similar trend was observed after stimulation with BmFE and BmFEtet (both 200 μg/ml); however, statistical significance was not reached. Results are shown in figure 4.8.

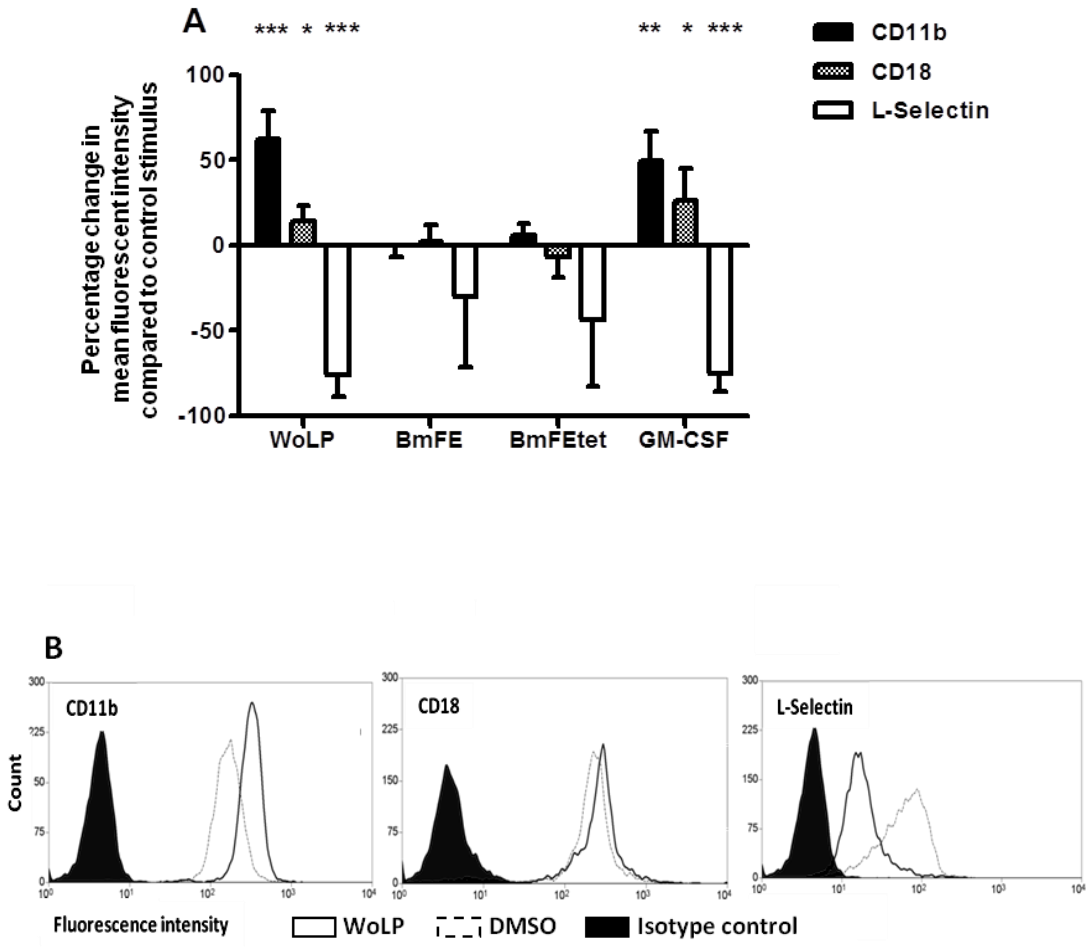

Figure 4-8. Modulation of surface adhesion molecules on PMN as assessed by flow cytometry after 1 hour stimulation. **A**, Bar graph represents percentage change (mean ± SD) in mean fluorescence intensity compared to stimulation with control DMSO (for WoLP [0.1 µg/ml]) and media alone (for GM-CSF [5 ng/ml], BmFE and BmFEtet [both 200 µg/ml]).PMN were cultured at 5 x 10^6 cells/ml. n = 5 donors for WoLP and GM-CSF; n = 3 donors for BmFE and BmFEtet, each tested in duplicate. *p = 0.025 WoLP and p = 0.033 GM-CSF; **p = 0.003; *** $p \leq 0.001$. **B**, Histograms show representative experiments carried out with WoLP.

4.3.6 WoLP modulates surface expression of Fcγ Receptors in neutrophils

FcγRII (CD32) and FcγRIII (CD16) are constitutively expressed receptors for IgG on PMN. They act cooperatively by binding immune complexes and activating cell functions such as respiratory burst, degranulation and phagocytosis. FcγRII can be up-regulated via the mobilization of sub-cellular pools. Similarly, upon activation FcγRIII is translocated from granules to the plasma membrane but at the same time is also shed from the cell surface; therefore the total surface expression of this receptor may increase, decrease or remain the same

upon activation.

As shown in figure 4.9, after 1 hour exposure to WoLP (0.1 μg/ml) levels of FcγRII were up-regulated, while FcγRIII was down-regulated (p = 0.023 and p = 0.019 respectively) compared to DMSO-stimulated cells. BmFE but not BmFEtet induced a net up-regulation of FcγRIII (p = 0.008), while levels of FcγRII did not change upon stimulation with filarial extracts (200 μg/ml).

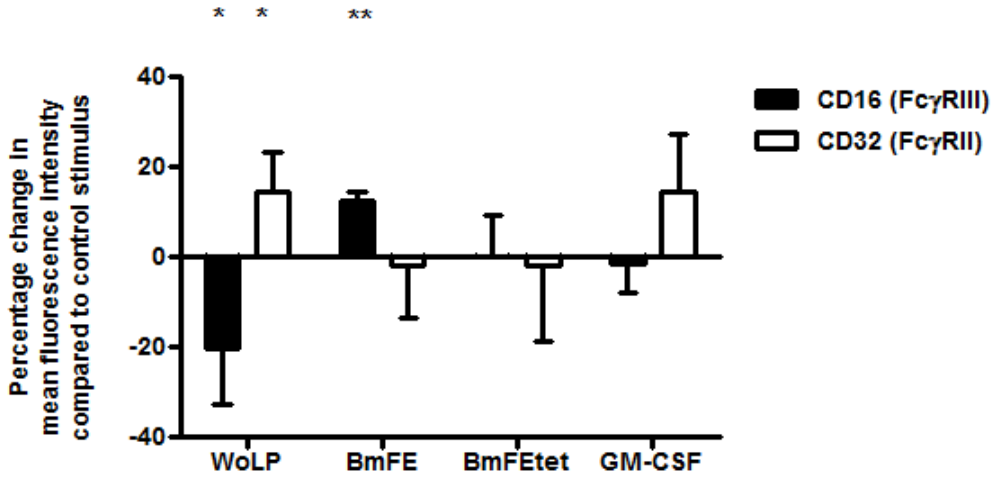

Figure 4-9. Modulation of surface Fcγ Receptors on PMN as assessed by flow cytometry after 1 hour stimulation. Bar graph represents percentage change (mean ± SD) in mean fluorescence intensity compared to stimulation with control DMSO (for WoLP [0.1 μg/ml]) and media alone (for GM-CSF [5 ng/ml], BmFE and BmFEtet [both 200 μg/ml]). PMN were cultured at 5 x 10^6 cells/ml. n = 5 donors for WoLP and GM-CSF; n = 3 donors for BmFE and BmFEtet, each tested in duplicate. *p = 0.019 WoLP-CD16 and p = 0.023 WoLP-CD32; **p = 0.008.

4.3.7 Apoptosis is delayed in neutrophils exposed to WoLP

While resting PMN are short-lived cells undergoing constitutive apoptosis after ~12 hours, their activation leads to an increase in lifespan. A marker of apoptosis is the rearrangement of molecules present on the inner and outer leaflet of the cell membrane. In particular, phosphatidylserine appears on the outer surface of the cell membrane of apoptotic cells, where it can be labelled with Annexin-V-FITC. Cells in late apoptosis and necrosis show an increased cell membrane permeability, which can be detected by PI staining.

After 15 hours culture in media alone (Figure 4.10 A), 51% (± 9.67%) PMN underwent apoptosis. At this time point, WoLP (1 ng/ml-5 μg/ml) induced a reduction in the percentage of PMN undergoing apoptosis (Annexin-V^+ PI^-) in a

concentration-dependent manner. A statistically significant difference in the percentage of apoptotic cells compared to control was found only for WoLP 0.5 μg/ml (p = 0.046) and BmFE 200 μg/ml (p = 0.036).

When the assay was repeated for 20 hours stimulation (Figure 4.10 B), similar results were found, with a statistically significant difference in the percentage of apoptotic cells compared to control found for WoLP 0.1 μg/ml (p = 0.040) and 1 μg/ml (p = 0.047). At this time point no differences were found between BmFE (200 μg/ml), BmFEtet (200 μg/ml) and media control and when the two filarial extracts were compared.

No differences were found in the percentage of cells in late apoptosis (Annexin-V^+ PI^+, range 0.06%-4.98%) or necrosis (Annexin-V^- PI^+, range 0.1%-2.84%) (Figure 4.11).

Taken together, the reproducibility of results at two different time points suggests that WoLP is able to protect PMN from undergoing apoptosis. The absence or poor reproducibility of statistical significance is most likely due to the large variability in the data (leading to large standard deviation data). Indeed, PMN obtained from blood are all at different stages of their natural life span, and exposed to different stimuli whilst in the blood. Therefore, this level of variability was expected.

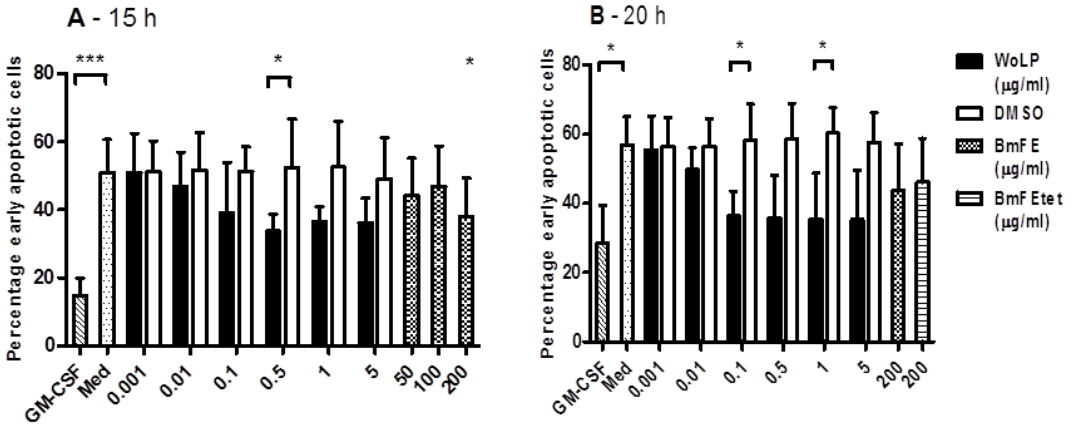

Figure 4-10. Proportion of early apoptotic (Annexin-V^+ PI^-) PMN after 15 hours (**A**) or 20 hours (**B**) exposure to increasing concentrations of WoLP (0.001 to 5 μg/ml, as indicated), filarial extracts (BmFE and BmFEtet both 200 μg/ml), and control stimuli GM-CSF (5 ng/ml), DMSO (same volume as WoLP) and media alone (Med). Bar graphs represent percentage of early apoptotic cells (mean ± SD). $n \geq 3$ donors, each tested in duplicate. PMN were cultured at 1 x 10^6 cells/ml. *p = 0.046 WoLP 0.5 μg/ml 15 h; p = 0.036 BmFE 200 μg/ml vs Med 15 h; p = 0.023 GM-CSF 20 h; p = 0.040 WoLP 0.1 μg/ml 20 h; p = 0.047 WoLP 1 μg/ml 20 h; ***p = 0.001 GM-CSF 15 h.

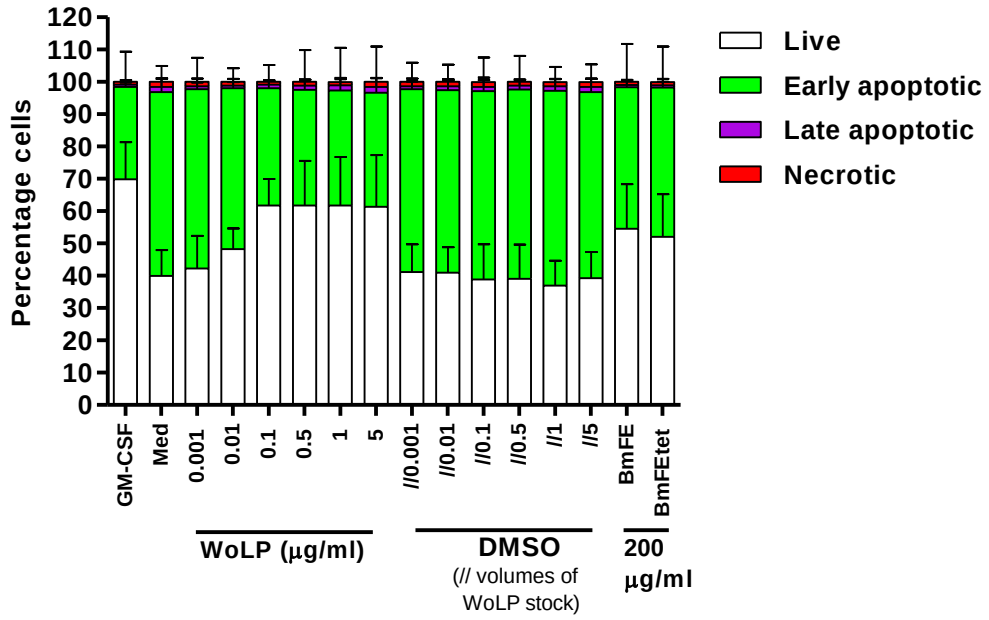

Figure 4-11. Distribution of PMN viability stages after 20 hours culture with the indicated stimuli. Live = Annexin-V$^-$ PI$^-$; Early apoptotic = Annexin-V$^+$ PI$^-$; Late apoptotic = Annexin-V$^+$ PI$^+$; Necrotic = Annexin-V$^-$ PI$^+$. ≥ 95% PMN were in the live or early apoptotic stage when examined. n ≥ 3 donors, each tested in duplicate. Bar graph represents percentage of cells (mean ± SD) in each viability stage.

4.3.8 WoLP directly induces and primes for the production of reactive oxygen species

The production of ROS in the respiratory burst is an important tool by which PMN kill phagocytosed microbes. ROS are produced by an enzyme complex whose components assemble during cell activation. ROS production can be stimulated by several agonists in a receptor-mediated (e.g. by fMLP) or receptor-independent (e.g. by PMA) manner. Generally, the production of ROS is quite low unless PMN have been pre-treated (i.e. primed) with other agents (e.g. TNFα), which up-regulate the level of expression of some complex components on the cell surface. Priming results in a greater ROS production (e.g. upon stimulation with fMLP) and/or in a quicker response to stimuli (e.g. upon stimulation with PMA).

Levels of ROS produced by PMN primed with WoLP (1 ng/ml-5 µg/ml) and subsequently not stimulated were significantly higher compared to media-primed cells and increased in a WoLP concentration-dependent manner,

reaching a plateau for concentrations of 0.5 μg/ml WoLP and above (Figure 4.12 A and A'). When cells were stimulated with fMLP (1 μM) after priming with WoLP, the production of ROS was approximately doubled compared to unstimulated PMN and unprimed cells, showing a similar concentration-dependency pattern (Figure 4.12 B and B'). Also, stimulation of WoLP-primed cells with PMA (100 ng/ml) induced a concentration-dependent reduction in the time needed to reach the peak of ROS production compared to unprimed cells (Figure 4.12 C and C'). When the three assays were carried out by priming PMN with BmFE (50-200 μg/ml), extremely low levels of total chemiluminescence were detected. Figure 4.13 shows a representative example of BmFE-primed fMLP-induced ROS production. Moreover, when increasing concentrations of BmFE (1-50 μg/ml) were added to WoLP 0.5 μg/ml and the mix was used as the priming stimulus, levels of ROS production in all assays were inversely correlated with the concentration of BmFE used (not shown). This apparent lack of ROS production was not due to a much higher percentage of dead cells after BmFE compared to WoLP stimulation, as assessed by trypan blue staining of PMN at the time point corresponding to peak production of ROS. The percentage of dead cells at this time point was between 5% and 7% for all the priming stimuli examined.

These results indicate that WoLP alone induces an oxidative burst in PMN, and primes PMN for fMLP- and PMA-induced ROS production. Although it is intriguing to think that the absence of ROS production upon priming with BmFE may be due to a suppressive effect exerted on PMN, the most likely explanation is that the worm extract quenched the reactive species, preventing them from interacting with luminol. Indeed, sulphydryl groups (-SH), present, for example, in cysteine and reduced glutathione, are able to quench free radicals and are likely to be abundant in the worm extract. Moreover, antioxidant enzymes are abundantly produced by parasitic nematodes [403]. In light of these considerations and of the scarcity of extracts available, this aspect of ROS production was not investigated further.

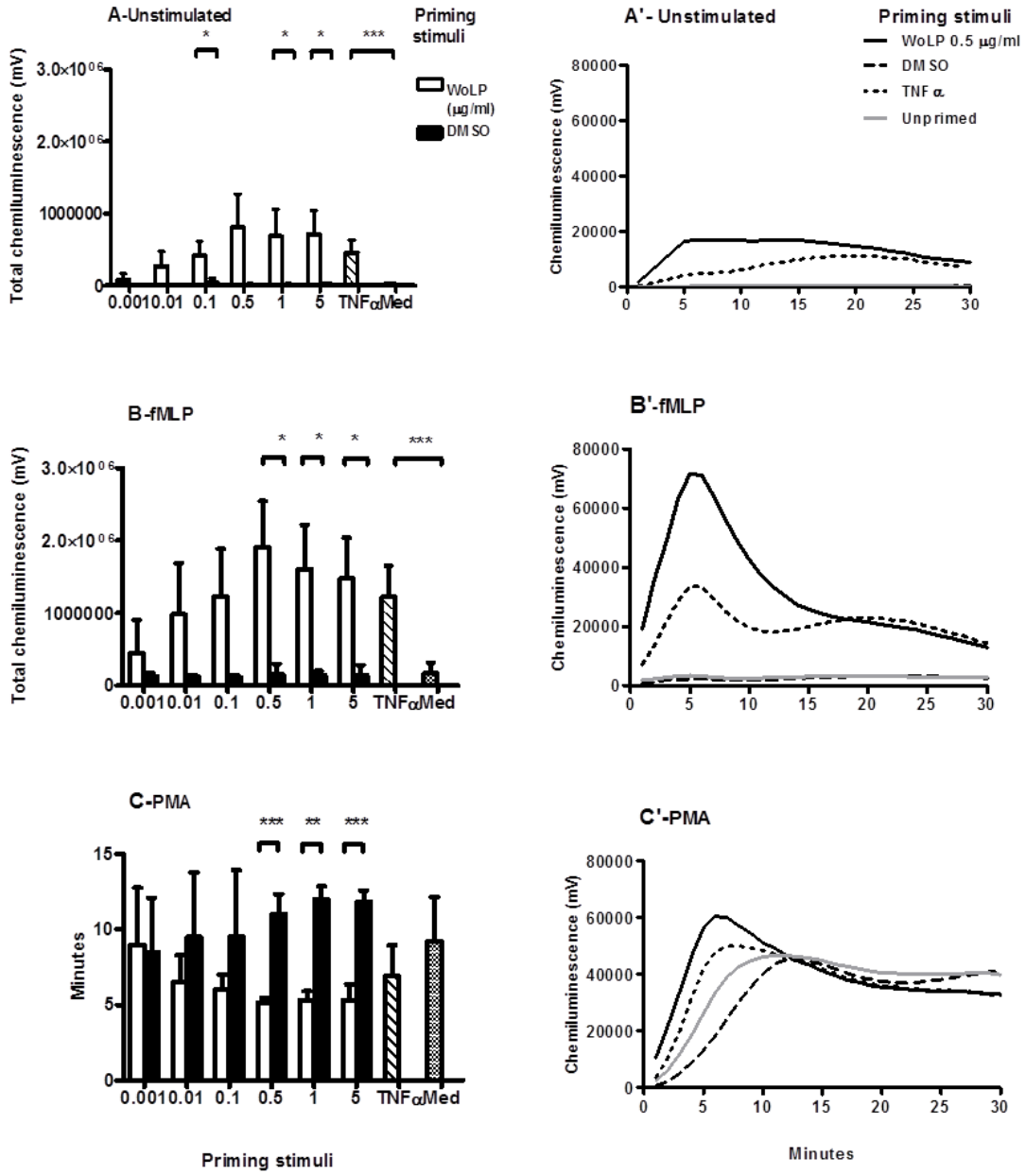

Figure 0-12. ROS production over 30 minutes exposure to Luminol (10 µM) and stimuli (fMLP, PMA or MSO) after 30 minutes priming with WoLP (0.001 to 5 µg/ml as indicated) or control stimuli DMSO (same volume as WoLP), TNFα (10 ng/ml) and media alone (Med). PMN were cultured at 5 x 10^6 cells/ml for priming and at 2 x 10^5 cells/40 µl for stimulation. Bar graph **A** represents total chemiluminescence (AUC mean ± SD; n = 3 donors, each tested in duplicate.) upon stimulation with DMSO (unstimulated); *p = 0.035 WoLP 0.1 µg/ml; p = 0.036 WoLP 1 µg/ml; p = 0.024 WoLP 5 µg/ml; ***p < 0.001. Bar graph **B** represents total chemiluminescence (AUC mean ± SD; n = 3 donors, each tested in duplicate.) upon stimulation with fMLP (1 µM); *p = 0.040 WoLP 0.5 µg/ml; p = 0.014 WoLP 1 µg/ml; p = 0.015 WoLP 5 µg/ml; ***p < 0.001. Bar graph **C** represents time-to-peak (mean ± SD; n = 3 donors, each tested in duplicate.) ROS production upon stimulation with PMA 100 ng/ml); **p = 0.002; ***p = 0.001. Histograms **A'**, **B'** and **C'** show mean ROS production over time.

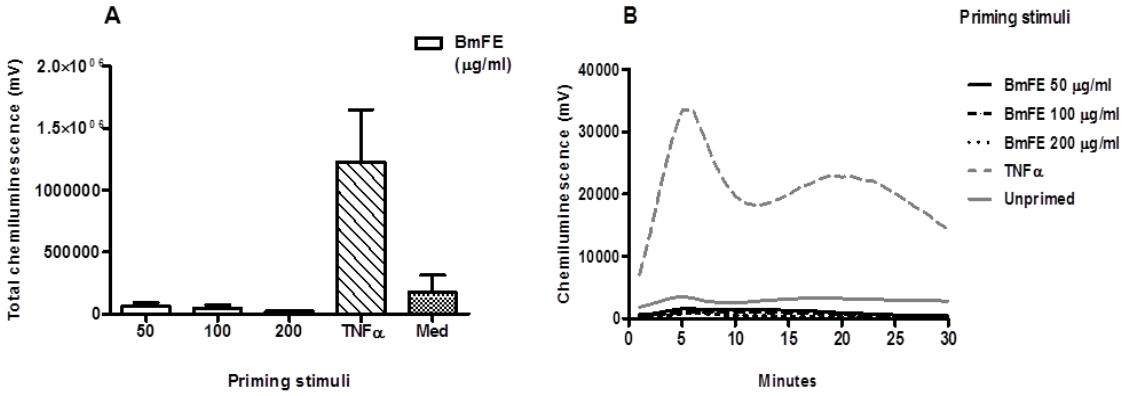

Figure 0-13. ROS production over 30 minutes exposure to Luminol (10 µM) and fMLP stimulus (1µM) after 30 minutes priming with BmFE (50-200 µg/ml) or control stimuli (TNFα 10 ng/ml, and media alone). **A**, Bar graph represents total chemiluminescence (AUC mean ± SD). **B**, Histograms show mean ROS production over time. *n* = 3 donors, each tested in duplicate. PMN were cultured at 5 x 10^6 cells/ml for priming and at 2 x 10^5 cells/40 µl for stimulation.

4.3.9 Exposure to WoLP induces the production of IL-8 by neutrophils

To investigate whether the activation of PMN *in vitro* was exclusively induced by a direct effect of WoLP or mediated by cytokines induced by WoLP, levels of IL-1β, IL-8, IL-12p70, GM-CSF and TNFα were assessed in the supernatant of PMN exposed to increasing concentrations of WoLP (1 ng/ml-5 µg/ml) for 30 minutes, 1 hour and 15 hours (i.e. at time points relevant for the functional assays performed). Only IL-8 was detected by ELISA in PMN culture supernatants, at 1 hour and 15 hours. Levels of IL-8 were higher after 15 hours incubation and increased in a concentration-dependent manner with increasing concentrations of WoLP (Figure 4.14).

These results suggest that WoLP-induced production of IL-8 may contribute to the activation of PMN. Neutrophils are the major source of IL-8. Nevertheless, it can not be ruled out that contaminating PBMC were activated and produced cytokines, thus contributing to the phenotype of WoLP-stimulated neutrophils.

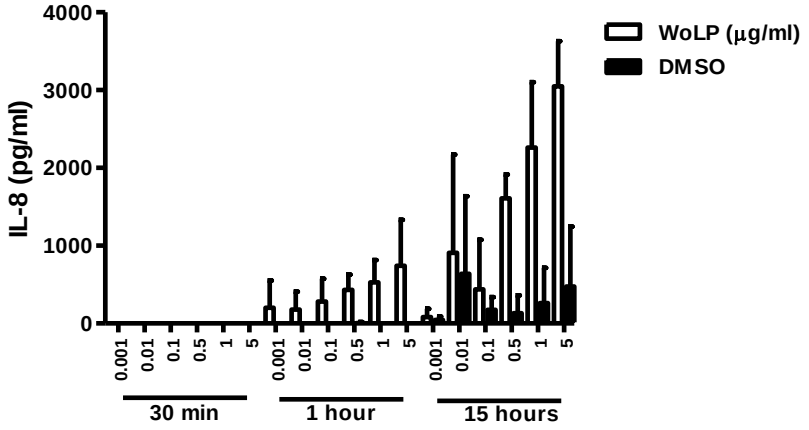

Figure 0-14. PMN secrete IL-8 upon stimulation with WoLP (0.001-5 µg/ml) or DMSO control (same volume as WoLP) for ≥ 1 hour. Bar graph represents levels of IL-8 in pg/ml (mean ± SD of n = 3 donors, each assessed in duplicate). PMN were cultured at 5 x 10^6 cells/ml for 30 minutes and 1 hour and at 1 x 10^6 cells/ml for 15 hours.

4.4 DISCUSSION

Neutrophils are pivotal effector cells in the pathogenesis of onchocercal disease and their presence in *O. volvulus* infected tissues depends upon that of *Wolbachia*. Nevertheless, their role in the broader *O. volvulus* parasitism is not clear. This work investigated the effects of WoLP and *Wolbachia*-containing or *Wolbachia*-depleted filarial extracts on the recruitment and activation of isolated human neutrophils *in vitro.*

The results obtained indicate that WoLP exerts an activating effect on human neutrophils, as shown by their activated cell shape and IL-8 production upon exposure to WoLP. Analysis of cell surface adhesion molecules and FcγR expression confirmed the activating effect of WoLP: L-Selectin and FcγRIII expression was down-regulated and CD11b/CD18 and FcγRII were up-regulated. These results are in accordance to what was reported for the TLR2/6 ligand MALP-2 by Wilde *et al* [316]. Shedding of L-Selectin was also reported in mouse neutrophils stimulated with isolated *Wolbachia* [154].
In contrast to MALP-2 [316], WoLP was found to both prime for and directly induce the production of ROS, and to exert a chemotactic rather than chemokinetic effect on neutrophils. These discrepancies could be possibly explained by the use of MALP-2 at 10 ng/ml: when WoLP was used at the same concentration, only a small direct induction of ROS was also observed. On the

other hand, this concentration of MALP-2 was sufficient to detect a small but significant direct induction of ROS by Francois *et al* [323]. The chemotactic/chemokinetic effect of WoLP was not assessed at concentrations lower than 0.5 μg/ml in the present work.

Contrasting results exist about the effect of TLR2-binding stimuli on PMN apoptosis delay. Sabroe and co-workers [313] found that in highly pure neutrophil cultures, short-term (4 hours) and long-term (22 hours) apoptosis was not delayed by stimulation with Pam_3CSK_4 (TLR2/1 ligand), also when neutrophil cultures were added with 5% PBMC, which instead mediated long-term LPS-induced apoptosis delay. They suggested that neutrophil survival over a prolonged time course in this latter case could be due to the induced monocyte synthesis of cytokines with pro-survival activity [321, 322]. In the work of Wilde and colleagues [316], MALP-2 was found to have direct effect on short-term but not on long-term apoptosis delay in highly pure neutrophil cultures, whereas MALP-2 induced long-term apoptosis delay on neutrophils which transmigrated through endothelial cells. The results presented here of WoLP being able to delay long-term (15 and 20 hours) neutrophil apoptosis are in line with the work of Francois *et al* [323], who found that stimulation with Pam_3CSK_4 and MALP-2 induced long-term apoptosis delay in highly purified neutrophils, and of Power *et al* [324], who reported long-term delayed PMN apoptosis upon stimulation with comparable concentrations of Pam_3CSK_4 of gradient-purified (i.e. non-highly pure < 5% PBMC contaminated) neutrophil cultures. The reported increased effect of MALP-2 on neutrophil apoptosis delay when cells were stimulated in whole blood cultures [323] suggests that other stimuli derived from other cells in co-culture may contribute to the increased life span of neutrophils. In the work presented here, neutrophils were ≥ 97% pure with ≤ 0.14% contaminating monocytes, therefore a role for WoLP-induced monocyte-derived pro-survival stimuli in the observed neutrophil apoptosis delay can not be completely excluded. The only measured cytokine that reached detectable levels in WoLP-stimulated neutrophil supernatants was IL-8, which is known to delay neutrophil apoptosis [275]. Levels of IL-8 in the supernatant of the same cell cultures used for the 20 hours apoptosis assay increased proportionally to the concentration of WoLP used and a moderate negative correlation was found between percentage of apoptotic cells and levels of IL-8 (R^2 = 0.446) (data not shown). As levels of IL-8 of the same magnitude can be produced by PBMC cell cultures stimulated with TLR2 agonists [313], it is unlikely that the small percentage of contaminating monocytes in neutrophil cultures would be the predominant source of IL-8 in this case.

IL-8 is a potent activator of neutrophils, and can induce up-regulation of CD11b and shedding of L-Selectin, therefore it is possible that this cytokine may contribute to the WoLP-induced activation of neutrophils in a paracrine way. IL-8 secreted by neutrophils may also contribute to the chemotaxis of these cells *in vitro*. However, no detectable levels of IL-8 were found in the supernatants of neutrophils in the chemotaxis and chemokinetic assays (data not shown), possibly because fewer cells were used in this assay (1 x 10^6 compared to 5 x 10^6 used in cell cultures for cytokine assessment). Levels of other measured cytokines (IL-1β, IL-12p70, TNFα and GM-CSF) were below the detection level of the ELISA assays. These results suggest that WoLP exerts a direct and/or partially IL-8-mediated effect on isolated human neutrophils, although it can not be completely ruled out that other cytokines and/or very low levels of tested cytokines produced by neutrophils or contaminating monocytes may contribute to the phenotype of WoLP-stimulated neutrophils.

When neutrophils were stimulated with *Wolbachia*-containing filarial extract, their phenotype did not match that induced by WoLP. Moreover, it did not differ from that found upon stimulation with the *Wolbachia*-depleted extract. These results are in marked contrast with what reported with murine DCs and human HEK cells by Turner *et al* [47], that showed a near-identical phenotype in response to BmFE and WoLP. It is possible that human neutrophils are less responsive than murine DCs and HEK cells to the stimulus provided by *Wolbachia* lipoproteins. Indeed, the reported optimal concentration of WoLP for the stimulation of these cells was 0.1 μg/ml [47], while here in most assays, optimal concentration for neutrophil stimulation was found to be higher. It is therefore possible that the concentration of filarial extracts used, and therefore that of *Wolbachia* lipoproteins in BmFE, was not sufficient to optimally stimulate human neutrophils in this study. Indeed, filarial adult extracts are a complex stimulus, whose net effect on cell activation depends on the relative quantity and potency of their components. Moreover, filarial extracts not necessarily mirror the stimulation provided by whole worms in different viability states.

It has been reported that *Wolbachia*-containing but not *Wolbachia*-depleted *O. volvulus* soluble extract was able to induce human neutrophil recruitment *in vitro* [44], and that this was due to chemotaxis rather than chemokinesis [375]. These results were not replicated in the present study, where BmFE and BmFEtet induced comparable levels of chemokinesis on PMN. In the light of the reported neutrophil chemotactic activity of soluble secreted products from adult female *O. volvulus* parasites [375], it would be interesting to assess whether the

use of culture supernatants of *Wolbachia*-depleted worms would drive a different response. Indeed, the use of worm culture supernatants, containing soluble secreted products, would constitute a closer stimulus to what neutrophils are exposed to *in vivo*.

In conclusion, the work presented here shows that *Wolbachia* activates human neutrophils and indentifies *Wolbachia* peptidoglycan-associated lipoprotein as a key molecule driving their recruitment and activation. This supports the evidence of neutrophils mediating *Wolbachia*-induced onchocercal pathology. Nevertheless, neutrophils do not seem openly detrimental to adult worms in onchocercomas and the diverse role of the *Wolbachia*-induced neutrophilia in the contexts of pathology and parasitism without pathology, if ever a difference exists, deserves further attention. It is possible that levels of *Wolbachia*-derived stimuli released by adult worms in nodules would be enough to recruit but not to fully activate neutrophils in onchocercomas. On the other hand, the massive release of *Wolbachia* after antifilarial therapy may more potently activate neutrophils, thus mediating onchocercal immunopathology.

The suggested role of *Wolbachia*-induced neutrophilia as a tactic developed by adult worms to protect themselves by the attack of potentially lethal eosinophils is intriguing [45, 370]. So far, no studies have assessed if the findings obtained in cattle infected with *O. ochengi* also apply to human onchocerciasis. These would shed light on the apparent contrasting results about the relative role of neutrophils and eosinophils in killing of adult parasites suggested by these studies compared to those conducted in the *L. sigmodontis* model. Interestingly, it has been reported that binding of eosinophil peroxidase to human neutrophils *in vitro* leads to reversible inhibition of its peroxidase activity [404], therefore the interaction between neutrophils and eosinophils, for example in co-cultures stimulated with WoLP, deserves further attention.

TLR2 activation of DCs and T cells has been found to induce cytokines that skew the adaptive immune response, with different outcomes depending on the system studied in terms of Th1, Th2, Th17 and Treg expansion [305, 405-408]. In the context of filariasis, Turner *et al* [47] showed that WoLP induced a predominant Th1 response in murine co-cultures of DCs and $CD4^+$ T lymphocytes. *Wolbachia*-containing *B. malayi* extracts induced a mixed Th1/Th2 response and *Wolbachia*-depleted extract a predominant Th2 response, as assessed by the balance between IL-4 and IFNγ in culture supernatants. Recent advances in the knowledge of neutrophil functions showed that these cells are able to shape the adaptive immune response through their interaction with DCs [263], and studies *in vivo* have linked the

presence or absence of neutrophils to the development of either Th1 or Th2 responses toward parasitic (*L. major*) and fungal (*C. albicans*) pathogens [399-401] depending on the strain used. Moreover, one recent study showed that neutrophils are able to directly instruct murine Th1 and Th17 differentiation *in vitro*, although a role for contaminating monocytes could not been completely excluded [267]. Thus, it is possible to speculate that *Wolbachia* may influence the type of cellular response toward different filarial parasite stages also *via* interaction with neutrophils.

The relative role of Th1 and Th2 responses in protection and disease in human onchocerciasis and animal models of filariasis is still not clear (see chapter 2 and 3). It would be interesting to investigate the role of *Wolbachia*-recruited and stimulated neutrophils in shaping the adaptive immunity to different stages of *O. volvulus*, to better understand the complex interaction between the symbiont, the worm and the host's immune system.

Chapter 5. CELLULAR SOURCE OF *WOLBACHIA*-DEPENDENT IL-17A PRODUCTION: INVESTIGATION IN ONCHOCERCOMAS AND IN VITRO

"In the age of monarchies, the expression "Le roi est mort; vive le roi" marked the passing of eras, ushering out one regime while introducing another. Analogously, it might be said, "T cell differentiation is dead; long live T cell differentiation." Suddenly, understanding the molecular regulation of T cell differentiation has become much more complicated. The simple notion of a dualistic view of Th1/Th2 cell differentiation is moribund, but the era of new complexities of immunoregulation promises to provide better understanding of mechanisms of host defense and immune-mediated disease."

Tato CM, Laurence A, O'Shea JJ

Helper T cell differentiation enters a new era: le roi est mort; vive le roi!

Joural of Experimental Medicine, 2006 [409]

ABSTRACT

The interaction between *Wolbachia* and the host's immune system plays an important role in the immunopathogenesis of filarial diseases. The main *Wolbachia*-dependent characteristic of *Onchocerca volvulus* infection, that is neutrophil recruitment in the cornea and in onchocercomas, is also typical of Type-17 immune responses, recently described in autoimmune and infectious diseases, and characterised by the production of pro-inflammatory interleukin-17 (IL-17). Moreover, the cytokine environment promoted by the combination of *Wolbachia* and filarial worms could favour the differentiation of Th17 cells, the best characterized source of IL-17. Although very limited, some evidence exists of the development of Th17 cells in lymphatic filariasis, but no studies so far addressed the specific question of the presence and induction of Type-17 immune responses in onchocerciasis and their relation with *Wolbachia*. The work presented here using immunohistochemistry (IHC) on onchocercomas from placebo and doxycycline-treated patients confirmed the association between presence of *Wolbachia* in nodules and recruitment of neutrophils, which were found in a NET-like structure. Most importantly, it described for the first time the presence of IL-17$^+$ cells and Th17 cells in *Onchocerca* nodules. In nodules, the percentage of Th17 on total CD4$^+$ T cells was higher than values reported in other pathologies such as psoriasis or rheumatoid arthritis, and their presence was associated with that of *Wolbachia*. Strikingly, >70% of IL-17 producing cells were neutrophils using IHC. All neutrophils in *Wolbachia*-containing onchocercomas were IL-17$^+$, and the neutrophil infiltrate virtually disappeared in *Wolbachia*-depleted nodules. IHC studies using purified neutrophils from healthy blood donors revealed that unstimulated neutrophils stained positive for IL-17, and staining intensity increased after stimulation with *Wolbachia* lipoprotein and LPS, suggesting that IL-17 may be constitutively expressed by these cells and induced upon bacterial stimulation. However, attempts to validate these results at the protein level (ELISA, Luminex, Western Blot, Immunoprecipitation followed by Mass Spectrometry) and at the transcription level (RT-PCR) failed to reach conclusive results about the presence and the expression of IL-17 in human neutrophils. Further studies are needed to confirm the presence of IL-17 in human neutrophils, and, subsequently, to clarify whether these cells are a source of IL-17A or clear this cytokine from the surrounding tissutal environment.

5.1 INTRODUCTION

5.1.1 Type-17 immune response: an overview

Brief history of the discovery of IL-17 and Th17 cells

The differentiation of $CD4^+$ effector T cells by cytokines produced by cells of the innate immunity arm stimulated by pathogens is the hallmark of adaptive immunity, which acts in a pathogen-specific way and establishes long-living memory for enhanced recall responses. With the advent of molecular immunology, Mosmann and Coffman in 1986 proposed the Th1/Th2 hypothesis, in which distinct subsets of $CD4^+$ T helper cells expressing distinct cytokine profiles were figured as responsible for discrete and mutually inhibited adaptive immune responses, namely delayed-type hypersensitivity for Th1 and B cell help for Th2 [410]. Notwithstanding its inability to fit in all models of organ-specific diseases and infections (reviewed in [411-413]), the Th1/Th2 hypothesis has been the backbone paradigm of immunology for about two decades. The first revision to the Th1/Th2 paradigm has been the re-discovery of T cells with suppressive features, now referred to as regulatory T cells (Tregs) (reviewed in [414]), but the first significant change to it has been the discovery of the subset of IL-17 producing T cells, named Th17 [415].

IL-17 was first identified in 1993 (named CTLA-8) in mouse hybridoma T cells as a homologue of a herpesvirus saimiri open reading frame [416]. Two years later, Yao and colleagues named it IL-17, cloned it from human T cells, and described for the first time one of its receptor subunits and some of its proinflammatory biological activities *in vitro* [417, 418]. In the following years, early studies investigated the role of IL-17 in the context of the pathogenesis of human and murine models of autoimmune diseases, firstly rheumatoid arthritis (RA) [419-424], and infections [425, 426]. In parallel, Oppmann and colleagues recognized that the p40 subunit of IL-12 (IL-12p40) could dimerize not only with the p35 subunit forming active IL-12 (IL-12p70), but also with a novel p19 subunit, forming the novel biologically active IL-23 [427]. This discovery provided the key to resolve the long lasting conundrum of why mice with defects in Th1 responses had exacerbated experimental autoimmune encephalomyelitis (EAE, murine model of human multiple sclerosis), a disease believed to be caused by a Th1 response, and *vice versa* injection of IFNγ and IL-12 blocked disease development [411]. Indeed, IL-12p19$^{-/-}$ (lacking IL-23) but not IL-12p35$^{-/-}$ (lacking IL-12) mice were resistant to EAE and collagen induced arthritis (CIA, mouse model of human RA) [428, 429], indicating that IL-23 and

not IL-12 (and therefore not Th1) were responsible for the pathogenesis of these autoimmune diseases. Protection from CIA in IL-23$^{-/-}$ mice was linked to absence of IL-17 and IL-17 producing T cells in the presence of normal Th1 responses [429]. The demonstration that IL-23 induced IL-17 production in mouse memory T cells [430], opened the way to the description of Th17 cells and their role in autoimmunity and infections.

IL-17 and IL-17 Receptor (IL-17R) family members

IL-17 (IL-17A) is the prototype member of a family of six cytokines named IL-17A to IL-17F. IL-17A and IL-17F are 50% identical in their amino acid sequence, while IL-17E (IL-25) is the most divergent. The other family members (IL-17B, IL-17C and IL-17D) have been identified through database searches based on homology with IL-17A (23-29%) and although their cellular sources, targets and functions are still poorly known, some evidence exists of their involvement in inflammation [431, 432].

In humans, IL-17A is a 155 amino acid glycoprotein of ~35 kDa secreted in a mixture of glycosylated and unglycosylated disulfide-linked homodimer or heterodimer with IL-17F [433-435], with the relative production of these forms by activated CD4^{+} T cells being IL-17F/F>IL-17A/F>IL-17A/A [435]. In non-reducing conditions, IL-17A is detected by Western Blot as two bands between 25 and 37 kDa, depending on glycosylation, when a homodimer, and as two bands close to 37 kDa when a heterodimer with IL-17F. In reducing conditions, IL-17A and IL-17F are detected as bands of 15-22 kDa (depending on glycosylation) and 17 kDa respectively [433, 434].

Five IL-17R subunits have been identified so far (IL-17RA to IL-17RE), structurally different from other receptors (reviewed in [432]). IL-17RA and IL-17RC are required for IL-17A and IL-17F signalling. In humans, IL-17RA binds with high affinity to IL-17A and with low affinity to IL-17F, while the contrary happens for IL-17RC [436]. Also, the relative distribution and abundance of these subunits differs among cells and tissues, possibly accounting for the different effects of IL-17A and IL-17F recently brought to attention [437]. IL-17RB binds to IL-25 (IL-17RB/IL-17RA) and IL-17B, while IL-17RE binds IL-17C. So far no receptor has been identified for IL-17D and no ligand for IL-17RD.

Induction of Th17 cells requires a combination of pro- and anti-inflammatory cytokines

The stimuli required for Th17 differentiation in the mouse and in humans have been a matter of much debate. In the mouse there has been general consensus on IL-6 plus TGFβ being required for the upregulation of STAT3, RORγt and RORα in naïve T cells, the master transcriptional regulators of Th17. IL-21 produced by Th17 themselves upon stimulation with IL-6 acts in an autocrine manner to promote Th17 amplification and maintenance, and together with TGFβ may also induce Th17 differentiation in the absence of IL-6. Finally, IL-23 although not needed for their differentiation, appears to be essential for the full and sustained differentiation and functionality of Th17 cells (reviewed in [438]). TGFβ appears indispensable for Th17 differentiation by inhibiting the upregulation of Th1 and Th2 transcriptional factors and allowing full IL-6 promoted RORγt induction [438-440]. Recently, however, Ghoreschi and colleagues also reported the differentiation, although less efficient, of a subset of RORγt$^+$ T-bet$^+$ Th17 cells from mouse naïve T cells upon stimulation with a combination of IL-6, IL-1β, and IL-23, that possess a more pathogenic phenotype than those differentiatied in the presence of TGFβ [441].

In humans, the picture is less clear. Initial studies from van Beelen and colleagues found that IL-1 in combination with IL-23 was able to induce IL-17 production from memory CD4$^+$ T cells, but not to induce Th17 cell differentiation from circulating naïve T cells [442]. Early data reporting no requirement for TGFβ in human Th17 differentiation from circulating naïve T cells [443, 444] created some debate in the immunology community by suggesting substantial difference between mice and humans. However, using naïve human umbilical cord blood CD4$^+$ T cells, Manel *et al* [445] found that TGFβ plus IL-1β in combination with either IL-6 and IL-21 or IL-23 were able to induce Th17 differentiation. Moreover, they found that TGFβ up-regulated the expression of RORγt but at the same time inhibited its ability to induce the expression of IL-17, ability that was rescued in the presence of other pro-inflammatory cytokines. Other studies reported different cocktails of cytokines being required for Th17 differentiation from human naïve T cells, but all requiring the presence of TGFβ [446, 447]. Recently Romagnani *et al* proposed that TGFβ may not play a direct role in the differentiation of Th17 cells, but rather favour their development by selectively inhibiting Th1 development [3] (Figure 5.1). Human Th17 cells have been recently described as originating from a subset of CD161$^+$ CD4$^+$ precursors expressing constitutively RORγt, CCR6, IL-23R and IL-12Rβ$_2$ [448]. Only CD161$^+$ but not CD161$^-$ CD4$^+$ naïve T

cells were able to express IL-17 upon stimulation with IL-1β plus IL-23, while no other cytokine combination, including TGFβ, could do so [448]. While $CD161^+$ cells could also differentiate into Th1 ($IFN\gamma^+$) and Th1/Th17 ($IFN\gamma^+$ $IL\text{-}17^+$) under these conditions, and to Th2 ($IL\text{-}4^+$) in the presence of IL-4, $CD161^-$ cells could only give rise to Th1 or Th2 but not Th17 cells [448, 449]. Of note, also $CD8^+$ T cells and γδT cells producing IL-17 express CD161 [450]. Blocking TGFβ or the use of serum-free cultures did not alter the effect of IL-1β plus IL-23, suggesting again that TGFβ may be dispensable for the induction of Th17 in humans [449, 451]. However, the addition of TGFβ inhibited the expression of T-bet (transcription regulator of Th1 cells) and IFNγ but not of RORγt and IL-17. This resulted in a much enriched Th17 culture in the presence of TGFβ [449, 451], suggesting an indirect role of this cytokine in Th17 induction, although it could not be completely excluded that the constitutive expression of RORγt in this small subset of $CD161^+$ $CD4^+$ T cells may reflect the *in vivo* activity of TGFβ before cell isolation [452]. This hypothesis of TGFβ regulating IL-17 indirectly by inhibiting Th1 and Th2 differentiation has also been suggested in the mouse [440].

With respect to inhibitory stimuli, Th1 and Th2 signature cytokines, namely IFNγ, IL-4, IL-13 and IL-25 (IL-17E), were found to directly inhibit Th17 differentiation and IL-17 production [415, 429, 444, 453-456]. Moreover, IL-27 and IL-10 have been reported to inhibit Th17 responses [457-460]. Finally, several cytokines may have a dose or time-dependent effects on Th17 differentiation: TGFβ although required (directly or indirectly) for optimal Th17 induction, at high doses is actually inhibitory [443, 461, 462], and IL-2 was found to be inhibitory at very early but not at late stages of Th17 development [443, 445, 453].

Figure 5-1. Proposed mechanism of differentiation of human Th17 cells. **A**, human Th17 cells and Th1 cells originate from a subset of $CD161^+$ $CD4^+$ naïve T cells constitutively expressing RORγt in response to IL-1β and IL-23. Under the same conditions, $CD161^-$ $CD4^+$ naïve T cells only develop into Th1 cells. **B**, TGFβ acts indirectly on Th17 differentiation by inhibiting Th1 development thus favouring Th17 relative expansion. Reproduced with permission (license number 2866060384565) from [3].

Biological functions of Th17 and IL-17 and the complex relationship with other immune cells

The Th17 lineage is characterized by the production of IL-17A, IL-17F, IL-21, and IL-22 [438, 444]. In addition, mouse Th17 have been reported to produce IL-6 and TNFα, while human Th17 cells also produce IL-26 and CCL20 [438, 444]. IL-17A induces stronger inflammatory responses compared to IL-17F [463], and, in addition, different roles for these two cytokines in pathology have been described [464] (Table 5.1). Although IL-17A and IL-17F are generally produced together, single-positive cells producing only IL-17A or IL-17F have been reported [463]. Moreover, evidence exists of their possible differential regulation [465, 466]. Similarly, although IL-22 is co-expressed with IL-17A in Th17 cells, evidence for a differential regulation of these cytokines exists: IL-23 is needed for the secretion of IL-22, whereas TGFβ inhibits its production [467, 468].

Th17 and IL-17-mediated responses are involved in protective immunity mainly to bacterial and fungal infections, especially at cutaneous and mucosal surfaces, and in the pathogenesis of autoimmune diseases and chronic infections in both mice and humans (Table 5.1). IL-17A and IL-7F through IL-17RA/C act on endothelial, epithelial and stromal cells and cells of the monocyte/macrophage lineage inducing the production of granulopoietic factors (G-CSF, GM-CSF), chemokines (CXCL1, CXCL2, CXCL5, CXCL8 [IL-8], MIP-1, CCL20), pro-inflammatory cytokines (IL-1, IL-6, TNFα), and molecules with antibacterial and tissue-remodelling functions (β-defensins, mucins, S100 proteins, matrix-metalloproteinases) (reviewed in [438, 469, 470]).

The characteristic feature of IL-17-mediated responses is the recruitment of neutrophils to the site of infection/inflammation (Table 5.1). IL-17A and IL-17F have similar potency in this respect [471]. Th17 cells and neutrophils co-localize in inflamed tissues [472] and a reciprocal recruitment between Th17 cells and neutrophils exists (Figure 5.2). IL-17 does not have a direct effect on these cells, which lack the IL-17RC receptor subunit [472], but mediate neutrophil recruitment by inducing chemokine production in stromal cells and by their endogenous production of IL-8 [472]. In addition, IL-17 promotes granulopoiesis via the induction of G-CSF [434]. IL-17 activates neutrophils indirectly through the induction of pro-inflammatory cytokines by other cells [473, 474] and directly through the endogenous production of GM-CSF, TNFα and IFNγ by Th17 cells [472]. Conversely, neutrophils induce Th17 chemotaxis through production of CCL2 and CCL20 [472]. Moreover, phagocytosis of infected apoptotic neutrophils by DCs induces a positive feedback on Th17 cells

through the production of IL-23 [475, 476], and it has recently been described that neutrophils behaving as fully functional APCs are able to induce Th17 differentiation *in vitro* [267].

Figure 5-2. Schematic view of Th17-neutrophil interaction at mucosal surface. **A**, Dendritic cells activated by pathogens produce IL-23, which induces IL-17 and IL-22 production by resident memort Th17 cells. **B**, IL-17 and IL-22 induces the secretion of antibacterial molecules by epithelial cells as well as the production of chemokines that recruit neutrophils to the site of infection (**C**). **D**, Dendritic cells phagocytosing infected apoptotic neutrophils secrete pro-inflammatory cytokines supporting further Th17 differentiation and recruitment. Reproduced with permission (license number 2867720311026) from [477].

Pathology	Effect	Remarks
Bacterial infections		
Klebsiella pneumoniae	Protection/ Exacerbation	Neutrophil recruitment; exacerbation in cystic fibrosis
Citrobacter rodentium	Protection	Neutrophil recruitment
Porphyromonas gingivalis	Protection	Neutrophil recruitment
Mycoplasma pneumoniae	Protection	Neutrophil recruitment
Listeria monocytogenes	Protection	When Th1 response impaired
Francisella tularensis	Protection	IL-23/IL-17 required for protective Th1
Streptococcus pneumoniae	Protection	IL-17 important for protection upon challenge after vaccination
Bordetella pertussis	Exacerbation	IL-17 important for Th1 protection upon challenge after vaccination.
Pseudomonas aeruginosa	Exacerbation	In cystic fibrosis
Helicobacter pylori	Exacerbation	Neutrophil infiltration
Mycobacterium tuberculosis and BCG vaccination	Protection	IL-17 response during primary infection promotes Th1 responses protective upon challenge
Fungal infections		
Candida albicans	Protection/ Exacerbation	Protection from oral muco-cutaneous, exacerbation of gastric candidiasis.
Pneumocistis carinii	Protection	
Aspergillus fumigatus	Protection/ Exacerbation	Exacerbation in conditions of impairement of neutrophil functions
Viral infections		
Herpes virus	Exacerbation	Neutrophil recruitment
Human rhinovirus	Exacerbation	Neutrophil recruitment
Parasitic infections		
Toxoplasma gondii	Protection	Neutrophil recruitment
Schistosoma spp	Pathogenic	Exacerbation of egg-induced inflammation
Leishmania spp	Protection/ Pathogenic	Persistance of skin lesions with neutrophil infiltration; protection in vaccine studies
Trypanosoma cruzi	Protection	Induction of protective Th1 response
Trypanosoma. congolense	Protection	Control of parasitemia
Nippostrongylus brasiliensis	Pathogenic	Lung damage with neutrophil infiltration during lung migration of larvae
Autoimmune diseases		
Experimental Autoimmune Encephalitis/ Multiple Sclerosis	Pathogenic	IL-17F not required; IL-17A not major pathogenetic factor; IL-23 indispensable for pathology; relative role of Th1 and Th17/IL-17 debated
Psoriasis	Pathogenic	Both Th1 and Th17 needed
Inflammatory Bowel Diseases	Protection/ Pathogenic	IL-23 induces IL-17 from non-T cells; IL-22 protective; IL-17A and IL-17F protective or pathogenic; IL-21 pathogenic
Collagen Induced Arthritis/ Rheumatoid Arthritis	Pathogenic	IL-17F not required; IL-22 pathogenic; IL-17A probably more important during sensitization than effector phase
Type I Diabetes	Pathogenic	Th17 cells re-programmed into Th1
Systemic Lupus Erythematosus	Pathogenic	IL-17A probably more important during sensitization than effector phase
Allergic reactions		
Asthma	Pathogenic	IL-17A or IL-17F depending on the allergen; post-infection sensitization; neutrophil/eosinophil infiltration or reduction in eosinophil infiltration
Other		
Ischemia-reperfusion injury	Pathogenic	Neutrophil-mediated kidney injury; induction of Th1
Transplant rejection	Protection/ Pathogenic	Neutrophil-mediated rejection of skin grafts; protection in Th2-mediated corneal allograft and GVHD
Tumors	?	Different roles depending on tumor

Table 5-1. Type-17 responses in infectious and autoimmune diseases [413, 438, 470, 477-489].

Other cytokines secreted by Th17 cells are involved in promoting humoral responses and cytotoxic cellular immunity (IL-21) and pro-inflammatory and tissue-repair responses from non-immune cells (IL-22) (reviewed in [438, 469, 470]).

Th17 cells also interact with APCs and other T cell lineages and B cells. Both reciprocal inhibition and potentiation has been described between Th17 and Th1, Tregs and also Th2 responses, and a high level of plasticity of these cells is increasingly observed, accounting for the complexity of their functions (reviewed in [2, 490]).

APCs can drive a Th17 response through the production of Th17-polarizing cytokines upon stimulation by PRR ligands [461, 491] and Th1-derived cytokines [492]. The production of TGFβ by APCs at the infection site may also be important in promoting Th17 differentiation [466].

IFNγ and T-bet expression directly inhibit Th17 differentiation [2, 415, 444, 453] but may indirectly induce Th17 memory responses through APCs stimulation [492]. IL-17 inhibits Th1 cells at an early but not late differentiation stage [493, 494]. IL-17$^+$ IFNγ$^+$ T cells are commonly found during differentiation of naïve T cells to Th17 and in tissues in both mouse and man [2]. Moreover, Th17 cells can be induced to a Th1 phenotype by IL-12 and chronic IL-23 stimulation *in vitro* [453, 495, 496] and *in vivo* [497]. These findings are consistent with the co-existence of Th1 and Th17 cells in inflamed tissues and the cooperation of these T cell lineages in the induction and sustainment of autoimmune pathologies and response to diseases (table 5.1) [498-501].

The interaction between Th17 and Th2/B cell responses has been less studied. Annunziato and colleagues found that human Th17 and Th17/Th1 clones induced the production of IgG, IgM, IgA but not IgE in co-cultures with B cells [453], but a similar study reported a direct induction of IgE by B cells stimulated with IL-17A or in co-culture with Th17 cells [502], and a link between IL-17A and IgE production has been observed in animal models of autoimmune diseases (ref. [502]). Th17 clones from RA patients express the B-cell chemoattractant CXCL13 [503].

Th2 signature cytokines IL-4, IL-13, IL-25 and GATA-3 expression inhibit Th17 induction [415, 444, 453-456, 504, 505]. IL-17 has been reported not to suppress Th2 responses [506], but a more complex lineage interaction has been found in asthma (table 5.1). The global picture from both mouse models and human disease is that IL-23/IL-17 axis contributes to asthma pathology [507] through neutrophil influx [508, 509] but also exacerbation of eosinophil infiltration possibly through activation of APCs and epithelial and stromal cells [509].

A protective role for IL-17 on eosinophil-induced pathology has also been reported [508]. The group of Romagnani found that a subset of $CD161^+$ $CD4^+$ memory T cells produced both Th17 and Th2 signature cytokines [510]. These Th17/Th2 cells were most abundant in patients with asthma and were able to induce IgE *in vitro* [510]. The generation of Th2 from Th17 cells has also been reported in mice [496].

Finally, the relationship between Th17 cells and regulatory T cells, highlighted by the communal requirement for TGFβ, has been the subject of much investigation and debate. It has been widely reported from both mice and humans that $FoxP3^+$ Treg can convert to Th17 cells in a pro-inflammatory cytokine (IL-1, IL-2, IL-6) rich environment, and the existence of $FoxP3^+$ $ROR\gamma t^+$ cells (reviewed in [511]). Also, TGFβ produced by Tregs may actually favour Th17 differentiation and IL-17 production [439, 512]. Although the reverse conversion to Tregs has not been documented, some data show that these cells may retain and restore suppressive functions [513, 514]. Th17 appear to be less efficiently suppressed by Tregs than Th1 and Th2 lineages (reviewed in [515]). In the same publication, the authors proposed that Treg-resistant Th17 may suppress Tregs favouring Th1 and Th2 activity at the infection/inflammation site; then Th17 may convert to Th1 cells in the presence of a constant pro-inflammatory environment, facilitating Treg-mediated suppression and inflammation resolution.

The study of Wei *et al* has shed light on the apparent increased plasticity of Th17 compared to cells of other lineages [516]. Using a genome analysis of permissive and repressive histone methylation marks, they found that genes encoding Th1 and Th2 transcription factors are incompletely silenced in $ROR\gamma t^+$ cells, while the RORγt promoter is repressively methylated in Th1 and Th2 cells. A schematic overview of the complex relationship between Th17 and other $CD4^+$ T cell lineages is shown in Figure 5.3.

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Peck A, Mellins ED: **Plasticity of T-cell phenotype and function: the T helper type 17 example**. *Immunology* 2010, **129**(2):147-153.

Figure 5-3. Schematic view of $CD4^+$ T cell development, reciprocal antagonisms and cell plasticity. Reproduced with permission (license number 2870870451561) from [2].

Other cell sources of IL-17

Non-Th IL-17 producing cells have been described in both mice and humans in the adaptive and the innate immune cell compartment, and can be the predominant source of these cytokines in some settings (Table 5.2).

Innate immune cells can mediate early Type-1 [517] and Type-2 immune responses [518], mirroring the role of Th1 and Th2 cells, respectively, albeit more quickly and less specifically. Similarly, a Type-17 immune response can be found after just a few hours from infection, prior to the development of Th17 cells, and an increasing number of IL-17 producing innate immune cells is being recognized (reviewed in [519, 520]). IL-17 expression is induced by IL-23 also in the absence of B and T cells in mice, and nearly all innate IL-17 producing cells, which predominantly reside in the skin and mucosal surfaces, express IL-23R (ref [438, 519]). The transcriptional factor/s involved in the production of IL-17 by myeloid cells are still not known, with the exception of mast cells in humans and macrophages and neutrophils in the mouse, which have been reported to express RORγt [458, 520-522].

Cell source	Model	Condition	Technique*	References**
CD8⁺ T cells	Mouse	*Ex vivo* *In vitro*	FC, IHC FC, RT-PCR	[523-528] [526-528]
	Human	*Ex vivo* *In vitro*	RT-PCR, IHC, FC ELISPOT, RT-PCR, FC	[529-532] [529, 531, 533]
γδ T cells***	Mouse	*Ex vivo* *In vitro*	FC, IHC, RT-PCR ELISA	[534-539] [535-537]
	Human	*Ex vivo* *In vitro*	IHC, FC ELISA, FC, RT-PCR	[535, 539, 540] [535, 540-542]
LTi cells***£	Mouse	*Ex vivo* *In vitro*	FC, RT-PCR FC, ELISA, RT-PCR	[543, 544] [543]
	Human	*Ex vivo* *In vitro*	FC ELISA	[545] [545]
iNKT cells$	Mouse	*Ex vivo* *In vitro*	FC, RT-PCR ELISA, FC, ELISPOT	[546-550] [547-551]
	Human	*Ex vivo* *In vitro*	- ELISA, FC	- [552-554]
NK cells¥	Mouse	*Ex vivo* *In vitro*	FC ELISA	[555] [555]
	Human	*Ex vivo* *In vitro*	FC	[556]
Macrophages	Mouse	*Ex vivo* *In vitro*	FC, WB, RT-PCR, IHC, cell depletion FC, ELISA, RT-PCR	[557-559] [458, 557, 559, 560]
	Human	*Ex vivo* *In vitro*	FC, IHC, RT-PCR -	[557, 561-563] -
Mast cells	Mouse	*Ex vivo* *In vitro*	- ELISA	- [564]
	Human	*Ex vivo* *In vitro*	FC (only one report [565]), IHC ELISA	[521, 563, 565-572] [521]
Neutrophils§	Mouse	*Ex vivo* *In vitro*	FC, RT-PCR FC, ELISA, IHC	[522, 558, 573-577] [522, 573, 574, 578]
	Human	*Ex vivo* *In vitro*	IHC, WB, RT-PCR, FC IHC, Luminex, ELISA, WB	[562, 563, 568-571, 579-585] [384, 568, 582, 585, 586]
Eosinophils	Mouse	*Ex vivo* *In vitro*	-	-
	Human	*Ex vivo* *In vitro*	IHC, WB, FISH (WB and FISH only one report [587]) Luminex	[587-589] [590]
Epithelial cells	Mouse	*Ex vivo* *In vitro*	IHC, IEM (Paneth cells) RT-PCR (gut), IHC (airways) -	[591] [464, 592] -
	Human	*Ex vivo* *In vitro*	IHC (airways) -	[563] -

Table 5-2. Non-Th17 IL-17 producing cells. *FC, Flow Cytometry; IHC, Immunohistochemistry; RT-PCR, Reverse-transcription PCR; WB, Western Blot; FISH, Fluorescence in situ hybridization; IEM, Immunoelectronmicroscopy. **Most recent publications comprehensive of the techniques used to detect IL-17 in the indicated cells, and not exhaustive of all the literature available. ***Extensive reviews addressing IL-17 producing γδ T cells and LTi cells in mice and humans have been recently published [593-595]. £LTi cells, Lymphoid Tissue inducer cells; $iNKT cells, invariant Natural Killer T cells. ¥NK cells producing the Type-17 cytokine IL-22 but not IL-17 have been reported in mice and humans [596-599]. §See discussion of this chapter.

Helmith-mediated modulation of Type-17 immunity

IL-17 plays a pivotal role in the development of autoimmune diseases (Table 5.1). On the other hand, heminth infections have been implicated in the reduction of allergic and autoimmune pathology, as thoroughly reviewed in a dedicated issue of Immunology (2009 Jan; 126[1]). Although helminths themselves are able also to induce IL-17 expression [128, 481, 483, 488, 600], increasing experimental evidence exists of this being mediated at least in part by the helminth-mediated down-regulation of Type-17 immune responses.

It has been reported that in experimental human hookworm infection systemic and mucosal Th17 responses are downregulated [601], and DCs differentiated in the presence of *Fasciola* secreted molecules induced a reduced production of IL-17 by T cells [602]. In animal models, presence of active helminth infection or exposure to helminth-derived products has been reported to ameliorate autoimmune diseases characterized by a Type-17 immune response. Infection with *H. polygyrus* and soluble proteins from *S. mansoni* and *Ancylostoma caninum* were reported to decrease the severity of Inflammatory Bowel Disease [603-605]. Also, infection with *Trichinella spiralis*, *Taenia crassiceps* and *Fasciola hepatica* attenuated disease manifestations of EAE [606-609]. Finally filarial soluble ES-62 and infection with *S. mansoni* controlled the development of CIA in mice [610, 611]. These results are mirrored in human studies, where it has been reported that infection with *Necator americanus* and *Trichuris trichiura* ameliorate disease manifestations of celiac disease and ulcerative colitis [612, 613]. In both animals and humans, these effects were found to be mediated by the suppression of Type-17 and Type-1 immune responses. This IL-17 down-regulation induced by helminths has also been linked to an exacerbation of mycobacterial infection in a mouse model of *M. bovis* and *Strongyloides* co-infection [614], and a decreased Th17 response has been reported also in humans co-infected with *W. bancrofti* and *M. tuberculosis* [259].

Finally, of particular interest is the observation that helminths are able to modulate the whole Type-17 immune response in both stimulatory and suppressive manners. Indeed, the same parasite (*S. mansoni*) is able to both induce IL-17-mediated pathology but also suppress the development of IL-17-mediated autoimmune diseases [481, 610], while, intriguingly, the infection with another helminth, *H. polygyrus*, is, in turn, able to reduce the IL-17-mediated egg-induced liver pathology in mice infected with *Schistosoma* [615]. Moreover, helminths are able to up-regulate the expression of the Type-17 key cytokine IL-22 [601, 612], known for mediating tissue-repair responses.

5.1.2 Type-17 immune response and filariasis

The features of type-17 immune responses parallel those found in *O. volvulus* infected tissues, and in particular those induced by *Wolbachia*, such as neutrophil recruitment in the cornea and in onchocercomas.
Moreover, filarial extracts and *Wolbachia*-derived molecules can induce a cytokine milieu favourable for Th17 development (Figure 1.4 in chapter 1). Murine DCs stimulated with *Wolbachia*-containing *O. volvulus* and *B. malayi* extracts, but not with extracts from *B. malayi* depleted of *Wolbachia* by tetracycline treatment, secreted IL-6 and IL-23 [47, 151], and this was mirrored by stimulation with *Wolbachia* diacylated lipopeptide WoLP [47]. Similarly, IL-6 was secreted by murine macrophages stimulated with *Wolbachia*-containing *O. volvulus* and *B. malayi* extracts and with isolated *Wolbachia* bacteria but not with extracts of *Acanthocheilonema viteae*, naturally devoid of *Wolbachia* [152, 153]. *In vivo*, *B. malayi* extracts induced high levels of circulating IL-1β and IL-6 when injected in mice [152]. PBMC from patients with lymphatic filariasis stimulated with filarial extracts produced IL-1β, IL-6 and TGFβ [121, 190], and TGFβ was also produced by murine macrophages stimulated with filarial extracts [152]. TGFβ has been found to be abundantly produced in *Onchocerca*-infected tissues (onchocercomas, skin and lymph nodes) by a variety of cells but mainly macrophages [88, 126]. It has also been suggested that filarial worms, independently of the presence of *Wolbachia*, may produce a TGFβ-like homolog of the human cytokine [192, 616], although no TGFβ-like molecules were found in the proteomic analysis of *B. malayi* carried out by Bennuru and colleagues [146, 157].

Wolbachia and *Wolbachia*-containing filarial extracts exert their stimulatory activity on host immune cells through the activation of TLR2/6 by WoLP [47]. APCs stimulated with TLR2 ligands secrete IL-1, IL-6 and IL-23 [443, 617-619], and several studies *in vitro* and *in vivo* reported the induction of IL-17 from Th17 and other cells upon activation of TLR2 [408, 425, 564, 617, 620-624] and TLR6 [625], although a negative regulatory role for TLR2 on Th17 has also been reported [626]. Moreover, a recent study reported the direct induction of Th17 differentiation *in vitro* by neutrophils [267], a cell population linked to the presence of *Wolbachia* and abundant in *Onchocerca* infected tissues. Thus, it appears that the system *Wolbachia*-filaria would provide a suitable milieu for the development of Th17, as Th17-inducing pro-inflammatory cytokines are produced by APCs stimulated by Wolbachia and TGFβ is also produced by these cells as wella as possibly the filarial worm itself.

In the light of the relation between Th17 and Tregs, the work of Korten *et al* [627] reporting a higher number of FoxP3$^+$ cells in *O. volvulus* nodules after depletion of *Wolbachia* is intriguing, and it appears to fit with the hypothesis that the worm's environment would favour, possibly via TGFβ, the induction of regulatory T cells, while *Wolbachia* would provide the stimulus for the production of pro-inflammatory cytokines, skewing the T cell response away from a Treg-dominated and, speculatively, toward a Th17. Nevertheless, a more careful analysis of these results reveals that the percentage of FoxP3$^+$ T cells of total CD4$^+$ T cells does not differ between nodules from doxycycline-treated and placebo-treated patients, and that in nodules from suramin treated patients (suramin kills the worms rapidly without depleting *Wolbachia*) FoxP3$^+$ T cells constitute up to 90% of all CD4$^+$ T cells. Therefore the relation between Tregs and Th17 cells, and the role of *Wolbachia*, should be studied further.

So far, few studies have investigated Th17 and Type-17 immune responses in filariasis. Babu and co-workers reported that IL-17A, IL-17F, IL-21 and IL-23 expression by PBMC in response to filarial extracts was significantly increased in patients with symptomatic filarial lymphoedema compared to asymptomatic infected subjects, suggesting for the first time an involvement of IL-17 in filarial pathology [190]. Further, two studies conducted in Mali reported higher frequencies of CD4$^+$ IL-17$^+$ T cells at homeostasis in people infected with filariasis [128, 145]. Finally, a recent study found that *L. sigmodontis* infection enhanced IL-17 production by splenocytes [193].

5.1.3 Scope of the experimental study

Type-17 immune responses induce pathologic features mirroring those of onchocerciasis. Moreover, the *Wolbachia*-nematode combination could promote a cytokine milieu favourable for the induction of a Type-17 immune response, but very little is known about the actual presence and role of such a response in filariasis, and no published data exists for onchocerciasis. Preliminary immunohistochemistry work conducted by Miss G. Smith in our laboratory found that IL-17$^+$ cells were present in *O. volvulus* nodules, and this presence correlated with that of *Wolbachia* [628].
The work presented here aimed at confirming these preliminary results, identifying the cellular source(s) of IL-17 in *O. volvulus* onchocercomas, and investigating the role of *Wolbachia* lipoprotein WoLP, if any, in the induction of IL-17 from human cells *in vitro*.

5.2 METHODS

5.2.1 Onchocercomas and cell pellets

Onchocercomas from patients infected with *O. volvulus* were from Cameroonian participants in a double-blind, randomized clinical trial of doxycycline for 6 weeks (200 mg/day) ± ivermectin (IVM, 150 μg/kg 4 months after the start of treatment) and placebo [186]. The onchocercomas were surgically removed after 21 months from the start of the treatment, fixed in 80% ethanol and paraffin-embedded.

Cell pellets were obtained by centrifugation at 300 g for 5 minutes, fixed in 80% ice-cold ethanol, and embedded in paraffin, mirroring the processing of onchocercomas. In some cases, pelleted cells were fixed in 4% formalin in PBS. Sections of 4 μm were cut by microtome and mounted by electrothermal bath at 45°C on Poly-L-lysine slides in the Department of Veterinary Pathology, Animal and Population Health, University of Liverpool. Slides were incubated overnight at 58°C before use.

All human material was handled and stored in accordance to the Human Tissue Act 2004.

5.2.2 Neutrophils and PBMC isolation and cultures

The use of white blood cells from adult healthy volunteers for this study was approved by the Research Ethics Committee of the University of Liverpool, UK.

Peripheral blood was processed as described in chapter 4. Polymorphonucleated cells were collected from the lower cell layer and processed as detailed in chapter 4. Unless specified otherwise, PBMC were collected from the first cell layer of the Polymorphprep preparations, washed twice sterile PBS, resuspended in 10 ml RPMI 1640 culture media (Gibco), and further isolated by Lymphoprep following manufacturer's instructions. After centrifugation at 500 g (break off) for 35 minutes at RT, PBMC were collected from the white cell layer, washed twice in sterile PBS, and resuspended in RPMI 1640 culture media. Cell viability was assessed by 0.2% trypan blue staining using a haemocytometer. Cell counting was carried out using a Beckman Coulter cell counter supporting Multinizer 3 software (Beckman Coulter). The purity of isolated cell populations was assessed by rapid Romanowsky staining

of cytospins, as detailed in chapter 4. PMN purity was always ≥ 97% and viability ≥ 98%.
PMN contamination of PBMC was ≤ 5% and viability ≥ 98%. In some cases, PBMC were isolated by Lymphoprep without previous processing on Polymorphprep and the percentage of contaminating PMN was up to 20%.
All cultures were performed at 37°C in a humidified incubator. PMN were cultured using the same culture conditions described in chapter 4. In some experiments, PMN cultures were spiked with 5% PBMC. PBMC were cultured at 1 x 10^6 cells/200 μl/well in U-shape 96-wells tissue culture plates (Nunc), in RPMI 1640 culture media supplemented with 10% heat inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Pen Strep, Gibco) and incubated in the presence of 5% CO_2.
Synthetic WoLP [47] was used in at 0.1 μg/ml and 1 μg/ml. Ultra-pure LPS was used at 0.1 μg/ml. DMSO in equal volume of WoLP was used as the vehicle control for WoLP. PMA and ionomycin were used at 50 ng/ml and 1 μg/ml respectively. Filarial extracts containing *Wolbachia* (BmFE) and depleted from *Wolbachia* (BmFEtet) (Appendix) were used at 200 μg/ml.

5.2.3 HL60 and HEK cell cultures

HL60 cells were purchased from the European Collection of Cell Cultures and maintained in culture as described in the Appendix. HL60 cells were differentiated in neutrophil-like cells by culture at 1 x 10^6 cells/ml/well in 24-well tissue culture plates in the presence of 1.3% DMSO, changing culture media with DMSO every other day, as described by Santos-Beneit and Mollinedo [629]. Final differentiation of HL60 cells was assessed by reduced expression of caspase-3 to levels found in human unstimulated PMN, using RT-PCR (detailed below), as described in [629].
HEK cells were purchased from Invivogen and maintained in culture as described in the Appendix.

5.2.4 Immunohistochemistry and cell imaging

Sections were re-hydrated by sequential passages of 2 minutes each in xylene (twice), 100%, 90%, 70% ethanol and distilled water. Antigen retrieval was performed in 1mM EDTA pH 8.0 by heat procedures, as specified below, followed by 10 minutes cooling in running tap water. Sections were then

encircled with a hydrophobic pen (Calbiochem).
All incubations were carried out at RT in a humidified box. After each step, sections were washed 3 times and soaked for 5 minutes in washing buffer (50 mM Tris + 0.05% v/v Tween20 in PBS, pH 7.6).

Staining for Wolbachia in onchocercomas

Presence of *Wolbachia* in sections of onchocercomas was shown using rabbit polyclonal affinity-purified IgG against wBmPAL [47], kindly provided by Dr K Johnston (Molecular and Biochemical Parasitology, LSTM) diluted 1:500 in Dako Antibody Diluent with Background Reducing Components and visualized with Ultra-Vision ONE Detection System AP Polymer & Fast Red Chromogen, as per manufacturer's instructions.
Briefly, after re-hydration and antigen retrieval (2 x 3.5 minutes in a 800 W microwave), sections were incubated for 5 minutes with the supplied Ultra-V blocking reagent. Primary anti-wBmPAL antibody at 1:500 in Dako Antibody Diluent was added for 2 hours. Sections were then incubated with Ultra-Vision AP-Polymer for 30 minutes and with Liquid Fast Red Chromogen in Naphtol Phosphate Substrate (1:300) for 5 to 10 minutes until optimal staining versus background was obtained. Slides were then rinsed in distilled H_2O, counterstained with Harris Haematoxylin (5 seconds) and Scott's Tap Water (30 seconds), dehydrated by rapid passages in 70%, 90%, 100% ethanol and xylene, and mounted with DPX mounting medium.
A slide from a placebo-treated nodule stained, omitting the primary antibody step, was included as control for unspecific binding of the AP polymer.

Staining for CD4

After re-hydration and antigen retrieval (10 minutes from steady state in a pressure cooker, as optimized experimentally), endogenous peroxidase was quenched by incubation for 30 minutes in 3% H_2O_2 in methanol, before sections were blocked for 30 minutes in TNB blocking buffer. Slides were then incubated for 2 hours at RT with mouse anti-human CD4 IgG_1 (clone 1F6, Novocastra) diluted in blocking buffer at 1:40. Goat anti-mouse IgG-HRP conjugated secondary antibody (NEF822, Perkin Elmer) was used at 1:800 in blocking buffer for 1 hour at RT. Both antibody dilutions were optimized experimentally. The Tyramide Signal Amplification (TSA) Plus FITC System was used as the revealing system. Slides were mounted in Pro-Long Gold anti-fade reagent or Vectashield Mounting Medium with DAPI.
Slides stained omitting the primary antibody were included as control for non-specific binding of the secondary antibody.

Staining for IL-17A

Single staining for IL-17A was performed as described above for CD4. Antigen retrieval was performed in an 800 W microwave with 2 cycles of 3.5 minutes. The primary antibody goat anti-human IL-17A IgG (AF-317-NA, R&D Systems) was used at a 1:10 for onchocercomas and 1:50 for cell pellets. The secondary antibody donkey anti-goat IgG-HRP conjugated (OBT1500P, AbD Serotec) was used at a 1:500 and 1:1000 dilution for onchocercomas and cell pellets respectively. All antibody concentrations were optimized experimentally. The TSA Plus Cyanine3 (Cy3) System was used as the revealing system. Slides were mounted in Pro-Long Gold anti-fade reagent or Vectashield Mounting Medium with DAPI.

Slides stained omitting the primary antibody were included as a control for non-specific binding of the secondary antibody. The use of normal goat IgG in place of the primary antibody controlled for non-specific binding of the goat anti-human IL-17A IgG antibody.

Double staining for CD4 and IL-17A

Sequential co-staining for CD4 and IL-17 was carried out as follows. Rehydration, antigen retrieval and blocking steps were performed as described above for CD4 single staining. Slides were incubated with both mouse anti-human CD4 IgG_1 and goat anti-human IL-17A IgG primary antibodies at the same final concentrations used for the respective single staining. After washing, the secondary antibody goat anti-mouse IgG-HRP conjugated was applied, followed by TSA Plus FITC for CD4 staining. Ten minutes incubation with 3% H_2O_2 in methanol was applied to quench residual HRP possibly not consumed by TSA Plus FITC, as this could have caused a false-positive co-staining. Slides were then incubated with the secondary antibody donkey anti-goat IgG-HRP conjugated and the TSA Plus Cy3 for IL-17A staining. Slides were mounted in Pro-Long Gold anti-fade reagent or Vectashield Mounting Medium with DAPI.

The following slides were included as controls: 1) omission of either of the primary antibodies but incubation with the respective secondary antibody to assess non-specific binding of the secondary antibody; 2) use of primary mouse anti-human CD4 IgG_1 followed by secondary donkey anti-goat IgG-HRP (used for IL-17A staining) to assess non-specific binding of this secondary antibody to anti-CD4 primary antibody, possibly causing a false-positive co-staining; 3) incubation with TSA Plus Cy3 after the quenching step with H_2O_2 , omitting the donkey anti-goat IgG-HRP secondary antibody, to assess the effective quenching of the HRP conjugated to the goat anti-mouse IgG (secondary antibody for CD4 staining), possible cause of false-positive co-staining; 4) single

staining for IL-17A followed by incubation with 3% H_2O_2 to assess the stability of the anti-IL-17A antibody in the procedure, a possible cause of false-negative co-staining; 5) use of donkey anti-goat FITC-conjugated IgG secondary antibody (sc-2024, Santa Cruz Biotechnology; 1:500, 1:300, 1:100) instead of donkey anti-goat HRP IgG followed by TSA Plus Cy3 for the detection of CD4; this control would further exclude false-positive co-staining.

Staining for CD68

Single staining for CD68 was performed as described above for CD4. Primary antibody mouse anti-human CD68 IgG_1 (clone KP1, Dako) was used at 1:1,200. Secondary antibody goat anti-mouse IgG-HRP conjugated was used at 1:500.
Slides from a nodule from placebo-treated patients were stained omitting the primary antibody as control for non-specific binding of the secondary antibody.

Haematoxylin-Eosin staining (H&E)

H&E staining was performed by serial passages in Harris Haematoxylin (2 minutes), 1% Acid Alcohol (5 seconds), Scott's Tap Water (30 seconds) and Eosin (2 minutes). Each step was followed by a rapid wash in running tap water. Slides were then dehydrated by rapid passages in 70%, 90%, 100% ethanol and xylene and mounted with DPX mounting medium.

Identification of IL-17A$^+$ cells in onchocercomas by sequential fluorescent and H&E staining

An attempt was made to detect neutrophils by staining of human neutrophil defensins (mouse anti-HNP1-3 IgG_1, clone D21, Leica Microsystems), but due to the difficulty in optimising the technique (high background and non-specific binding in all conditions tested) and the limitation of nodule material, this approach was abandoned.
Sections of placebo-treated nodules were stained for IL-17A with Cy3 as previously described and mounted in glycerol:PBS 1:1 v/v. Pictures of IL17$^+$-dense clearly identifiable fields were recorded. Following removal of the coverslip, the same section was stained with H&E and mounted with DPX mounting medium, as previously described. Pictures of the same fields were recorded. Merging of IL-17A Cy3 and H&E stained images was carried out in Adobe Photoshop CS4 Extended software (Adobe Systems).
This procedure allowed the staining of granules of PMN cells, therefore differentiating between neutrophils and eosinophils.

Microscopes and software

Single-stained slides were analysed using an Olympus BX60 fluorescent microscope supporting SPOT RT Colour camera and software (Diagnostics Instruments) or Nikon DS-Fi1c camera with NIS Elements Imaging software (Nikon).

Double-stained slides were evaluated by Zeiss LSM5 Pascal confocal laser scanning microscope (Carl Zeiss). Excitation was performed at 488 and 543 nm. Emitted light was collected through a 560 nm low pass filter and a 505-530 band pass filter from a 488/543/633 nm main dichroic beam splitter and a 560 nm secondary dichroic beam splitter. Data capture and extraction was carried out by Zeiss Pascal software.

5.2.5 Cell lysis

For assays carried out in native conditions, cells were lysed using Pierce IP Lysis buffer supplemented with 0.5 M mM EDTA and 1x final concentration protease and phosphatase inhibitors included in Halt Protease and Phosphatase Inhibition Cocktail, following manufacturer's instructions. Briefly, 100 µl complete lysis buffer was added to 5 x 10^6 pelleted cells, incubated on ice for 5 minutes with periodic mixing, and the supernatant collected and stored at -20°C after a centrifugation step of 10 minutes at 13,000 g at 4°C. For the production of cell lysates for the immunoprecipitation assay, the protocol was slightly modified by using 1 ml lysis buffer per 5 x 10^6 PMN and 200 µl lysis buffer per 1 x 10^6 PBMC. For the production of cell lysate for Luminex assays, the Bio-Plex Cell Lysis kit was used, according to manufacturer's instructions. Briefly, after a wash in ice-cold wash buffer, cells were pelleted and incubated in rotation for 20 minutes at 4°C with 250 µl lysis buffer/5 x 10^6 cells. The supernatant was collected and stored at -20°C after a centrifugation step of 20 minutes at 10,000 g at 4°C.

For Western Blot analysis of whole cell lysate in reducing conditions, 5 x 10^6 cells were washed in PBS, pelleted by centrifugation for 3 minutes at 900 g, and lysed in 100 µl boiling Laemmli buffer for 3-5 minutes at 100°C with occasional vortexing. When completely lysed, samples were stored at -20°C.

5.2.6 Protein precipitation

The supernatant from PBMC stimulated for 12 hours with PMA/ionomycin was

added with ice-cold acetone (1:3 v/v supernatant:acetone) and incubated overnight at -20°C. The precipitate was centrifuged at 900 g for 3 minutes and the pellet boiled in Laemmli buffer as described above.

5.2.7 Analysis of IL-17A production by ELISA and Luminex assays

Levels of IL-17A in cell lysates and culture supernatants were measured using two different ELISA kits (DuoSet ELISA Development kit, R&D Systems, and ELISA Ready-SET-Go! Kit, eBiosciences) and two different Luminex assay kits (Bio-Plex Pro Reagent kit, Bio-Rad, and Milliplex MAG Immunoassay kit, Millipore), according to manufacturers' instructions. For analysis by ELISA, cells were lysed using Pierce IP Lysis buffer as described. For the Luminex assays, cells were lysed with Bio-Plex Cell Lysis kit and compatibility with the Millipore kit assessed on a subset of samples. In ELISA, each sample was tested in duplicate, while in Luminex individual samples from 3 donors were assessed singularly. Absorbances of the ELISA assays were read using a FLUOstar Omega plate reader supporting MARS data analysis software 1.20 (BMG Labtech). Luminex assays were analysed in a Bio-Plex system (Luminex xMAP technology) machine supporting Bio-Plex Manager software 4.1.1 (Bio-Rad). The minimum detection limits for ELISA and Luminex assays were 7.8 pg/ml and 3.2 pg/ml respectively.

5.2.8 SDS-PAGE gel electrophoresis and Western Blot

Cell lysates from 5 x 10^6 cells were obtained in boiling Laemmli buffer. The supernatant from PBMC stimulated for ≥ 12 hours with PMA/ionomycin was incubated in acetone (1:3 v/v) overnight at -20°C and the pelleted precipitate diluted in boiling Laemmli buffer. Recombinant human IL-17A (rhIL-17A) diluted in Laemmli buffer (100 ng in 30 µl Laemmli buffer) was used as a positive control.

Protein samples were fractionated using SDS-PAGE electrophoresis in vertical mini-gels. Resolving gels (12% or 14% bis-acrylamide) were casted and overlayed with isopropanol to ensure an even surface of the gel. After polymerization, the isopropanol was removed and the gel surface washed with water. The 4.5% stacking gel was cast on top of the resolving gel and the appropriate casting comb inserted until fully polymerized.

Samples in Laemmli buffer were denatured at 100°C for 5 minutes and loaded

in the wells as follows: 20 µl/lane of cell lysate from 5 x 10^6 cells, 30 µl/lane of PBMC supernatant precipitate, 5 µl/lane of recombinant human IL-17A (100 ng in 30 µl Laemmli buffer). Biotinylated Protein Ladder (M.W. range 9 – 200 kDa, Cell Signaling Technology) and Precision Plus Kaleidoscope Standard (M.W. range 10 – 250 kDa, Bio-Rad) were included in each run as molecular weight markers.

After elecrophoretical fractioning in SDS running buffer (1 hour at 200 V), proteins were electrophoretically transferred to methanol-activated Polyvinylidene Difluoride (PDVF) membranes (Immobilon-P membrane 0.45 µm, Millipore) using a wet blotting technique by applying 100 V for 1 hour. Successful transfer and equal protein loading was rapidly assessed by Ponceau S staining.

After washing in wash buffer (0.1% v/v Tween20 in TBS), membranes were blocked with 5% (w/v) skimmed milk in wash buffer for 1 hour at RT on an orbital shaker. After a rapid wash to eliminate excess blocking buffer, membranes were probed overnight at 4°C on an orbital shaker with primary antibodies in blocking buffer (Table 5.3). After 3 x 10 minutes washes, membranes were incubated for 1 hour at RT on an orbital shaker with the appropriate HRP-conjugated secondary antibody in blocking buffer (Table 5.3). Blots probed with normal goat IgG control, mouse IgG_1 isotype control or secondary antibodies alone were used to confirm the specificity of the primary antibodies binding. Equal protein load was assessed by Ponceau S or nigrosin staining and/or by probing the membranes for β-actin. After washing as above, bound antibodies were visualized by enhanced chemiluminescence (ECL) on X-ray films.

In some cases, goat anti-human IL-17A and mouse anti-human IL-17A from R&D Systems were used at 1:2,500 and 1:5,000 respectively and detected with HRP-conjugated goat anti-mouse IgG/IgM (M30907, Caltag; 1:50,000) and mouse anti-goat IgG (31400, Thermo Scientific; 1:100,000). In these cases, bound antibodies were visualized using SuperSignal West Dura Extended Duration Substrate for HRP (Thermo Scientific) with exposure times between 2 hours and 24 hours. Western Blots in these latter conditions were kindly performed by Dr L Haines (Vector Group, LSTM).

Primary antibody			Secondary antibody HRP-conjugated		
Antibody	**Product**	**Dilution**	**Antibody**	**Product**	**Dilution**
Goat anti-human IL-17A IgG	AF-317-NA, R&D Systems	1:500	Donkey anti-goat IgG	OBT1500P, AbD Serotec	1:5,000
Mouse anti-human IL-17A IgG_1	Clone 41802, R&D Systems	1:500	Goat anti-mouse IgG	NEF822, Perkin Elmer	1:5,000
Rabbit anti-human IL-17A Ig	PRS4877, Sigma Aldrich	1:500	Goat anti-rabbit IgG	NEF812, Perkin Elmer	1:5,000
Goat anti-human IL-17A IgG	sc-6077, Santa Cruz Biotechnology	1:100	Donkey anti-goat IgG	sc-2020, Santa Cruz Biotechnology	1:2,000
Mouse anti-human β-Actin IgG_1	Clone ab8226, Abcam	1:10,000	Sheep anti-mouse IgG	A6782, Sigma Aldrich	1:10,000
Normal goat IgG control	AB-108-C, R&D Systems	1:500	Donkey anti-goat IgG	OBT1500P, AbD Serotec	1:5,000
Mouse IgG_1 isotype control	Clone P3.6.2.1, eBioscience	1:500	Goat anti-mouse IgG	NEF822, Perkin Elmer	1:5,000

Table 5-3. Primary and corresponding secondary antibodies used for IL-17A detection in Western Blot.

5.2.9 RNA extraction and RT-PCR

Total RNA was extracted with TRIzol-Chloroform method followed by purification with RNeasy Mini Kit from 1 x 10^7 PMN and PBMC, and from 1 x 10^6 HL60 cells. Briefly, pelleted cells were lysed in 1 ml TRIzol at RT for 5 minutes before 200 µl chloroform was added and mixed thoroughly. After 3 minutes incubation at RT, samples were centrifuged at 12,000 g for 15 minutes at 4°C and the upper phase containing total RNA collected. Total RNA was precipitated in isopropanol (1:1 v/v RNA-containing phase:isopropanol) for 24 hours at -20°C. RNA was resuspended in 500 µl 70% ethanol in DEPC-treated water after a centrifuging step of 30 minutes at 4°C at 12,000 g, and finally resuspended in 100 µl RNase-free water after centrifugation at 12,000 g for 5 minutes at RT. Total RNA was then further purified using RNeasy Mini Kit including a DNase digestion step performed with RNase-free DNase Set, and quantified using a NanoDrop spectrophotometer.
Total RNA (0. 25 µg for PMN and PBMC, 0.18 µg for HL60 cells) was reverse-transcribed into cDNA into cDNA using SuperScript III First Strand Synthesis System, as per manufacturer's instructions.

Caspase-3 and β-actin (housekeeping gene) in HL60 cells were amplified as

described in Santos-Beneit and Mollinedo [629] using Taq 2X Master Mix. Primers sequences were:

human caspase-3:

GenBank accession mRNA U26943.1

5'-TTTGTTTGTGTGCTTCTGAGCC-3' (forward)

5'-ATTCTGTTGCCACCTTTCGG-3' (reverse)

human β-actin:

GenBank accession mRNA X00351.1

5'-CTGTCTGGCGGCACCACCAT-3' (forward)

5'-GCAACTAAGTCATAGTCCGC-3' (reverse)

Primers were used at a final concentration of 400 nM. PCR conditions were: initial denaturation step 1 cycle at 95°C for 5 minutes, then 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 90 seconds followed by 1 cycle of extension at 72°C for 15 minutes.

For the assessment of IL-17A and ribosomal protein S12 (housekeeping gene) expression, oligonucleotides used in the reactions were:

human IL-17A [489]:

GenBank accession cDNA/gDNA NM_002190/NC_000006

5'-ACTACAACCGATCCACCTCAC-3' (forward),

5'-ACTTTGCCTCCCAGATCACAG-3' (reverse);

ribosomal protein S12:

GenBank accession cDNA/gDNA NM_001016/NC_000006.10

5'-GAATTCGCGAAGCTGCCAAA-3' (forward),

5'-GACTCCTTGCCATAGTCCTT-3' (reverse).

Amplification was performed using QuantiTect SYBR Green PCR kit with primers in a final concentration of 300 nM. PCR conditions were: initial denaturation step 1 cycle at 95°C for 15 minutes, then 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes.

Each sample was amplified in duplicate. RNase and DNAase-free H_2O was included in each assay as negative control. PCR reactions were performed on a Chromo 4 DNA Engine Pelthier Thermal Cycler (Bio-Rad). Melting curves and standard curves for calculation of amplification efficiencies were obtained for the IL-17A amplifications. Standard curves were obtained by 10 fold dilutions of cDNA from positive control sample (PBMC stimulated for 1 hour with PMA/ionomycin).

After amplification PCR products were separated electrophoretically and visualized in 1% or 1.8% agarose gel in Tris-Borate-EDTA buffer with 0.004% v/v ethidium bromide using UV light. DNA ladders were included in each run, according to the expected product size.

For the sequencing of PCR products from the IL-17A amplification, bands were excised with a scalpel and DNA extracted using QIAEX II Gel Extraction Kit as per manufacturer's protocol. Extracted DNA in distilled water was shipped at RT to Beckman Coulter Genomics (Essex, UK) for sequencing and the results analysed using Chromas Lite software (Technalysim Pty).

5.2.10 Immunoprecipitation and mass spectrometry analysis

Immunoprecipitation followed by mass spectrometry analysis was performed to investigate the binding specificity of goat anti-human IL-17A IgG and mouse anti-human IL-17A IgG_1 (R&D Systems).
Analysed samples were cell lysate and supernatant of PMN stimulated for 4 hours with 1 μg/ml WoLP, supernatant of PBMC stimulated for 12 hours with PMA/ionomycin, and recombinant human IL-17A. PMN and PBMC samples were tested for IL-17A in ELISA, with a positive result only for the supernatant of PBMC stimulated with PMA/ionomycin (IL-17A 2 μg/ml).

Immunoprecipitation

For immunoprecipitation, antibodies (0.6 mg goat IgG and 1 mg mouse IgG_1) were coupled to 1 ml HiTrap NHS-activated HP columns (GE Healthcare) following manufacturer's instructions, and maintained at 4°C in storage buffer (0.05 M Na_2HPO_4, 0.1% w/v NaN_3, pH 7.0) until used. Before coupling, antibodies diluted in PBS were processed through Slide-A-Lyzer Dialysis Cassette (2000 MWCO, Thermo Scientific) to exchange buffer to coupling solution (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).
Binding capacity for coupled antibodies were 100 μg IL-17A for the mouse monoclonal antibody and 60 μg IL-17A for the goat polyclonal antibody, assuming a binding efficacy of only 1% due to incorrect binding orientation, antibody denaturation, inefficient binding, and lack of specific antibodies in the polyclonal pool.
Samples (2.5 ml) were processed using a low-pressure affinity chromatography system (ÄKTAprime plus, GE Healthcare) allowing 25 minutes contact between sample and coupled antibodies. Fractions of 0.5 ml were collected during 10

minutes elution in 100 mM glycine pH 3.0, and neutralized using 10% v/v 1 M Tris pH 8.8. Recombinant human IL-17A (200 μg) was used as the positive control.

Fractions of each sample corresponding to those containing eluted rhIL-17A were pooled and processed for mass spectrometry analysis using an in-solution tryptic proteolysis. Briefly, protein concentration and buffer exchange to 25 mM ammonium bicarbonate (AmBic) was performed using Amicon Ultra-4 3K Centrifugal Filter Devices (Millipore), following manufacturer's instructions. Samples concentrated in 100 μl 25 mM AmBic were reduced for 10 minutes at 60°C with 2.5 mM (final concentration) DTT to break S-S bonds and alkylated for 30 minutes at RT in the dark with 7.5 mM (final concentration) iodoacetamide to block cystine residues. Protein digestion was performed with 4 μl 0.25 mg/ml trypsin in 25 mM AmBic (1:50 trypsin:protein ratio) for 12 hours at 37°C. Trypsin digestion was stopped by freezing at -80°C. Samples were maintained at this temperature until further processed.

The day before mass spectrometric analysis, samples were dried in a centrifugal evaporator at 38°C (RVC 2-25, Christ) and maintained at -20°C overnight. For mass spectrometry, samples were reconstituted in 40 μl of 3% acetonitrile and 1% fluoroacetic acid in HPLC water, and further centrifuged for 20 minutes at 13000 rpm at 4°C. Mass spectrometry analysis was performed on 30 μl supernatant.

Reversed-phase HPLC (High Performance Liquid Chromatography)

Peptide samples (10 μl loaded on column) underwent chromatographic separation prior to mass spectrometric analysis using an Untimate™3000 chromatography system (Dionex). Reversed-phase chromatographic separation was conducted at nanoflow rate (0.3 μl/min) using a C18 reverse phase column (3μm particle size (100), 75 μm diameter x 150 mm long). The chromatographic gradient employed for the separation of peptides was composed of buffer A (2.5% acetonitrile: 0.1 % formic acid) and buffer B (90% acetonitrile: 0.1% formic acid). The 60 minute gradient consisted of the following phases: 0-45 min, 0-50% buffer B (linear); 45-45.1 min, 50-100% buffer B (linear); 45.1-50 min, 100% buffer B; 50.1-60 min, 0% buffer B.

Mass Spectrometry

Electrospray ionisation tandem mass spectrometry (ESI-MSMS) was performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Finnigan) coupled upstream to a RP chromatography system (see above). The instrument was operated using Xcalibur 2.0 software (Thermo Scientific). A data-dependent

Top20CID method was used with intact peptides detected in the Orbitrap at a resolution of 30,000. Ions were scanned between 350-2000 *m\z*. The ion-trap operated at CID MS/MS with wide band activation on the 20 most intense ions. Dynamic exclusion was enabled to avoid repeatedly selecting intense ions for fragmentation and this was set at 500 with exclusion duration of 30.0 seconds. Charge states of 1 were rejected. The minimum MS signal threshold was set at 500 counts and the MS/MS default charge state was 4 with a 2.0 *m/z* isolation width, normalised collision energy of 35.0 and an activation time of 10 ms.

MSMS database search

MSMS spectra data were processed using Proteome Discoverer 1.1™ software and searched against a non-redundant human database consisting of a FASTA text files of concatenated protein sequences, from Uniprot, using the MASCOT search engine (Matrix science Ltd). Using a decoy strategy, scoring criteria was selected yielding 1% estimated false positive peptide identification rate. Searches were preformed with variable oxidation of methionine (16 Da) and N/Q deamidation (1 Da). Fixed modification was set for carbamidomethyl cysteines (57 Da). Up to 1 missed cleavage was allowed. A fragment ion mass tolerance of 0.5 Da was used together with a parent ion mass tolerance of 10 ppm. The spectra were filtered at a minimum precursor mass of 350 Da and a maximum of 5000 Da. The *in silico* digestion enzyme was trypsin.

Analysis of samples with reversed-phase HPLC and mass spectromentry was performed by Dr G Laing and Dr G Molyneux (Molecular and Biochemical Parasitology, LSTM). Dr G Molyneux also assisted in setting the MSMS database search parameters.

5.2.11 Statistical analysis

Statistical analysis was conducted on samples from ≥ 3 donors. For ELISA, and RT-PCR assays, samples from each donor were processed in duplicate; for Luminex assay samples fron each donor were processed singularly. Means were compared using independent-samples *t* test. Medians were compared using Mann-Whitney *U* test. For the statistical analysis of labelled cells in onchocercomas, cells were counted in up to 20 randomly selected fields of view (number and magnification as specified in the results section). The distributions of the mean number of labelled cells per field were positively skewed, therefore a Poisson regression model was applied and the

corresponding p-values adjusted for clustering of replicates within nodules. A *p*-value ≤ 0.05 was considered significant. Computations were done in SPSS Statistics 17.0 (IBM) and Stata 11.0 statistical computer package.

5.3 RESULTS

5.3.1 *Wolbachia* is present in nodules from patients treated with placebo and absent in those from doxycycline ± IVM treated patients

All nodules from placebo-treated patients contained worms positive for *Wolbachia*, as assessed using anti-wBmPAL antibodies. On the contrary, all nodules from doxycycline ± IVM treated patients were *Wolbachia*-negative, with the exception of one nodule from the doxycycline-treated group, which contained a worm positive for the endosymbiont. This result is not surprising, and likely reflects reinfection rather than failure of treatment, as described in [185]. This nodule was excluded from the statistical analysis. Representative images of wBmPAL staining are shown in figure 5.4.

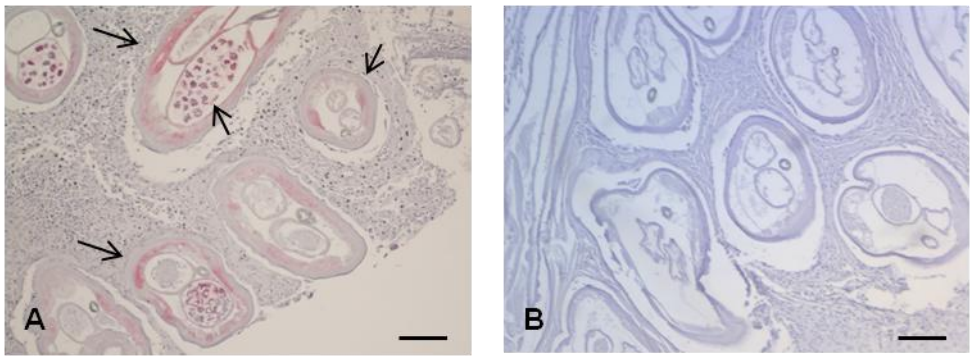

Figure 5-4. wBmPAL staining (red) of representative placebo (**A**) and doxycycline-treated (**B**) nodules. *Wolbachia* stained by anti-wBmPAL antibodies is present in the lateral chords and embryos of adult worms in the placebo-treated nodule (black arrows), while no staining is detected in the doxycycline-treated nodule, indicating clearance from *Wolbachia*. A, Original magnification 20x. Scale bar, 100 μm. B, Original magnification 10x. Scale bar, 200 μm.

5.3.2 The inflammatory cellular infiltrate in onchocercomas depends upon the presence of *Wolbachia*

A total of 28 *O. volvulus* nodules (13 from placebo [P], 7 from doxycycline [D],

and 8 from doxycycline + IVM [D1] treated patients) were investigated by IHC. Individual nodules were from a different subject. The nodule from the doxycycline-treated group that was identified as *Wolbachia*-positive was not included in the statistical analysis to avoid confounding between categorization as *Wolbachia*$^+$ and doxycycline-treated. A rich inflammatory cell infiltrate was present in the centre of nodules from placebo-treated patients around adult worms, and was less abundant in nodules from doxycycline ± IVM treated patients. Macrophages, identified as CD68$^+$ cells, were present both in the centre and in the periphery of nodules, in higher numbers in placebo compared to D1 and D+D1 treated groups (p = 0.003 in both comparisons) (Figure 5.5 A and figure 5.6 A and B). CD4$^+$ T cells were mostly found in the nodules centre around worms but not in close contact with them. The CD4$^+$ T cell infiltrate was also significantly less abundant in doxycycline ± IVM treated nodules compared to placebo (p < 0.001 P vs D; p = 0.022 P vs D1; p = 0.001 P vs D+D1 combined) (Figure 5.5 B and figure 5.6 C and D). By H&E staining, polymorphonucleated cells in nodules were identified as neutrophils, while eosinophils were almost absent in all examined nodules. Neutrophils were found almost exclusively in placebo-treated nodules, around and in close contact with adult worms, while their number was dramatically decreased in the doxycycline ± IVM treated groups, with virtual disappearance of these cells (p < 0.001 P vs D, D1, and D+D1 combined) (Figure 5.5 C and figure 5.6 E).

No staining was found in control slides, confirming the specificity of the staining. Test statistics are detailed in tables 5.4.

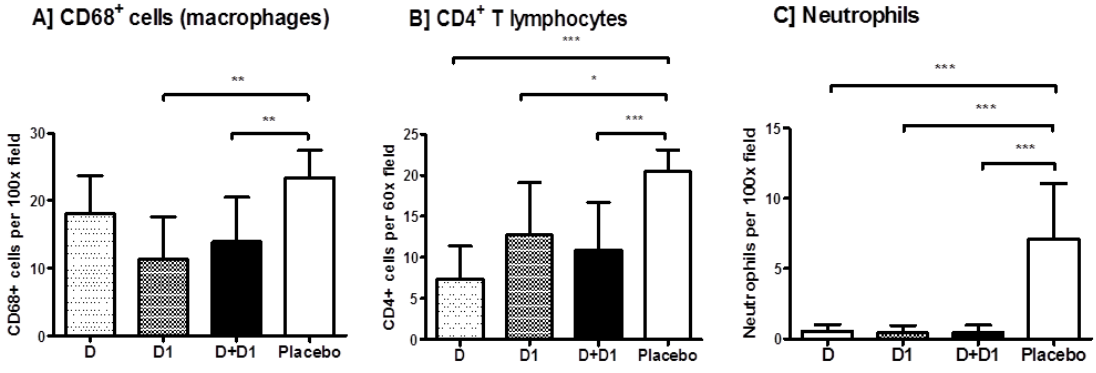

Figure 5-5. Cell populations in *O. volvulus* nodules from patients treated with doxycycline (D, n = 3 nodules), doxycycline + IVM (D1, n = 5 nodules), the two treatment groups combined (D+D1, n = 8 nodules), and placebo (n = 5 nodules). Cells were counted in 20 randomly selected fields of the indicated magnification within the whole nodule section. Bar graphs represent number of cells (mean ± SD) per field. *p = 0.022; **p = 0.003; ***$p \leq 0.001$.

Group	Mean	Standard Deviation		IRR (Incident Rate Ratio) [95% confidence intervals]	p-value
		Between nodules	Within nodules		
CD68⁺ cells (macrophages)					
P	23.44	4.02	12.18	P vs D: 1.29 [0.93-1.79]	0.125
D	18.13	5.59	10.10	P vs D1: 0.48 [0.30-0.78]	0.003
D1	11.38	6.20	0.76	D vs D1: 0.63 [0.37-1.07]	0.087
D+D1	13.91	6.57	9.82	P vs D+D1: 1.68 [1.19-2.38]	0.003
CD4⁺ T Lymphocytes					
P	20.5	2.62	11.1	P vs D: 0.36 [0.22-0.58]	<0.001
D	7.42	3.99	12.6	P vs D1: 0.62 [0.42-0.93]	0.022
D1	12.8	6.28	12.4	D vs D1: 0.58 [0.31-1.09]	0.091
D+D1	10.9	5.79	12.4	P vs D+D1: 0.53 [0.36-0.77]	0.001
Neutrophils					
P	7.10	3.96	9.87	P vs D: 12.95 [5.68-29.52]	<0.001
D	0.55	0.47	1.53	P vs D1: 0.006 [0.02-0.20]	<0.001
D1	0.42	0.53	1.70	D vs D1: 0.77 [0.21-2.81]	0.692
D+D1	0.49	0.48	1.61	P vs D+D1: 14.61 [6.81-21.30]	<0.001

Table 5-4. Statistical analysis of CD68⁺ cells, CD4⁺ T cells, and neutrophils in *O. volvulus* nodules (P, n = 5, D, n = 3; D1, n = 5 nodules) by Poisson regression model. Cells were counted in 20 randomly selected fields (100x magnification for CD68⁺ and neutrophils, 60x magnification for CD4⁺ cells) within the whole nodule section. Results are reported as mean counts per group, with within-nodule and between-nodule standard deviations. Differences between groups are reported as incidence rate ratios (IRR) with 95% confidence intervals; these intervals and *p*-values are adjusted for clustering of replicates within nodules.

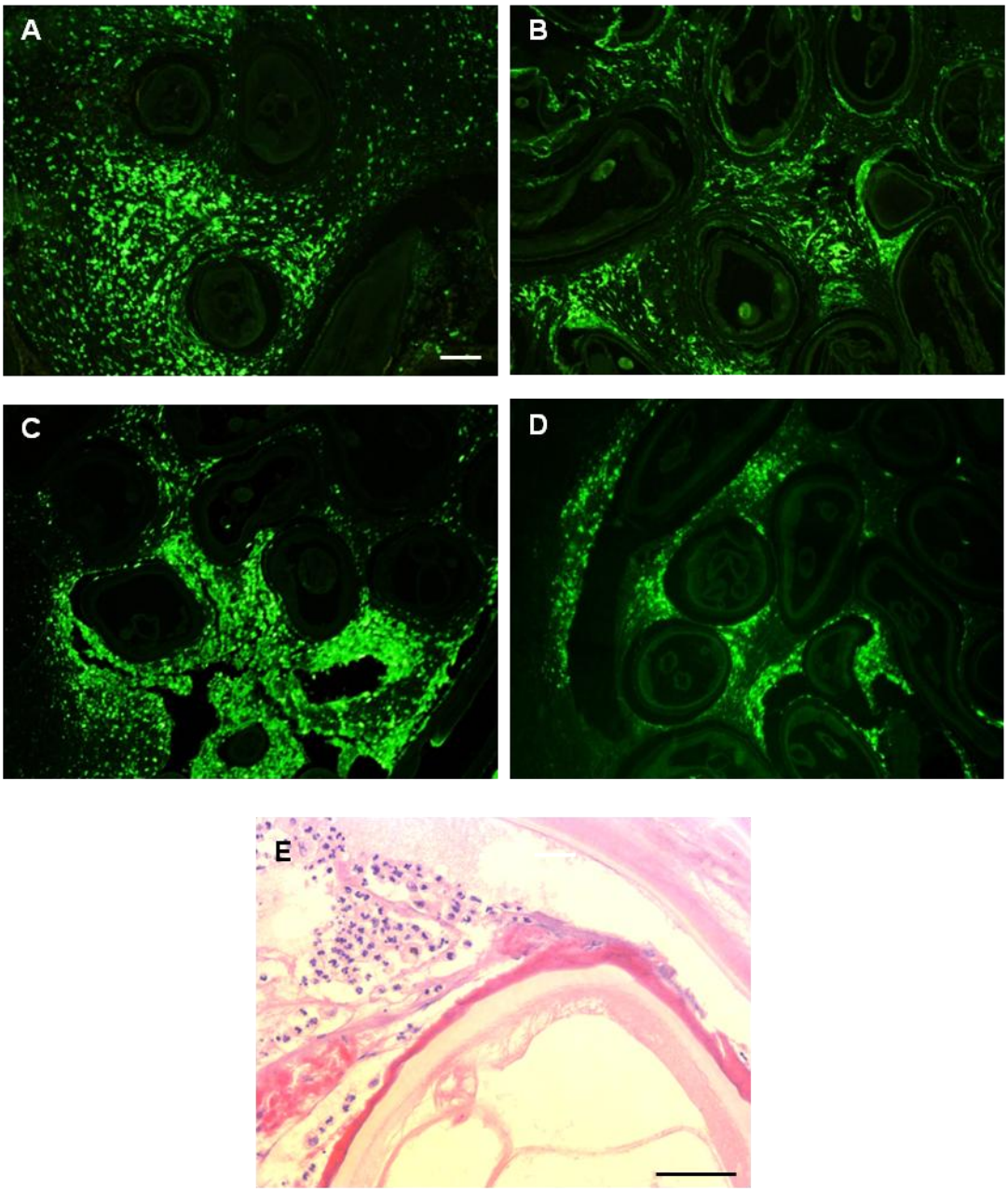

Figure 5-6. Representative images of *Wolbachia*-containing (A, C, E) and *Wolbachia*-depleted (B and D) onchocercomas stained for CD4 (A and B), CD68 (C and D), and with H&E (E). FITC-labelled $CD4^+$ cells (A-B, green) and FITC-labelled $CD68^+$ cells (C-D, green), are more abundant in *Wolbachia*-containing compared to *Wolbachia*-depleted nodules. Image E shows a rich neutrophil infiltrate surrounding a worm from a placebo-treated patient. A-D, fluorescent microscopy, original magnification 10x; scale bar 200 μm. E, bright field, original magnification 20x; scale bar 100 μm.

5.3.3 *Wolbachia*-dependent IL-17A$^+$ cellular infiltration in onchocercomas

Results from previous studies suggest that the combination of stimuli from filarial worms and *Wolbachia* can induce a cytokine milieu favourable for induction of a Type-17 immune response. It was therefore investigated whether IL-17A was produced in *O. volvulus* nodules and if *Wolbachia* could play a role in its production.

Seventeen onchocercomas (8 P, 4 D, and 5 D1) were used for the investigation of IL-17A by IHC. The nodule from the doxycycline treated group that resulted *Wolbachia*-positive was investigated by IHC but excluded from the statistical analysis to avoid confounding between categorization as *Wolbachia*$^+$ and doxycycline-treated. Staining for IL-17A revealed that an abundant IL-17A$^+$ cell infiltrate was present in *Wolbachia*-containing nodules, dramatically decreasing in *Wolbachia*-depleted onchocercomas (p < 0.001 P vs D, D1, and D+D1 combined) (Figure 5.7 and 5.8 A-D, and table 5.5). In *Wolbachia*-containing nodules, the IL-17A$^+$ cell infiltrate was present most abundantly in the nodules centre around and in contact with the adult worms (Figure 5.8 A, C, and E), while scattered IL-17A$^+$ cells were found in the nodules periphery. Of note, a rich IL-17A$^+$ cell infiltrate was also present around the *Wolbachia*-containing worm of the doxycycline treated group (Figure 5.8 G). In *Wolbachia*-depleted nodules, only scanty IL-17A$^+$ cells were scattered in all nodules areas (Figure 5.8 B, D, and F). Absence of staining in control slides confirmed the specificity of the staining.

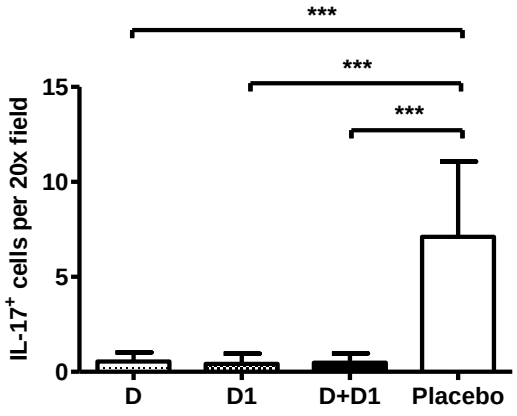

Figure 5-7. IL-17A$^+$ cell infiltrate in *O. volvulus* nodules from patients treated with doxycycline (D, n = 3 nodules), doxycycline + IVM (D1, n = 5 nodules), the two treatment groups combined (D+D1, n = 8 nodules) and placebo (n = 8 nodules). Cells were counted in 12 randomly selected fields of 20x magnification within the whole nodule section. Bar graphs represent number of cells (mean ± SD) per field. *** p < 0.001.

Group	Mean	Standard Deviation		IRR (Incident Rate Ratio) [95% confidence intervals]	p-value
		Between nodules	Within nodules		
IL-17A⁺ cells					
P	16.5	6.80	13.0	P vs D: 0.12 [0.05-0.25]	<0.001
D	1.92	1.59	2.40	P vs D1: 0.09 [0.05-0.19]	<0.001
D1	1.53	1.17	1.93	D vs D1: 1.25 [0.47-3.34]	0.649
D+D1	1.67	1.26	2.10	P vs D+D1: 0.10 [0.06-0.18]	<0.001

Table 5-5. Statistical analysis of IL-17A⁺ cells in *O. volvulus* nodules (P, n = 8; D, n = 3; D1, n = 5) by Poisson regression model. Cells were counted in 12 randomly selected fields of 20x magnification within the whole nodule section. The findings are reported as mean counts per group, with within-nodule and between-nodule standard deviations. Differences between groups are reported as incidence rate ratios (IRR) with 95% confidence intervals; these intervals and p-values are adjusted for clustering of replicates within nodules.

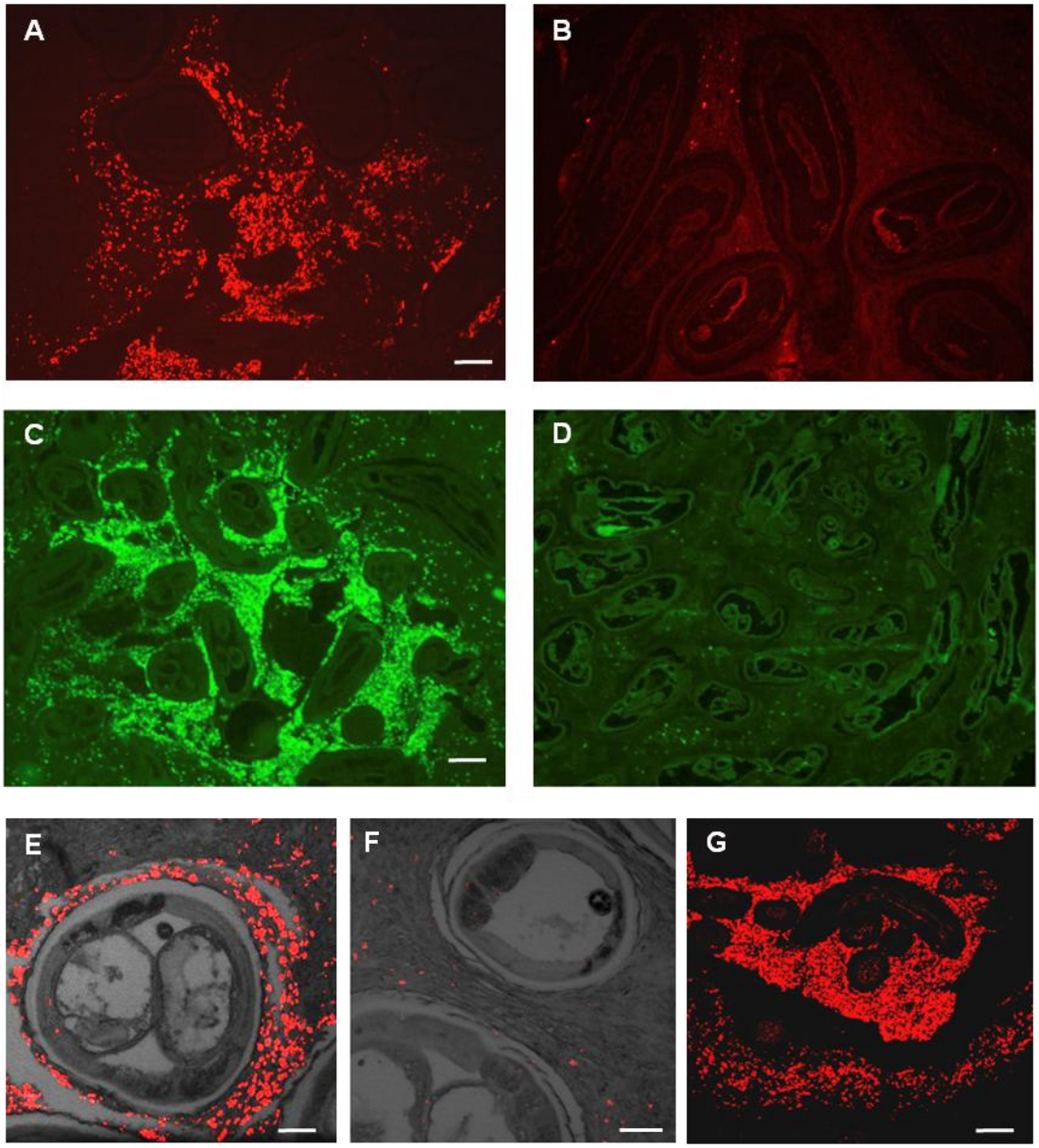

Figure 5-8. Cyanine3 (red) or FITC (green) labelled IL-17A$^+$ cell infiltrate in *Wolbachia*-containing (A, C, E) and *Wolbachia*-depleted (B, D, F) *O. volvulus* nodules. In *Wolbachia*-containing nodules, a rich IL-17A$^+$ cell infiltrate is present in the nodule centre around and attached to adult worms, while only few IL-17A$^+$ cells are present in *Wolbachia*-depleted nodules, scattered in the nodules centre and periphery. Image G: *Wolbachia*-containing worm in a nodule from a doxycycline-treated patient; an abundant IL-17A$^+$ cell infiltrate surrounds the *Wolbachia*$^+$ worm. A-D, fluorescent microscopy, original magnification 10x; scale bar 200 μm. E and F, confocal microscopy, original magnification 20x; scale bar 100 μm. G, confocal microscopy, original magnification 10x, scale bar 500 μm.

5.3.4 Investigation of the cellular sources of IL-17A in onchocercomas

Polymorphonucleated and mononucleated IL-17A⁺ cells are present in nodules and decrease upon Wolbachia depletion

To investigate the possible cellular sources of IL-17A in onchocercomas, nodule sections were stained for IL-17A and the nuclei stained with DAPI to evaluate nuclear morphology. DAPI staining of nuclei showed that IL-17⁺ cells were a heterogeneous population composed of both polymorphonucleated and mononucleated cells (Figure 5.10). IL-17A⁺ cells were counted in 15 random fields of 100x magnification. In *Wolbachia*-containing nodules, IL-17⁺ polymorphonucleated cells constituted 73.24% (IQR 65.36%-74.73%) of the total IL-17⁺ cell population and were virtually exclusively present in the nodules centre (Figure 5.9 B), around and in close contact with worms (Figure 5.10 B), while they virtually disappeared in doxycycline ± IVM treated nodules (p = 0.002 P vs D+D1, figure 5.9 A). IL-17⁺ mononucleated cells were present in both the nodule centre and periphery (Figure 5.9 B), and were also significantly reduced in *Wolbachia*-depleted nodules (p = 0.039 P vs D+D1, Figure 5.9 A). Interestingly, IL-17A⁺ polymorphonucleated cells were often immersed in a DAPI-stained material with a net-like structure (Figure 5.10 A, C, D). Representative images of IL-17A⁺ polymorphonucleated and mononucleated cells are shown in figure 5.10. Test statistics are detailed in table 5.6.

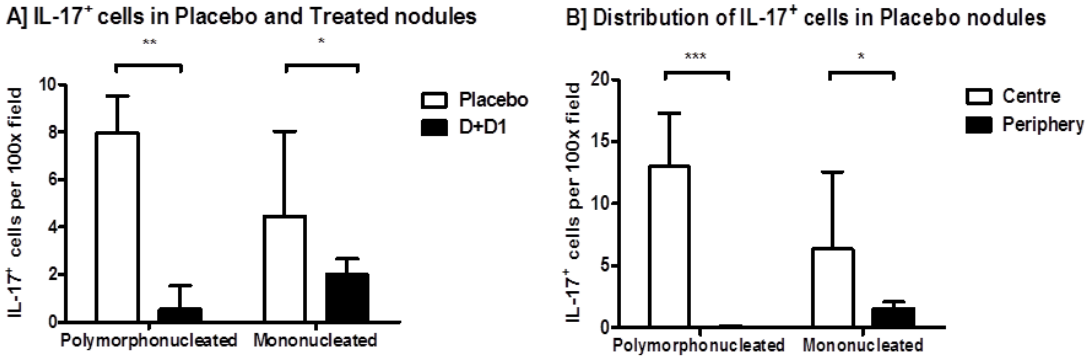

Figure 5-9. Distribution of IL-17⁺ polymorpho- and mono-nucleated cells in onchocercomas, as assessed by IL-17A staining and DAPI-stained nuclear shape. **A**, The IL-17⁺ polymorpho- and mono-nucleated cell infiltrate in nodules from doxycycline and doxycycline + IVM treated patients (*Wolbachia*-negative) is significantly reduced compared to placebo (*Wolbachia*-positive). *p = 0.039, **p = 0.002. n = 5 nodules per treatment group. **B**, Polymorphonucleated IL-17⁺ cells are virtually only present in the nodule centre, while mononucleated IL-17⁺ cells are in the centre and periphery. *p = 0.011, ***p < 0.001. n = 5 nodules. Cells were counted in 15 randomly selected fields of 100x magnification. Bar graphs represent number of cells (mean ± SD) per field.

Group	Mean	Standard Deviation		IRR (Incident Rate Ratio) [95% confidence intervals]	p-value
		Between nodules	Within nodules		
IL-17A⁺ polymorphonucleated cells					
P	7.95	1.56	9.35	14.76 [2.62-83.22]	0.002
D+D1	0.54	1.00	2.46		
Centre*	12.99	4.31	8.11	0.00 [0.00-0.03]	<0.001
Periph*	0.06	0.10	0.23		
IL-17A⁺ mononucleated cells					
P	4.46	3.58	5.43	2.23 [1.04-4.76]	0.039
D+D1	2.00	0.66	1.68		
Centre*	6.32	6.24	4.98	0.24 [0.08-0.73]	0.011
Periph*	1.54	0.53	0.81		

Table 5-6. Statistical analysis of IL17A⁺ polymorphonucleated and mononucleated cells in *O. volvulus* nodules by Poisson regression model. Cells were counted in 15 randomly selected fields of 100x magnification in 5 nodules per treatment groups. 5 nodules of the placebo group were used for IL-17A⁺ cells distribution evaluation (*****). The findings are reported as mean counts per group, with within-nodule and between-nodule standard deviations. Differences between groups are reported as incidence rate ratios (IRR) with their 95% confidence intervals; these intervals and the corresponding p-values are adjusted for clustering of replicates within nodules.

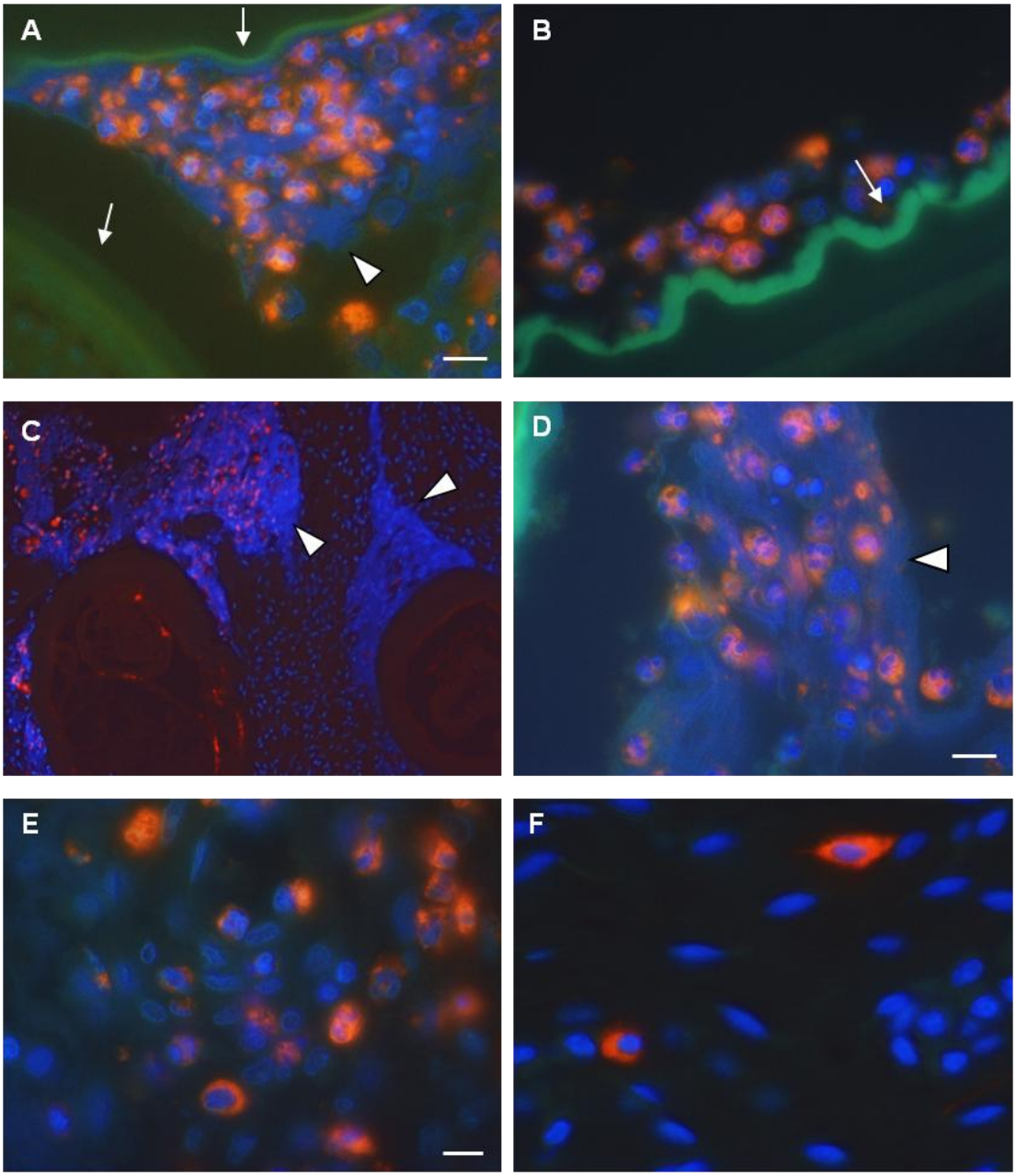

Figure 5-10. Representative images of polymorphonucleated and mononucleated IL-17A$^+$ cells in *O. volvulus* nodules. Sections were stained for IL-17A (Cyanine3; red) and DNA stained with DAPI (blue). **A-C**, IL-17A$^+$ polymorphonucleated cells around and attached to adult worms (arrows), often immersed in a DAPI$^+$ net-like structure (arrow heads). Fluorescent microscopy, original magnification 100x; scale bar 20 μm. **D**, DAPI$^+$ net-like structure with IL-17$^+$ cells around adult worms. Original magnification 20x; scale bar 200 μm. **E**, Polymorphonucleated and mononucleated IL-17A$^+$ cells. Original magnification 100x; scale bar 10 μm. **F**, particular of mononucleated IL-17A$^+$ cells. Original magnification 100x; scale bar 10 μm.

CD4+ T cells but not macrophages are IL-17A⁺ in onchocercomas

The best characterised source of IL-17A in humans are Th17 cells. Therefore, a sequential co-staining of nodule sections for CD4 and IL-17A was performed to address the question whether IL-17⁺ cells in onchocercomas were Th17 lymphocytes. Analysis of placebo nodules revealed that both CD4⁺ and IL-17⁺ cells were distributed around *O. volvulus* worms, but the two populations were largely distinct (Figure 5.11 A). Th17 cells in *Wolbachia*-containing nodules constituted 5.16% (IQR 4.65%-7.83%) of all IL-17A⁺ cells, and a high proportion of all CD4⁺ cells (3.72%, IQR 2.98%-10.11%), while virtually disappeared in doxycycline ± IVM treated nodules (p = 0.014 P vs D; p = 0.003 P vs D1; p = 0.001 P vs D+D1) (Figure 5.11 B and C). Statistics are detailed in table 5.7.
Figure 5.12 shows a representative image of a double-positive Th17 cell. The use of FITC-labelled primary antibody for IL-17A ruled out false-positive staining due to residual non quenched HRP (see methods).

Previous work in our lab found that CD68⁺ cells did not co-stain for IL-17A, excluding macrophages as a source of IL-17A in *O. volvulus* nodules (G. Smith MSc thesis [628]). This result was confirmed by re-assessment of previously co-stained slides from this previous work, but original reproduction of CD68-IL-17A co-staining was not possible due to scaricity of nodule material. Other possible mononucleated cellular sources of IL-17A were not investigated also due to the limitation of material.

Group	Mean	Standard Deviation		IRR (Incident Rate Ratio) [95% confidence intervals]	p-value
		Between nodules	Within nodules		
Th17 cells					
P	1.19	0.85	1.36	P vs D: 0.03 [0.01-0.16]	<0.001
D	0.04	0.06	0.19	P vs D1: 0.02 [0.00-0.11]	<0.001
D1	0.02	0.04	0.14	D vs D1: 1.81 [0.16-2.06]	0.633
D+D1	0.03	0.05	0.16	P vs D+D1: 0.02 [0.01-0.08]	<0.001

Table 5-7. Statistical analysis of CD4⁺ IL17A⁺ double-positive Th17 cells in *O. volvulus* nodules (P, n = 8; D, n = 3; D1, n = 5; nodules) by Poisson regression model. Cells were counted in 12 randomly selected fields of 60x magnification within the whole nodule section. The findings are reported as mean counts per group, with within-nodule and between-nodule standard deviations. Differences between groups are reported as incidence rate ratios (IRR) with their 95% confidence intervals; these intervals and the corresponding p-values are adjusted for clustering of replicates within nodules.

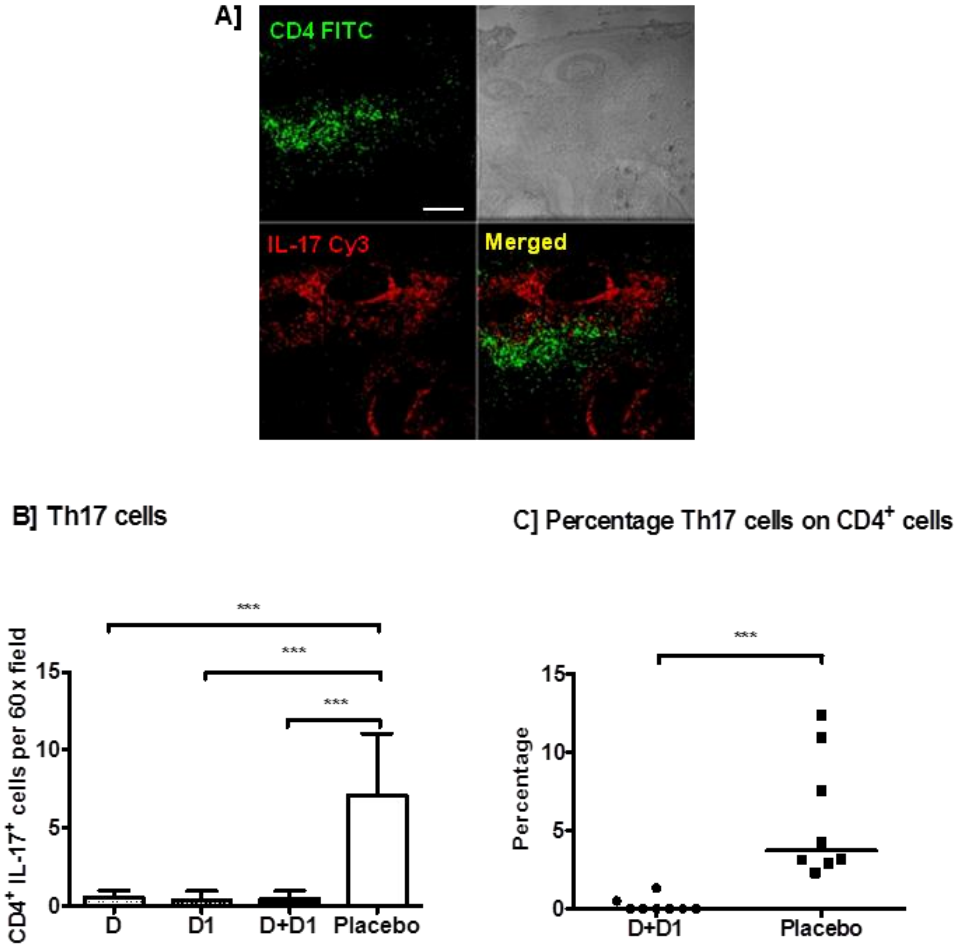

Figure 5-11. Th17 cells are present virtually only in nodules with *Wolbachia*-containing worms. **A**, Representative images of IL-17$^+$ (Cyanine3, red) and CD4$^+$ (FITC, green) cell populations around *Wolbachia*-containing worms. The two populations occupy the same area of the nodule around adult worms but are largely not overlapping (merged). Confocal microscopy, original magnification 10X. Scale bar, 500 μm. **B**, Th17 cells were counted in up to 12 randomly selected fields of 60X magnification. Bar graph represents IL-17$^+$ CD4$^+$ cells (mean ± SD) per field of view in nodules from patients treated with doxycycline (D, n = 3), doxycycline + IVM (D1, n = 5), the two groups combined (D+D1, n = 8), and placebo (n = 8). ***p < 0.001. **C**, Th17 cells represent a high proportion of total CD4$^+$ T lymphocytes. Placebo median Th17 cells on total CD4$^+$ T cells per nodule = 5.16% (IQR 4.65%-7.83%); combined treated groups median Th17 cells on total CD4$^+$ T cells per nodule = 0% (IQR 0%-1.32%). Scatter graph indicates medians. ***p < 0.001.

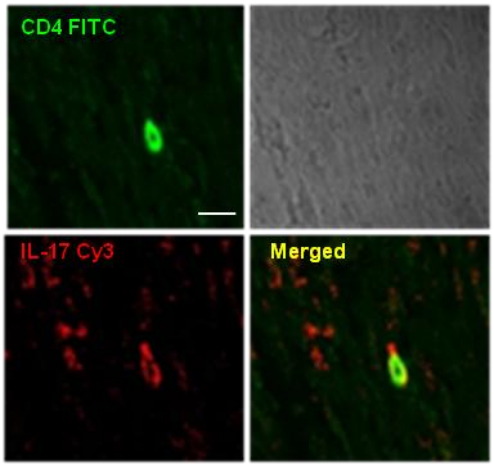

Figure 5-12. Representative image of an IL-17A$^+$ CD4$^+$ Th17 lymphocyte in an onchocercoma from a placebo-treated patient. CD4 is labelled with FITC (green); IL-17 is labelled with Cyanine3 (red). Overlapping staining is shown in yellow (merged image). Confocal microscopy, original magnification 60x. Scale bar 20 μm.

The majority of IL-17A$^+$ cell in onchocercomas are neutrophils

DAPI staining of cell nuclei showed that IL-17A$^+$ cells were a heterogeneous population composed of both polymorphonucleated and mononucleated cells. In *Wolbachia*-containing nodules, IL-17$^+$ polymorphonucleated cells constituted the majority of the total IL-17A$^+$ cell population and were almost exclusively present in the nodules centre, around and in close contact with worms, while virtually absent from doxycycline ± IVM treated nodules (Figure 5.5).

By sequential staining of sections of placebo nodules with anti-IL17A antibodies and H&E, it was confirmed that the IL-17A$^+$ polymorphonucleated cell population was composed of neutrophils (Figure 5.13). Strikingly, virtually all PMN in *O. volvulus* nodules were IL-17A$^+$, in both placebo and doxycycline ± IVM treated nodules. These new and unexpected results suggest that neutrophils may be one of the major sources of IL-17A in *O. volvulus* infection.

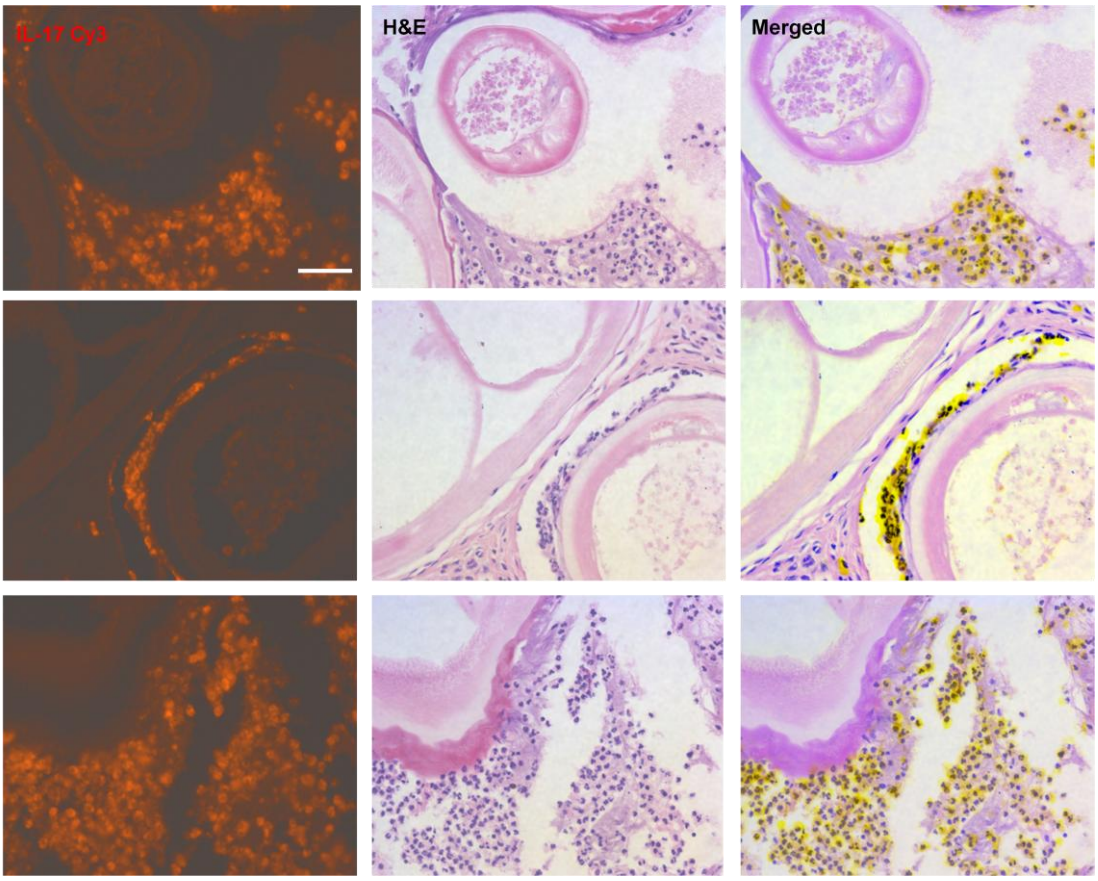

Figure 5-13. Representative images of IL-17A$^+$ (Cyanine3, red) neutrophil (identified by nuclear shape and light blue cytoplasmic stain by H&E) infiltrate around and attached to adult worms in placebo nodules (*Wolbachia*-positive). Merged images show IL-17A signal (yellow) in virtually all neutrophils. The same nodule section was sequentially stained with anti-IL-17 antibodies and H&E. Original magnification 40x, bar scale 200 μm.

5.3.5 Isolated neutrophils but not HL60 and HEK cells are IL-17A$^+$ by immunocytochemistry

In *O. volvulus* nodules, neutrophils were present virtually only in *Wolbachia*-containing nodules, making it difficult to assess the relation between presence of *Wolbachia* and IL-17A staining of these cells.

Isolated human neutrophils were pelleted and prepared with the same procedures as used for onchocercomas (80% ethanol fixed and embedded in paraffin), to investigate IL-17A staining of these cells upon stimulation with WoLP and filarial extracts.

Cells were processed immediately after isolation (T0) and after 4 hours incubation with WoLP (1 μg/ml), LPS (0.1 μg/ml), BmFE and BmFEtet (200

μg/ml), DMSO and medium alone. Strikingly, all T0 neutrophils stained positive for IL-17A, with a specific granule-like pattern of staining (Figure 5.14 A and B). Preliminary immune-transmission electron microscopy (ITEM) analysis of T0 neutrophils confirmed a specific IL-17A signal that appeared in clusters in the cell cytoplasm (Figure 5.15, courtesy of Dr D Voronin, Molecular and Biochemical Parasitology Group, LSTM).

To investigate the hypothesis that IL-17A may be produced and stored in the neutrophils granules before the release of mature granulocytes into the blood, IL-17A staining of HL60 cells during their differentiation to neutrophil-like cells was assessed. This endpoint was reached after 4 days of culture with 1.3% DMSO, as shown by reduction of caspase-3 expression as described in [629] (Figure 5.16). HL60 cells were collected daily and processed as described above. No IL-17A staining was detectable at any time point during the differentiation of HL60 into neutrophil-like cells (Figure 5.17).

Upon stimulation with WoLP and LPS the intensity of the IL-17A signal increased, while no change in signal intensity was induced by incubation with filarial extracts, DMSO or medium alone (Figure 5.17).

When PBMC pellets were analysed, only contaminating neutrophils were positive for IL-17A, as assessed by DAPI staining of nuclei, excluding the binding of anti-IL-17A antibodies to cell structures present in all white blood cells populations (Figure 5.14 C and D). Moreover, after stimulation with PMA/ionomycin (8 hours), rare $CD4^+$ $IL\text{-}17A^+$ Th17 cells were observed in fixed cells (Figure 5.17). No IL-17A staining was observed in HEK cells processed as described above (Figure 5.17). Absence of staining for IL-17A upon probing with secondary antibody alone or goat IgG control confirmed the specificity of primary antibody binding (Figure 5.17). Comparable results were obtained in ethanol-fixed and formalin-fixed cells, excluding artifact effects due to the fixative used (data not shown).

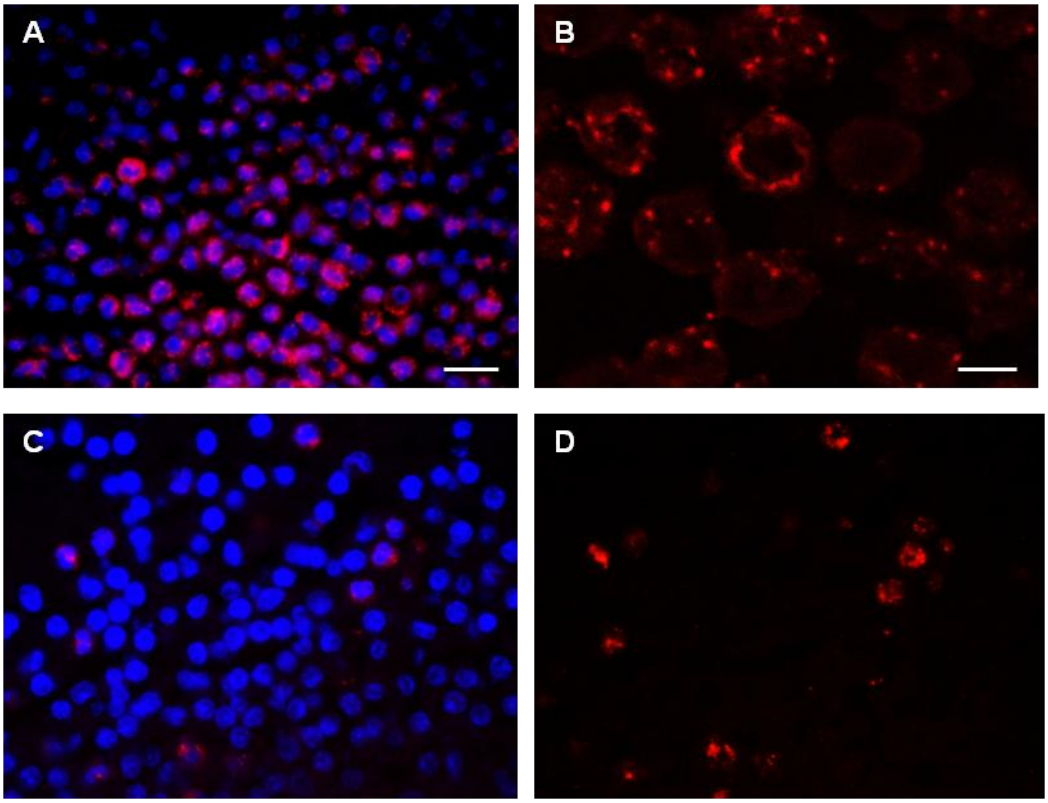

Figure 5-14. Neutrophils immediately after isolation from peripheral blood of healthy volunteers (T0) stain positive for IL-17A (Cyanine3, red). Neutrophils and mononucleated cells are identified by the shape of their nucleus stained with DAPI (blue) in ethanol-fixed paraffin-embedded cell pellet sections. **A**, All neutrophils processed at T0 are IL-17A^{+} (PBMC contamination ≤3%). **B**, The IL-17A staining in T0 neutrophils has a granule-like pattern. **C** and **D**, Images of the same field of T0 PBMC (PMN contamination ≤20%). Isolated T0 PBMC are IL-17A^{-}, the only cells IL-17A^{+} in these preparations being contaminating neutrophils, as evidenced by nuclear shape stained with DAPI (blue). A, C, D, fluorescent microscopy, original magnification 100x, scale bar 20 μm. B, confocal microscopy, original magnification 240x, scale bar 10 μm. Images are representative of 3 donors. Control sections (stained with secondary antibody only and with isotype goat antibody) did not show any staining (see Figure 5-16).

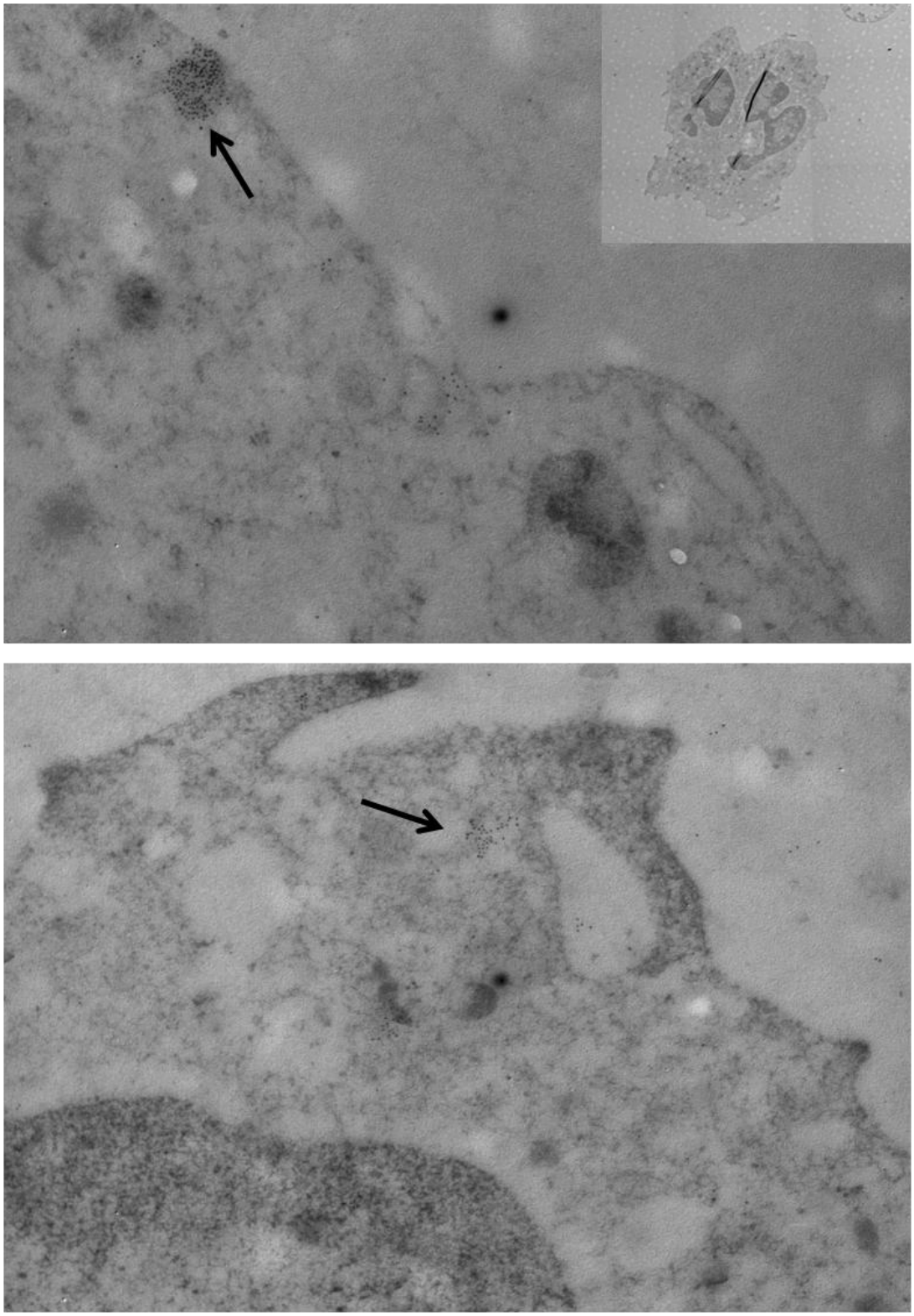

Figure 5-15. Immuno-transmission electron microscopy showing the binding pattern of anti-IL-17A antibodies in isolated human neutrophils (small insert). Arrows indicate specific Ab binding, with signals detected in clusters in cell cytoplasm. The presence of membrane around the antibody signal is not clear. PMN were sections from pelleted unstimulated (T0) cells from blood of healthy volunteers, fixed in 4% formaldehyde 0.1% glutaraldehyde, embedded in lowicryl gold plastic resin and stained with anti-goat IL-17 primary antibody and gold particles-labbelled rabbit anti-goat detecting antibody. Courtesy of Dr D Voronin (Molecular and Biochemical Parasitology, LSTM).

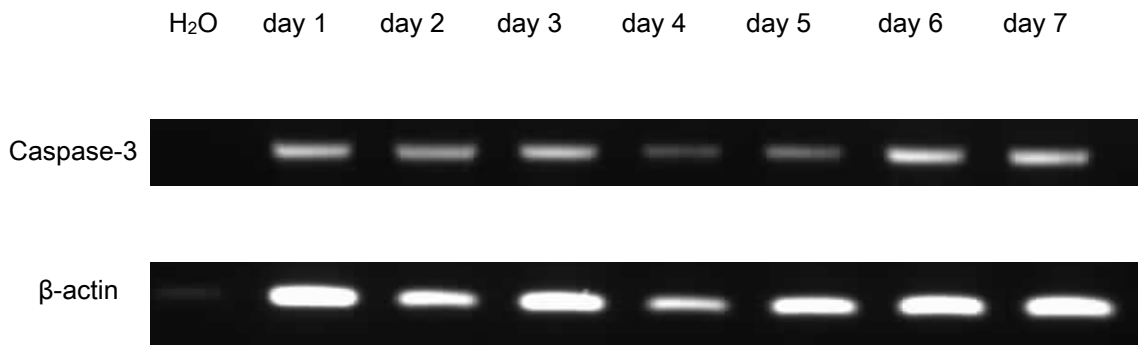

Figure 5-16. HL60 cells differentiate in neutrophil-like cells in 4 days culture with 1.3% DMSO as assessed by minimum expression of caspase-3. Higher expression of caspase-3 at later time points likely indicates that differentiated neutrophils had died by apoptosis, while only differentiation-resistant HL60 cells survived and proliferated. β-actin = housekeeping gene. The figure shows 20 μl PCR product per lane in a 1% agarose gel and is representative of duplicate experiments.

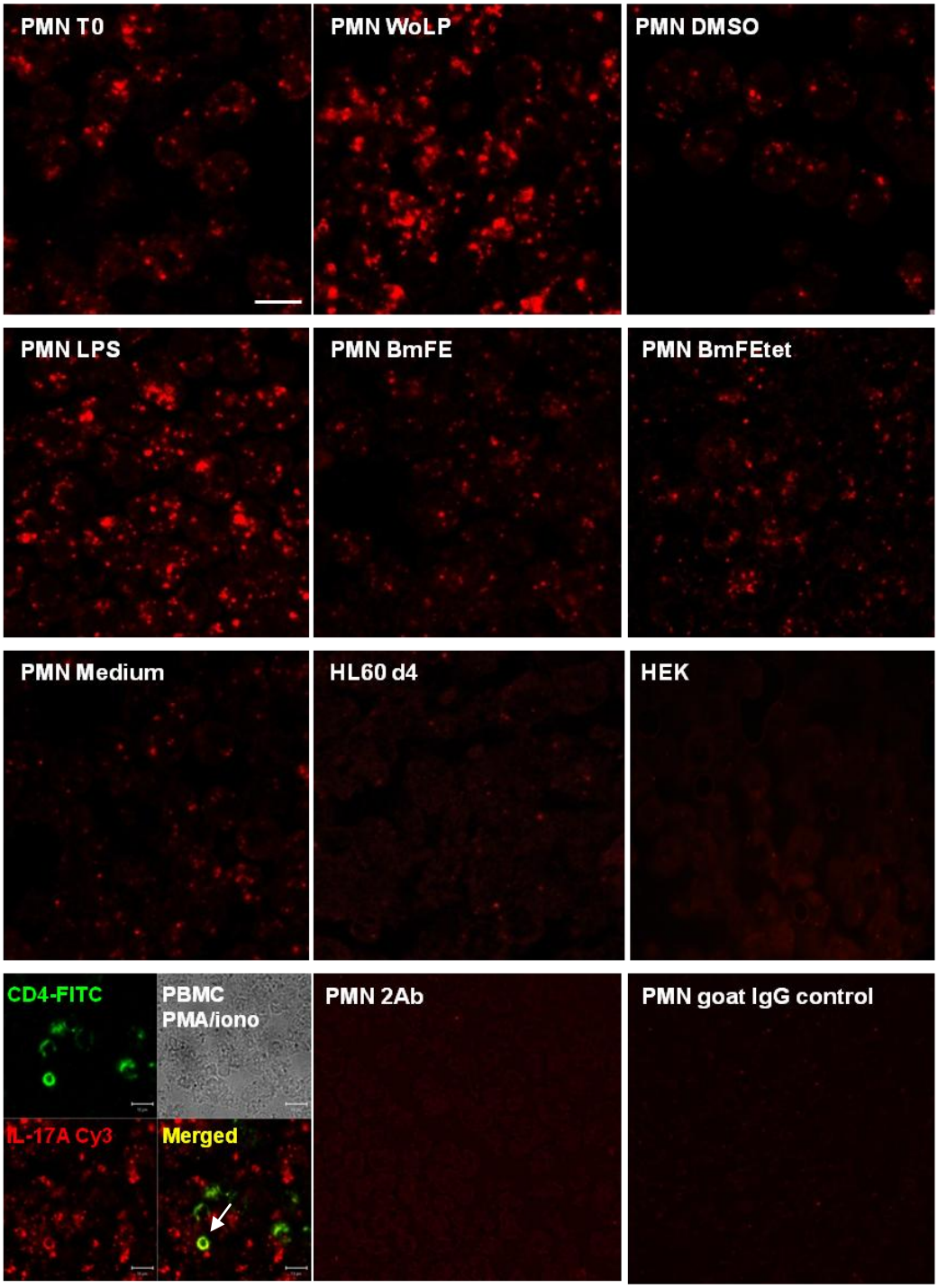

Figure 5-17. IL-17A staining (Cyanine 3, red) in ethanol-fixed paraffin-embedded pelleted cells. PMN from blood of healthy volunteers were processed after isolation (T0) and 4 hours stimulation with WoLP (1 µg/ml), LPS (0.1 µg/ml), BmFE and BmFEtet (both 200 µg/ml), DMSO (same volume as WoLP) and media. HL60 cells were at day 4 of differentiation. PBMC were isolated by Lymphoprep only: IL-17A$^+$ cells were contaminating PMN (≤20%), as assessed by DAPI-stained nuclei in a separate experiment. One CD4$^+$ IL-17A$^+$ Th17 cell is shown (yellow; white arrow). Lack of IL-17A staining in control sections (stained with secondary antibody only or isotype goat antibody control) confirms the specificity of goat anti-human IL-17A antibody. Images are representative of 3 donors (PMN, PBMC) or 3 technical replicates (HL60, HEK), captured with identical settings. Confocal microscopy, original magnification 200x. Scale bar 10 µm.

5.3.6 Expression of IL-17A in neutrophils at the protein level: results of ELISA, Luminex and Western Blot assays

The results of IHC of isolated neutrophils suggested that IL-17A may be present in circulating unstimulated cells (T0), and increase after stimulation with WoLP and LPS. Expression of IL-17A at the protein level by T0 neutrophils has so far only been reported once using Western Blot [571] but never IHC, and it was a surprising finding. It was therefore investigated, with other techniques and different sources of anti-IL-17A antibody, whether IL-17A was detectable at the protein level in isolated neutrophils at T0 and after stimulation. PBMC were stimulated with WoLP (1 μg/ml), LPS (0.1 μg/ml) and DMSO for 4 and 12 hours, while stimulation with PMA/ionomycin (12 hours) was used as the positive control. HL60 and HEK cells were also investigated.

By ELISA and Luminex assays, no detectable levels of IL-17A were found in supernatants or cell lysate of neutrophils at T0 or stimulated for 1 hour, 4 hours and 15 hours with WoLP (1 μg/ml), LPS (0.1 μg/ml), BmFE and BmFEtet (200 μg/ml), as well as with DMSO and media control, with neither of the techniques used. Also, no IL-17A was detectable in HEK (not shown) and HL60 cell culture supernatants and cell lysates at any time point during differentiation. Detectable levels of IL-17A were found in PBMC stimulated with PMA/ionomycin but not with WoLP, LPS, DMSO or media control, in all assays. Results are shown in figures 5.18 to 5.22.

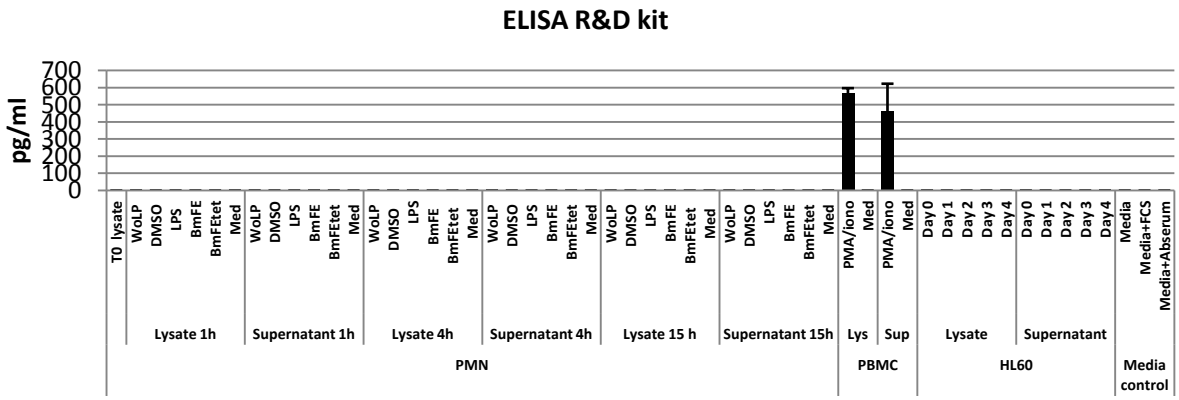

Figure 5-18. IL-17A levels in cell lysate and supernatants as assessed by R&D ELISA kit. PMN (PBMC contamination ≤3%) and PBMC (PMN contamination ≤5%) were isolated from blood of healty volunteers PMN were cultured for the indicated times at 5 x 10^6 cells/ml (1h and 4h cultures) or at 1 x 10^6 cells/ml (15h cultures) in the presence of WoLP (1 µg/ml), LPS (0.1 µg/ml), BmFE and BmFEtet (both 200 µg/ml), DMSO (same volume as WoLP) and media (Med). PBMC at 5 x 10^6 cells/ml stimulated for 12 hours with PMA (50 ng/ml) and ionomycyn (10 µg/ml) were used as positive controls. HL60 cells through their differentiation into neutrophil-like cells upon stimulation with 1.3% DMSO over 4 days were used as bona fide control. Samples of culture media were also included as control. Bar graphs represent mean ± SD of 3 donors, each tested in duplicate (PMN and PBMC) or 3 technical replicates (HL60 and media controls).

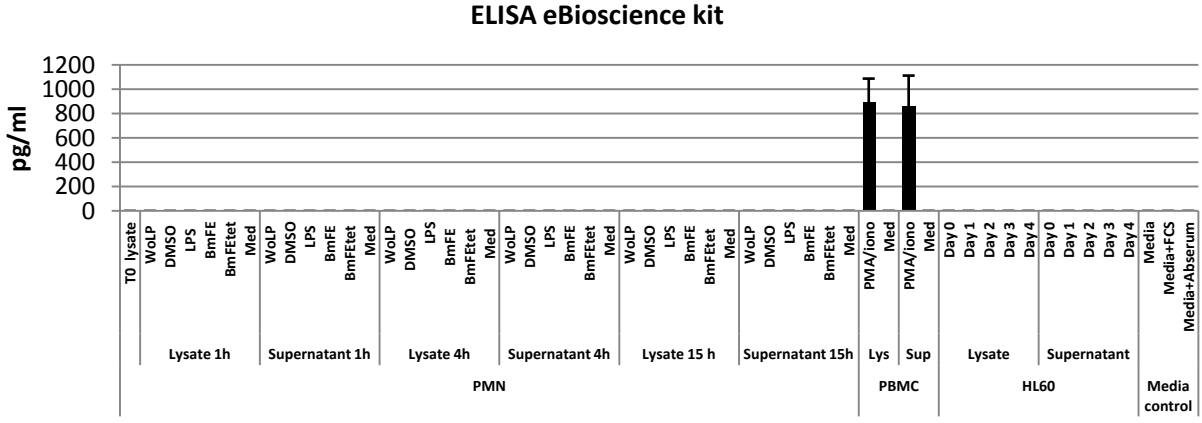

Figure 5-19. IL-17A levels in cell lysate and supernatants as assessed by eBiosciences ELISA kit. PMN (PBMC contamination ≤3%) and PBMC (PMN contamination ≤5%) were isolated from blood of healty volunteers PMN were cultured for the indicated times at 5 x 10^6 cells/ml (1h and 4h cultures) or at 1 x 10^6 cells/ml (15h cultures) in the presence of WoLP (1 µg/ml), LPS (0.1 µg/ml), BmFE and BmFEtet (both 200 µg/ml), DMSO (same volume as WoLP) and media (Med). PBMC at 5 x 10^6 cells/ml stimulated for 12 hours with PMA (50 ng/ml) and ionomycyn (10 µg/ml) were used as positive controls. HL60 cells through their differentiation into neutrophil-like cells upon stimulation with 1.3% DMSO over 4 days were used as bona fide control. Samples of culture media were also included as control. Bar graphs represent mean ± SD of 3 donors, each tested in duplicate (PMN and PBMC) or 3 technical replicates (HL60 and media controls.

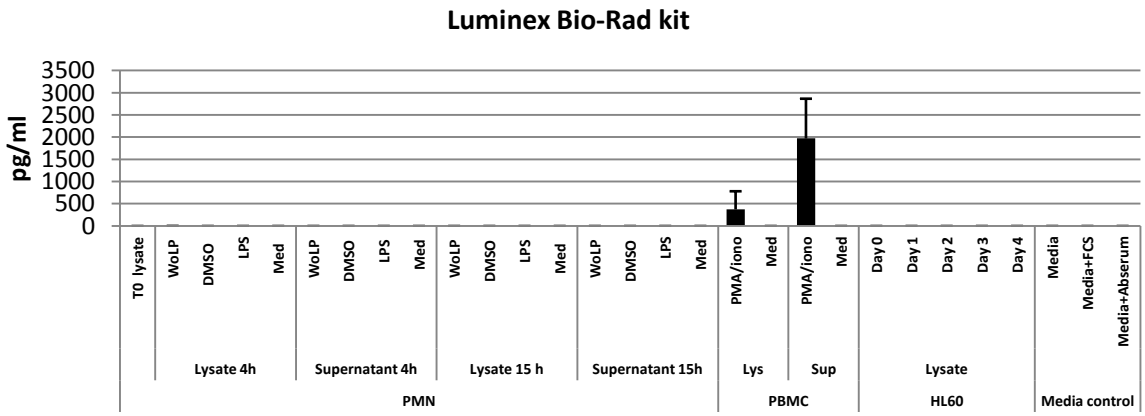

Figure 5-20. IL-17A levels in cell lysate and supernatants as assessed by Biorad Luminex kit. PMN (PBMC contamination ≤3%) and PBMC (PMN contamination ≤5%) were isolated from blood of healty volunteers PMN were cultured for the indicated times at 5 x 10^6 cells/ml (1h and 4h cultures) or at 1 x 10^6 cells/ml (15h cultures) in the presence of WoLP (1 µg/ml), LPS (0.1 µg/ml), BmFE and BmFEtet (both 200 µg/ml), DMSO (same volume as WoLP) and media (Med). PBMC at 5 x 10^6 cells/ml stimulated for 12 hours with PMA (50 ng/ml) and ionomycyn (10 µg/ml) were used as positive controls. HL60 cells through their differentiation into neutrophil-like cells upon stimulation with 1.3% DMSO over 4 days were used as bona fide control. Samples of culture media were also included as control. Bar graphs represent mean ± SD of 3 donors, each tested singularily (PMN and PBMC) or 3 technical replicates (HL60 and media controls).

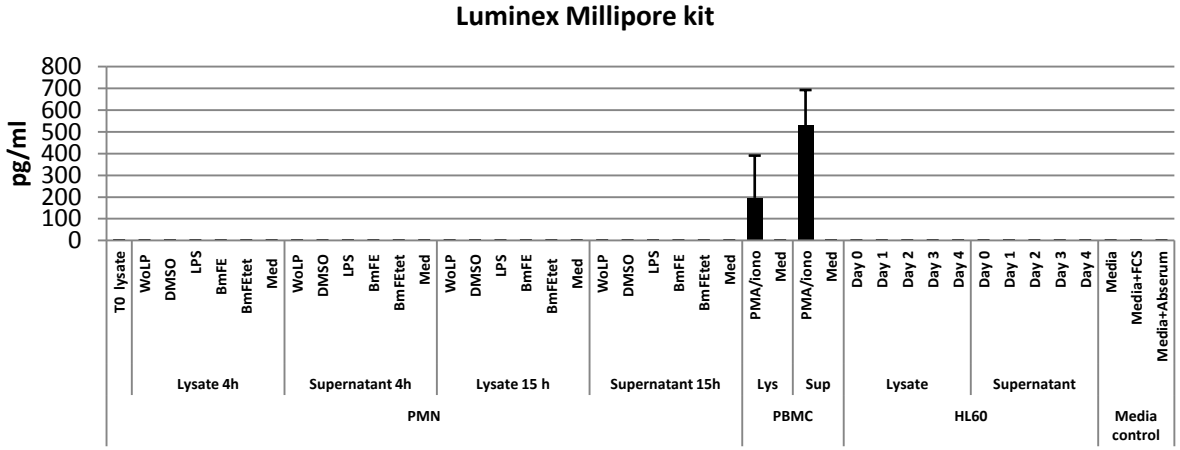

Figure 5-21. IL-17A levels in cell lysate and supernatants as assessed by Millipore Luminex kit. PMN (PBMC contamination ≤3%) and PBMC (PMN contamination ≤5%) were isolated from blood of healty volunteers PMN were cultured for the indicated times at 5 x 10^6 cells/ml (1h and 4h cultures) or at 1 x 10^6 cells/ml (15h cultures) in the presence of WoLP (1 µg/ml), LPS (0.1 µg/ml), BmFE and BmFEtet (both 200 µg/ml), DMSO (same volume as WoLP) and media (Med). PBMC at 5 x 10^6 cells/ml stimulated for 12 hours with PMA (50 ng/ml) and ionomycyn (10 µg/ml) were used as positive controls. HL60 cells through their differentiation into neutrophil-like cells upon stimulation with 1.3% DMSO over 4 days were used as bona fide control. Samples of culture media were also included as control. Bar graphs represent mean ± SD of 3 donors, each tested singularily (PMN and PBMC) or 3 technical replicates (HL60 and media controls).

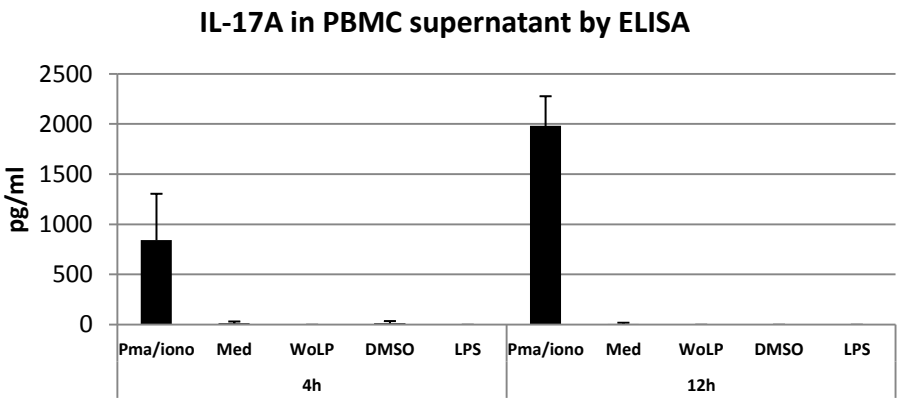

Figure 5-22. IL-17A levels in PBMC (PMN contamination ≤5%) supernatants as assessed by R&D ELISA kit. PBMC were obtained from healthy volunteers and cultured at 5 x 106 cells/ml for the indicated times in the presence of WoLP (1 μg/ml), LPS (0.1 μg/ml), DMSO (same volume as WoLP) and media (Med). Stimulation with PMA (50 ng/ml) and ionomycyn (10 μg/ml) was used as positive control. Bar graphs represent mean ± SD of 3 donors, each tested in duplicate.

Cell lysates in Laemmli buffer were tested for IL-17A by Western Blot under denaturing and reducing conditions. Recombinant human IL-17A (rhIL-17A) and acetone-precipitated supernatant of PBMC stimulated with PMA/ionomycin for 12 hours (Sup^{n}) were used as positive controls. Four different anti-IL-17A antibodies were used: i) goat anti-human IL-17A, AF-317-NA, R&D Biosystems (used for IHC and included as the capture antibody in the R&D ELISA kit); ii) mouse anti-human IL-17A, clone 41802, R&D Biosystems (later referred to as mouse R&D); iii) goat anti-human IL-17A, Sc-6077, Santa Cruz Biotechnology; iv) rabbit anti-human IL-17A, PRS4877, Sigma Aldrich. Details of primary and secondary antibody combinations and dilutions are described in table 5.3.
Different antibody sources were used to control for non IL-17A-specific binding of goat anti-human IL-17A, AF-317-NA, R&D Biosystems (later referred to as goat R&D), the rationale being that non-specific bands would be different upon use of different antibodies. This was important also in the light of recent changes in the commercialization of goat R&D antibody that withdrew Western Blot from the list of the recommended use of this antibody. It is however to note that anti-IL-17A antibodies from Sigma Aldrich and Santa Cruz Biotechnology were anyway raised against IL-17A peptides that partially overlapped with rhIL-17A from R&D, used to produce the R&D anti-human IL-17A antibody sources. This implies that any possible non-IL-17A-specific binding for these former antibodies could be present also with the use of the other anti-human IL-17A an-

tibody sources.

As shown in figure 5.23 C and D, a band of ~16 kDa was detected by both goat and mouse R&D antibodies in rhIL-17A control samples; goat R&D antibody also detected several other bands of higher and lower molecular weight in these samples. Both antibodies also recognised a faint but clear band of the same size in the sample obtained from the supernatant of PBMC stimulated with PMA/ionomycin, suggesting the ability to recognise both recombinant and native IL-17A. However, different results were obtained for cell samples (Figure 5.23 C and D)

Goat R&D antibodies recognised a consistent pattern of multiple bands in all neutrophil samples tested (T0 and stimulated for 1 hour, 4 hours and 12 hours with WoLP (1 μg/ml), LPS (0.1 μg/ml), DMSO and media). One of these bands, of ~17-18 kDa was possibly compatible with non-glycosylated IL-17A (indeed, due to the chemical composition of the supernatant precipitate sample, natural IL-17A band in this sample was detectable at 16 to 18 kDa in different experiments). On the contrary, no bands were detected in any of the PBMC, HL60 and HEK cell samples (Figure 5.23 A and B figure 5.24). Only when membranes were processed with long-exposure ECL, a band of the same size was detectable in PBMC samples, that was more intense in the sample from cells stimulated with PMA/ionomycin than in unstimulated cells. Moreover, with this technique the same band pattern of the neutrophil samples was detected in HL60 cell lysates (Figure 5.25).

In contrast to goat R&D antibodies, mouse R&D antibodies recognised a single band of ~17-18 kDa in all samples, included those from T0 and media-stimulated PBMC, HL60 and HEK cells (Figure 5.25).

No bands of ~22-28 kDa, compatible with glycosylated IL-17A were detected by mouse R&D antibodies, while a compatible band was found in goat R&D antibodies primed membranes (Figure 5.23 to 5.25)

For both antibodies, no difference in band intensity was detectable between samples, when evaluated in the light of β-actin loading control. Also, no differences were found when cells were stimulated with the lower dose of 0.1 μg/ml WoLP (data not shown). Of note, band intensity in goat R&D primed blots was different even when the same sample was analysed in different occasions, while mouse R&D gave more reproducible results.

Specific binding of primary antibodies was controlled for with the use of secondary antibody only, goat IgG and mouse IgG_1 isotype control, which did not result in any band detection (Figure 5.24). Also, no bands were detectable when only the secondary antibodies were applied (data not shown).

The use of anti-human IL-17A rabbit IgG from Sigma Aldrich gave results overlapping with those obtained with goat R&D antibodies (data not shown). The use of goat anti-human IL-17A IgG from Santa Cruz Biotechnology was difficult to optimise and gave poor reproducibility, and was therefore abandoned.

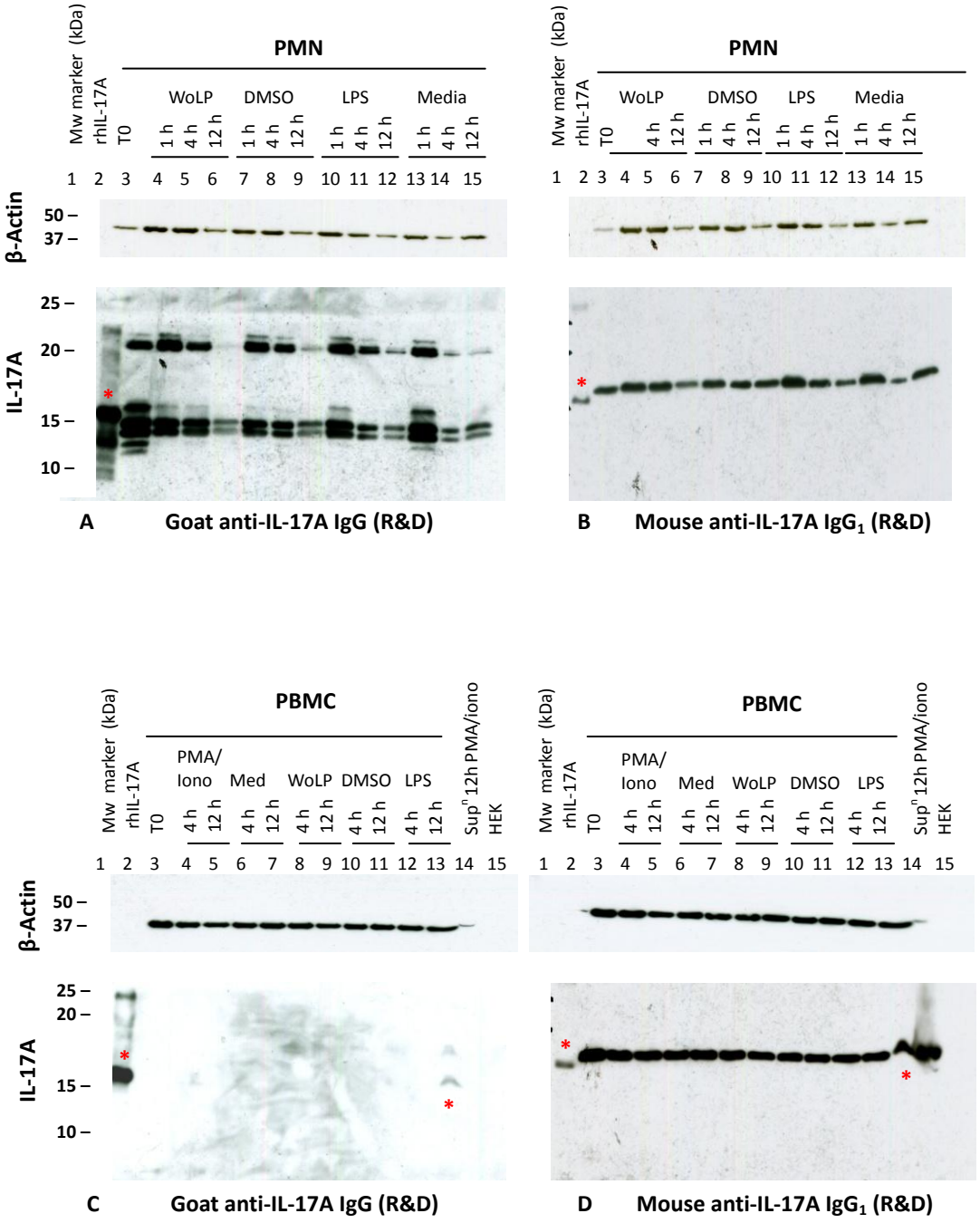

Figure 5-23. Detection of IL-17A by Western Blot using goat anti-human IL-17A IgG (A and C) and mouse anti-human IL-17A IgG_1 (B and D) (R&D Systems) in PMN (A and B), PBMC and HEK (C and D) cell lysates at the indicated time points. PMN (≤3% PBMC contamination) and PBMC (≤5% PMN contamination) from blood of healthy volunteers were cultured at 5 x 10^6 cells/ml in the presence of WoLP (1 µg/ml), LPS (0.1 µg/ml), DMSO (same volume as WoLP) and Media. HEK cells were unstimulated and used as bona fide negative control. Recombinant human IL-17A (rhIL-17, R&D 16.6 ng/lane) and supernatant precipitate (Sup^n) from PBMC stimulated at 5 x 10^6 cells/ml with PMA (50 ng/ml)/ionomycin (10 µg/ml) for 12 hours, used as positive controls, are indicated as bands of ~16-18 kDa (red *). β-Actin indicates protein load per lane. The figures are representative of at least 3 donors (PMN and PBMC) or 3 technical replicates for HEK cell samples.

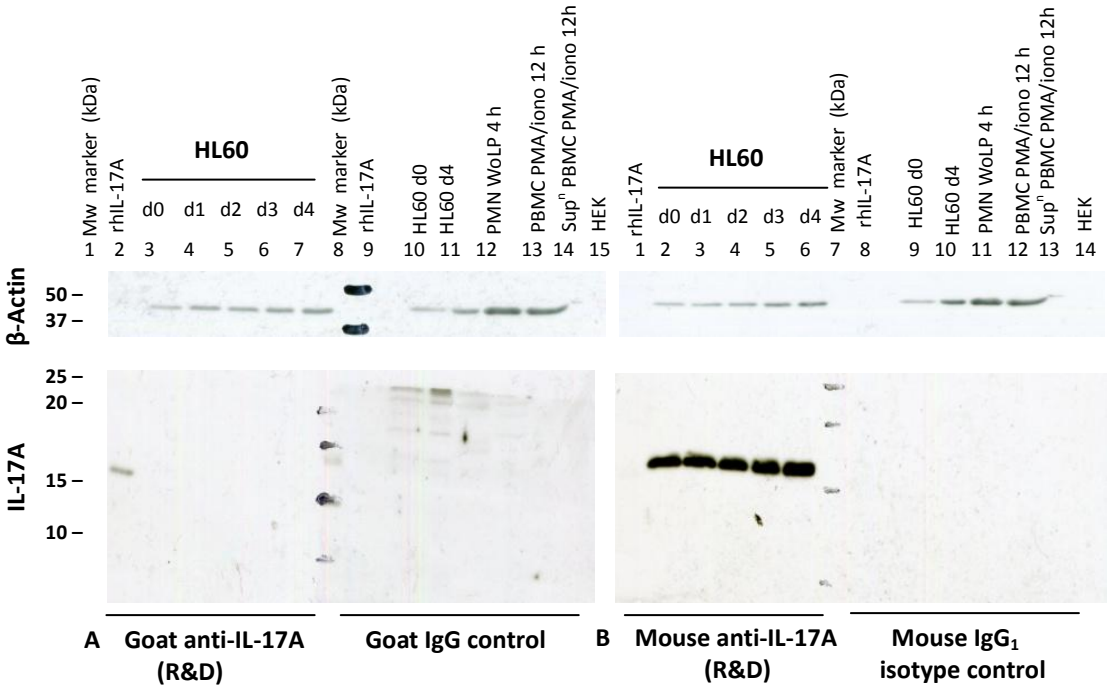

Figure 5-24. Detection of IL-17A by Western Blot using goat anti-human IL-17A IgG (A) and mouse anti-human IL-17A IgG_1 (B) (R&D Systems) in HL60 cell lysates at day 0 (d0) to day 4 (d4) of differentiation to neutrophil-like cells in culture with 1.3% DMSO at 1 x 10^6 cells/ml. Recombinant human IL-17A (rhIL-17, R&D, 16.6 ng/lane) was used as positive control. Absence of bands when control antibodies were used (A for goat and B for mouse antibodies on PMN, PBMC, HEK and HL60 cell samples –ref to figure 5.23 for details of stimulation) indicates specificity of the primary antibody used. β-Actin indicates protein load per lane. The figures are representative of at least 3 technical replicates for HL60 and HEK cell samples and 3 patients for PMN and PBMC samples.

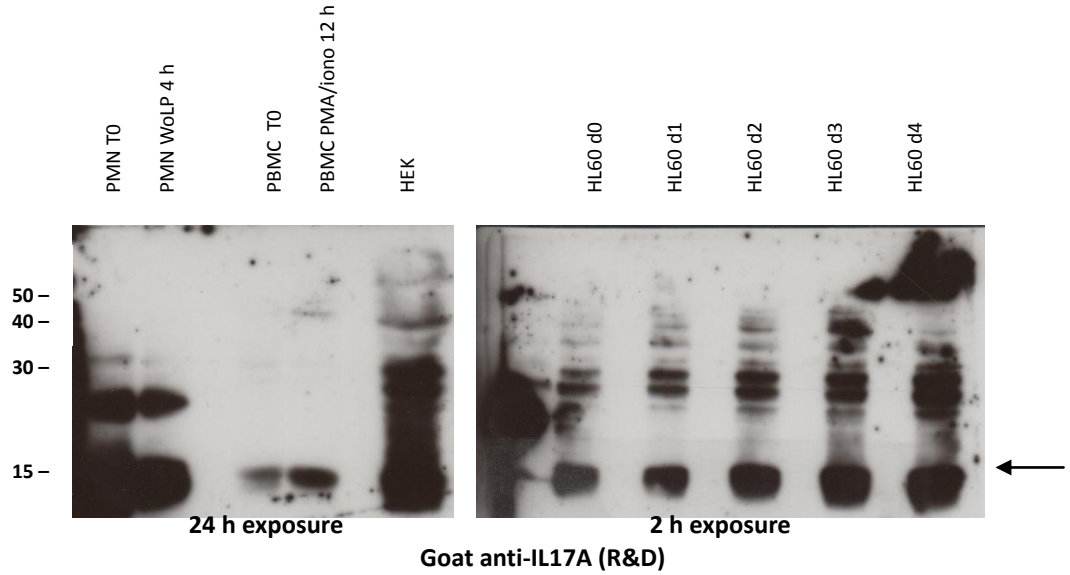

Figure 5-25. Detection of IL-17A by Western Blot using goat anti-human IL-17A IgG (R&D) in PMN, PBMC, HL60 and HEK cell lysates at the indicated time points, using a prolonged exposure method. PMN at 5 x 10^6 cells/ml were stimulated with WoLP (1 µg/ml); PBMC stimulated at 5 x 10^6 cells/ml with PMA (50 ng/ml)/ionomycin (10 µg/ml) were used as positive control; HEK cells were ustimulated and used as bona fide negative control; HL60 cells were differentiated in neutrophil-like cells upon stimulation with 1.3% DMSO for 4 days at 1 x 10^6 cells/ml. Multiple bands are visible in PMN, HEK and HL60 lanes. One band of ~ 16-18 kDa, compatible with IL-17A is visible in all samples and is the only detected in the PBMC samples (arrow). Courtesy of Dr L Haines (Vector group, LSTM).

A schematic overview of the results obtained by IHC, ELISA, Luminex and WB investigating the presence of IL-17A in human neutrophil samples from healty volunteers is given in table 5.8.

Sample	Stimulus	IHC	ELISA		LUMINEX		Western Blot	
			R&D kit	eBiosc. kit	BioRad kit	Millipore kit	Goat anti-hIL17	Mouse anti-hIL17
PMN	**Pellet** All*	+						
	Sup^n All*		-	-	-	-	nd	nd
	Lysate All*		-	-	-	-	+	+
PBMC	**Pellet** PMA/iono	+						
	Media	-						
	Sup^n PMA/iono		+	+	+	+	+	+
	Media		-	-	-	-	nd	nd
	WoLP/LPS		-	nd	nd	nd	nd	nd
	Lysate PMA/iono		+	+	+	+	+ (ony long exp)	+
	Media		-	-	-	-	-	+
	WoLP/LPS		nd	nd	nd	nd	-	+
HL60 cells	**Pellet** All days§	-						
	Sup^n All days§		-	-	-	-	nd	nd
	Lysate All days§		-	-	-	-	-	+
HEK cells	**Pellet**	-						
	Lysate		nd	nd	nd	nd	-	+

Table 5-8. Summary of results of IL-17A production at the protein level by isolated blood-derived human PMN, HL60 cells and control cells: human PBMC, and HEK cells. IL-17A in cell lysates and culture supernatants was investigated by ELISA, Luminex and Western Blot. IHC was carried out on ethanol-fixed paraffin-embedded cell pellets. *PMN were stimulated for 0, 1, 4, and 15 hours with WoLP (1 µg/ml), DMSO (same volume as WoLP), LPS (0.1 µg/ml), BmFE and BmFEtet (200 µg/ml) and media at 5 x 10^6 cells/ml. PBMC were stimulated for 4 and 12 hours with PMA/ionomycin (50 ng/mg and 1 µg/ml respectively) at 1 x 10^6 cells/ml. §HL60 cells were assessed daily during their 1.3% DMSO-induced differentiation to PMN-like cells from day 0 to day 4 cultes at 1 x 10^6 cells/ml. nd = not determined.

5.3.7 Expression of IL-17A in PMN at the mRNA level: results of RT-PCR

The results obtained upon investigation of IL-17A in neutrophils at the protein level were inconclusive, and did not support those of IHC showing an increased IL-17A signal in neutrophils upon stimulation with WoLP and LPS. Therefore, the expression of IL-17A at the mRNA level in neutrophils was investigated after 1 hour and 4 hours stimulation with WoLP (1 μg/ml), LPS (0.1 μg/ml), DMSO and media control, and HL60 cells (day 0-4 of differentiation). PBMC were stimulated with WoLP, LPS and DMSO at the same concentrations for 4 hours, and stimulation for 1 hour with PMA/ionomycin was used as the positive control. In some cases, neutrophil cultures were supplemented with 5% PBMC.

A total of 3 to 4 bands of ~60, 80, 120 and 180 bp were present in PMN and PBMC samples, with the exception of the positive control sample (PBMC stimulated 1 hour with PMA/ionomycin), where a single band of ~80 bp was obtained. The band at ~80 bp was compatible with IL-17A amplification product (expected size 84 bp), and was present in all PMN and PBMC samples. The ~ 60 bp band was present in all samples, included H_2O, with the exception of the positive control, and was compatible with primer-dimers. The bands of higher size were present only in some samples, and were not consistent between samples of different donors and duplicate amplification of the same sample. Also, band intensity was not consistent between samples of different donors and duplicate amplification of the same sample, despite equal PCR product loaded, as assessed by housekeeping S-12 band intensity. Examples of gel electrophoresis of 2 duplicate amplifications of the same samples are shown in figure 5.26 and are representative of 3 donors. No IL-17A expression was found in HL60 cells, at any time point during differentiation (Figure 5.27).

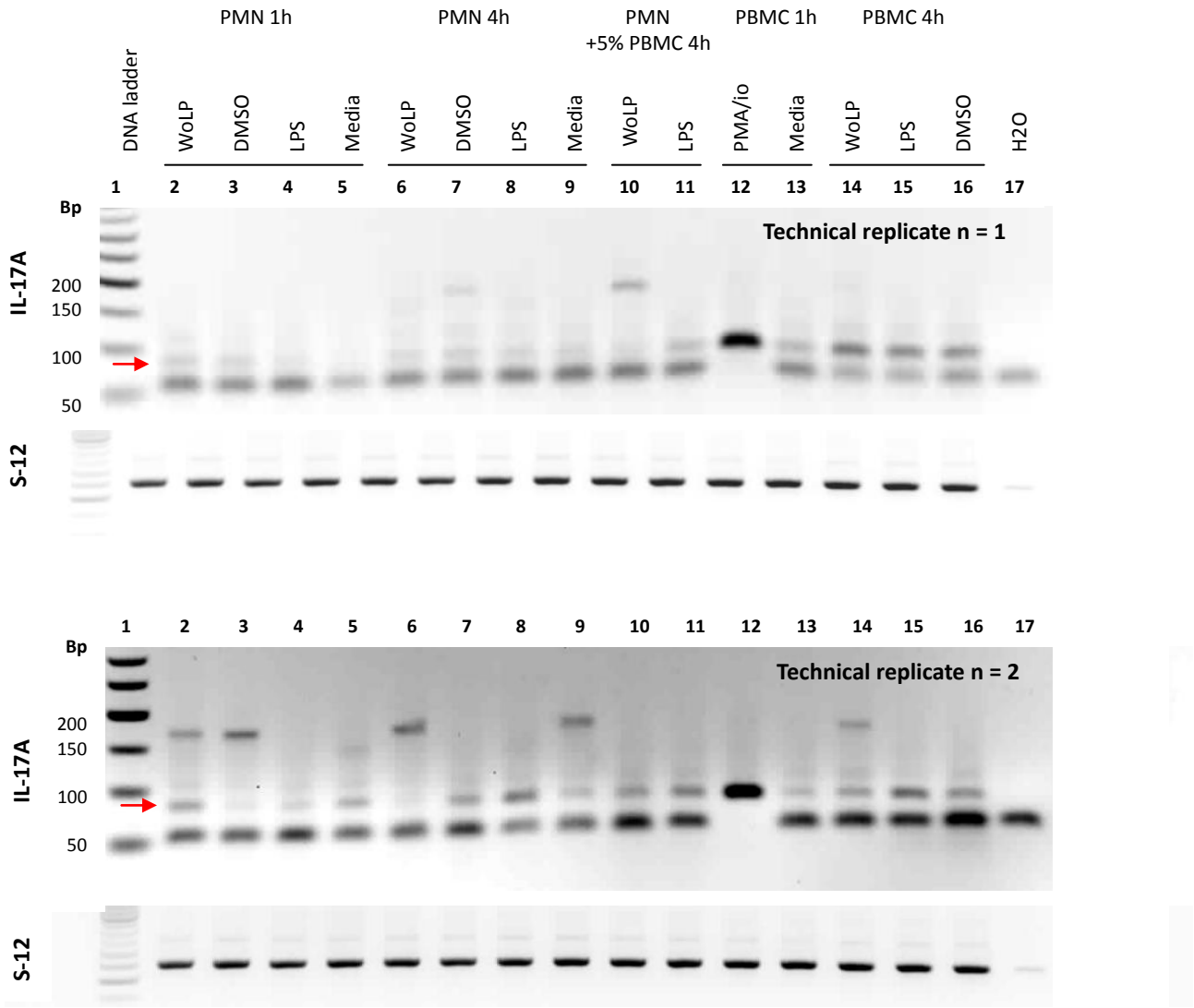

Figure 5-26. Examples of agarose gel electrophoresis of 2 duplicate amplifications of the same donor sample. PMN (≤3% PBMC contamination) and PBMC (≤5% PMN contamination) from blood of healthy volunteers were cultured at 5 x 10^6 cells/ml for the indicated times. Stimuli were WoLP (1 µg/ml), LPS (0.1 µg/ml) DMSO (same volume as WoLP) and media. PBMC stimulated with PMA (50 ng/ml)/ionomycin (10 µg/ml) were used as positive control. H_2O was used as negative control. Red arrows indicate IL-17A PCR products. Examples are representative of 3 donors.

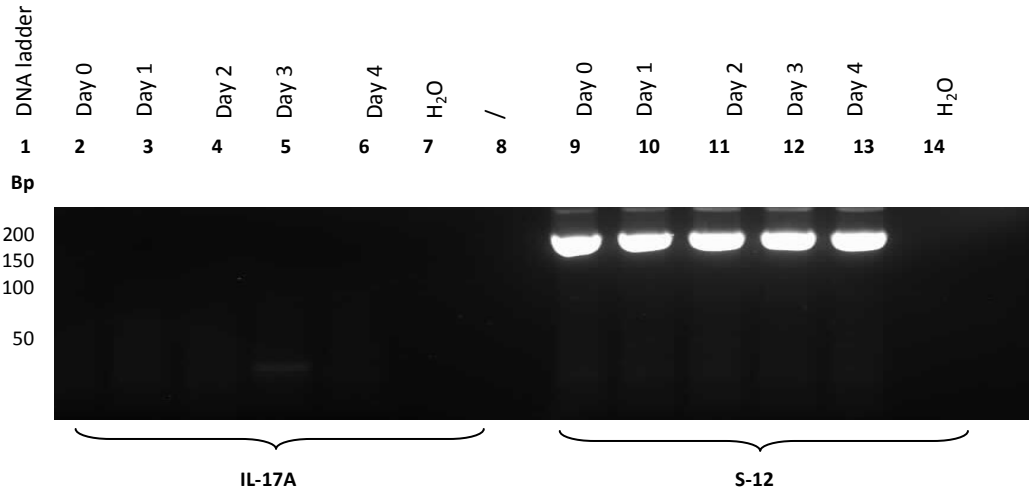

Figure 5-27. Representative agarose gel electrophoresis of IL-17A and housekeeping S-12 PCR amplification products of HL60 cells during differentiation (day 0 to day 4) to neutrophil-like cells in culture with 1.3% DMSO at 1 x 10^6 cells/ml. H_2O was used as negative control.

Quality of PCR was assessed by linear standard curve r^2, amplification efficiency and analysis of melt-curves. R^2 was 0.995-1 in all runs. Amplification efficiency was 91-133%. The >105% efficiency was compatible with co-amplification of nonspecific products such as primer-dimers. These results were supported by the analysis of the melt-curves that showed > 1 peak in some samples. Probes were considered specific for human IL-17A on the basis of BLAST (Basic Local Alignment Search Tool) sequence analysis against human DNA. Moreover, contamination with genomic DNA (gDNA) was excluded on the basis of possible amplification of gDNA by the probes used but lack of bands of compatible size upon amplification. Finally, co-amplification of non-specific products or primer-dimers was absent in the amplification of the positive control sample (PBMC stimulated with PMA/ionomycin for 1 hour), used for PCR optimization. In the light of these results, bands obtained from the electrophoretical separation of PCR products in agarose gel were excised (Figure 5.28) and the extracted amplification products sequenced by Beckman Coulter Genomics (UK). Results of BLAST sequence analysis are summarized in table 5.9. They confirm the presence of primer-dimers and the amplification of human IL-17A cDNA, although the reason why, for this gene, 2 products of different size were obtained is difficult to explain. Indeed, the presence of alternatively spliced isoforms, which could have explained this result, has been recently reported to be absent in both mouse and human IL-17A, contrary to other IL-17 family cytokines and IL-17Rs [630]. When analysed by BLAST the

primers sequences against the sequence of NM_004699.2 (Band 1 accession number), no significant similarity was found, making it difficult to explain its amplification by the primers.

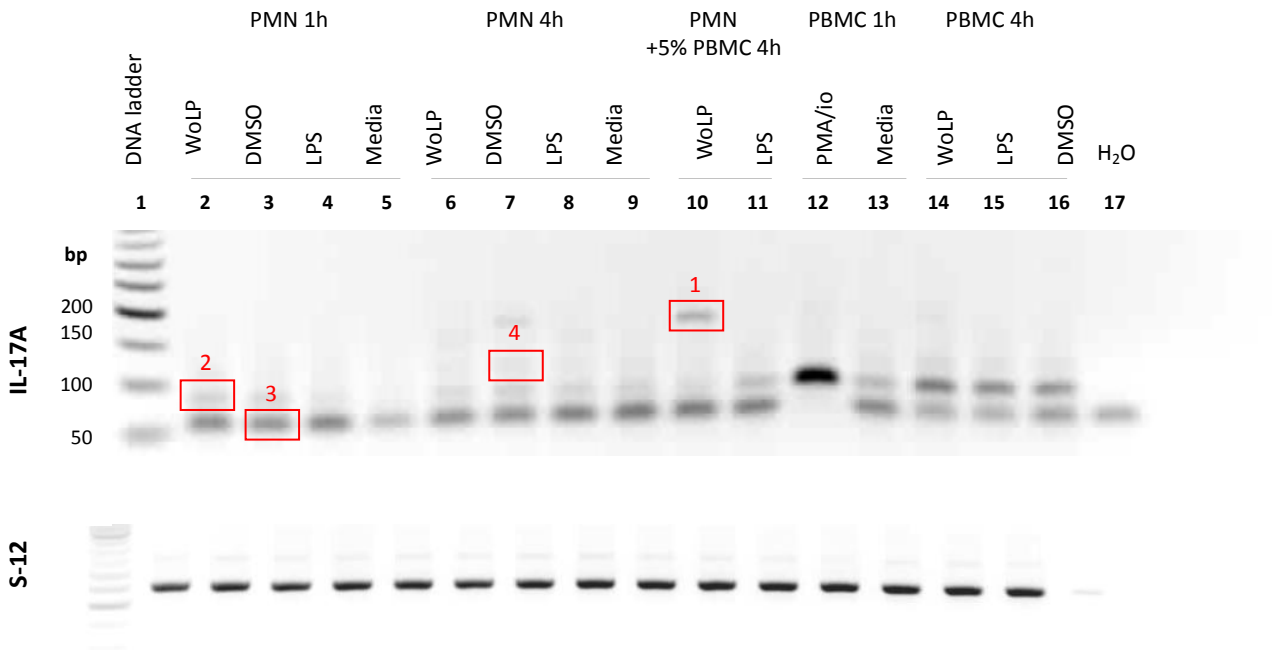

Figure 5-28. Sequenced PCR products. Red boxes indicate bands excised and analysed. For culture details refer to figure 5.26.

Band	Size	Accession	Description	Summary (RefSeq)
Band 1	~180 bp	NM_004699.2	Homo sapiens family with sequence similarity 50 (FAM50A), mRNA	Encoded protein highly conserved across species. It is a basic protein containing a nuclear localization signal and may function as DNA-binding protein or transcriptional factor
Band 2	~80 bp	NM_002190.2	Homo sapiens interleukin 17A (IL-17A), mRNA	Proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2, as well as enhance the production of nitric oxide
Band 3	~60 bp	Forward sequence alignment with IL-17A reverse primer: E=6e-4; Identities = 100%; Compatible with primer-dimer		
Band 4	~120 bp	NM_002190.2	Homo sapiens interleukin 17A (IL-17A), mRNA	Ref band 2

Table 5-9. Results of sequences of PCR products obtained from PNM cDNA amplified using probes specific for IL-17A cDNA/gDNA [489].

5.3.8 Immunoprecipitation

To assess the presence of IL-17A in human neutrophil samples and to further characterize the binding specificity of goat anti-human IL-17A IgG and mouse anti-human IL-17A IgG_1 (R&D Systems), an immunoprecipitation procedure followed by mass spectrometric analysis was performed.

Due to technical reasons and time constraint, samples from one single donor was available for the procedure. Both PMN and PBMC were from the same donor. Samples were analysed by ELISA prior to immunoprecipitation and detectable levels of IL-17A were only present in the supernatant of PBMC stimulated with PMA/ionomycin for 12 hours (2 ng/ml; not shown).
Upon analysis by low-pressure affinity chromatography, detectable levels of proteins were present only in the 5^{th} to 9^{th} 0.5 ml elution fraction of the mouse monoclonal anti-IL-17A antibody-coupled column precipitating rhIL-17A. Therefore, after immunoprecipitation, corresponding eluted fractions of each other sample were pooled together for mass spectrometric analysis. Samples were processed through the same antibody-coupled column in the following order due to contingency reasons: 1) PMN cell lysate, 2) PBMC supernatant, 3) rhIL-17A, 4) PMN supernatant. Due to concerns about the stability of bound antibodies, no control elutions with glycine pH 3.0 only were performed after immunoprecipitation of each sample. However, with the exception of PMN supernatant processed after rhIL-17A, no concern was present about contamination of samples with IL-17A bound molecules not eluted during the elution of the preceding sample. Each biological sample was divided into two aliquots to produce two technical replicates for mass spectrometric analysis. Unfortunately the first technical repeat was lost due to technical problem of the mass spectrometer, therefore data are representative of only one technical replicate.

Mass spectrometry data were submitted via Proteome Discoverer 1.1™ software to the MASCOT search engine and searched against the Uniprot database using the parameters discussed in paragraph 2.10.4. One missed cleavage and 1% false discovery rate were allowed. High confidence proteins with two or more peptides were considered identifications.

Results are shown in table 5.10 and 5.11.
Most identified proteins in all samples could be ascribed to human skin contaminantion from manipulation of labware (e.g. keratin, dermcidin, protein S100-A7 and -A8, profilin), to components of the PBMC culture media or other

circulating corpuscles present among PBMC (e.g. bovine serum albumin, bovine alpha-2-HS-glycoprotein, bovine complement factors, human thrombicidin), to the protein carrier of rhIL-17A (bovine serum albumin, bovine alpha-2-HS-glycoprotein), and trypsin residues. The possible contamination of samples during the elution step with proteins that had remained in the column from the preceding sample elution can be suggested, for example, by the presence of bovine serum albumin in the PMN supernatant samples, a protein not used in PMN cell cultures.

No IL-17A was identified in PMN cell lysate and PBMC supernatant even when all protein identification were considered, removing search filters (not shown). Both anti-human IL-17A were able to immunoprecipitate rhIL-17A. IL-17A was detected in the PMN supernatant.

Sample*	Uniprot acces-sion	Protein description	Protein accession	MASCOT score	Cov-erage %	N of pep-tides
1 PMN cell lysate	P05164-2	Myeloperoxidase Isoform H14	PERM_HUMAN	556.76	12.00	9
	A5A6M6	Keratin, type II cytoskeletal 1	K2C1_PANTR	431.18	16.64	8
	H2Q7J2	Uncharacterized protein	H2Q7J2_PANTR	402.60	16.43	7
	P35908	Keratin, type II cytoskeletal 2	K22E_HUMAN	329.34	11.11	6
	B7Z4X2	Lactoferroxin-C	B7Z4X2_HUMAN	301.00	9.01	4
	H2QCX3	Uncharacterized protein	H2QCX3_PANTR	222.35	6.41	3
	B3KSI4	Uncharacterized protein	B3KSI4_HUMAN	181.12	6.30	2
	P07737	Profilin-1	PROF1_HUMAN	171.92	28.57	3
	F8VV32	Lysozyme C	F8VV32_HUMAN	105.66	10.19	2
2 PBMC super-natant	P02769	Serum albumin	ALBU_BOVIN	9524.76	67.87	174
	P12763	Alpha-2-HS-glycoprotein	FETUA_BOVIN	1189.73	34.54	23
	D3JV41	Thrombocidin-2 antimicrobial variant	D3JV41_HUMAN	218.57	26.19	3
	P81187	Complement factor B	CFAB_BOVIN	81.38	2.89	2
3 rhIL-17A	P02769	Serum albumin	ALBU_BOVIN	3427.49	33.77	61
	P12763	Alpha-2-HS-glycoprotein	FETUA_BOVIN	798.75	30.36	11
	P35908	Keratin, type II cytoskeletal 2	K22E_HUMAN	552.45	18.15	11
	H2QCX3	Uncharacterized protein	H2QCX3_PANTR	501.38	19.58	10
	Q16552	Interleukin-17A	IL17_HUMAN	362.27	14.84	5
	P35527	Keratin, type I cytoskeletal 9	K1C9_HUMAN	314.28	11.24	7
	P00761	Trypsin	TRYP_PIG	300.58	16.45	8
	P02533	Keratin, type I cytoskeletal 14	K1C14_HUMAN	280.98	13.77	6
	E7EUE8	Keratin, type II cytoskeletal 6A	E7EUE8_HUMAN	176.41	7.50	4
	H2Q5Z8	Uncharacterized protein	H2Q5Z8_PANTR	160.00	6.79	4
	A0PJG0	THBS1 protein	A0PJG0_HUMAN	103.60	5.99	2
	P81605	Dermcidin	DCD_HUMAN	83.78	20.00	2
	P31151	Protein S100-A7	S10A7_HUMAN	79.41	22.77	2
4 PMN super-natant	P04264	Keratin, type II cytoskeletal 1	K2C1_HUMAN	1100.34	33.85	21
	P35908	Keratin, type II cytoskeletal 2	K22E_HUMAN	742.20	24.26	13
	H2QCX3	Uncharacterized protein	H2QCX3_PANTR	732.62	31.89	16
	P35527	Keratin, type I cytoskeletal 9	K1C9_HUMAN	679.67	22.47	13
	P02769	Serum albumin	ALBU_BOVIN	631.09	27.35	13
	B7Z4X2	Lactoferroxin-C	B7Z4X2_HUMAN	573.63	21.92	11
	Q16552	Interleukin-17A	IL17_HUMAN	407.50	19.35	9
	P05164-2	Myeloperoxidase Isoform H14	PERM_HUMAN	363.10	10.46	6
	P06702	Protein S100-A9	S10A9_HUMAN	357.32	37.72	6
	P00761	Trypsin	TRYP_PIG	353.38	31.60	8
	F6KPG5	Albumin	F6KPG5_HUMAN	278.70	9.06	4
	P02533	Keratin, type I cytoskeletal 14	K1C14_HUMAN	256.20	12.92	7
	P81605	Dermcidin	DCD_HUMAN	113.27	20.00	2
	P20160	Azurocidin	CAP7_HUMAN	104.25	12.35	3

Table 5-10. Identification of proteins precipitated by goat anti-human IL-17A IgG using MASCOT search engine against the Uniprot database. Proteins with high coverage and at least 2 peptides per proteins were considered identifications. * Samples were processed through the antibody-coupled column in the stated order. PMN were stimulated for 4 hours at 5 x 10^6 cells/ml with WoLP 1 µg/ml; PBMC were stimulated for 12 hours with PMA (50 ng/ml)/ionomycin (10 µg/ml) at 5 x 10^6 cells/ml and used as positive control after testing positive for IL-17A in ELISA (2 µg/ml); recombinant human IL-17A (rhIL-17A, R&D, 200 µg) was used as positive control.

Sample*	Uniprot accession	Protein description	Protein accession	MASCOT score	Cov-erage %	N of pep-tides
1 PMN cell lysate	P13645	Keratin, type I cytoskeletal 10	K1C10_HUMAN	918.76	35.95	15
	P04264	Keratin, type II cytoskeletal 1	K2C1_HUMAN	880.01	27.95	16
	P35908	Keratin, type II cytoskeletal 2	K22E_HUMAN	771.04	24.41	12
	P59666	Neutrophil defensin	DEF3_HUMAN	702.51	19.15	2
	P05164-2	Myeloperoxidase Isoform H14	PERM_HUMAN	682.52	18.46	13
	P20160	Azurocidin	CAP7_HUMAN	355.82	21.91	5
	P35527	Keratin, type I cytoskeletal 9	K1C9_HUMAN	347.65	9.47	4
	B2MUD5	Neutrophil elastase	B2MUD5_HUMAN	124.32	9.38	2
2 PBMC super-natant	P02769	Serum albumin	ALBU_BOVIN	4922.78	39.70	61
	P12763	Alpha-2-HS-glycoprotein	FETUA_BOVIN	1088.47	31.75	17
	P81187	Complement factor B	CFAB_BOVIN	125.67	3.29	3
3 rhIL-17A	P02769	Serum albumin	ALBU_BOVIN	1896.64	29.98	40
	Q16552	Interleukin-17A	IL17_HUMAN	1365.83	60.00	23
	P12763	Alpha-2-HS-glycoprotein	FETUA_BOVIN	470.13	16.71	5
	A5A6M6	Keratin, type II cytoskeletal 1	K2C1_PANTR	455.92	19.31	10
	P35527	Keratin, type I cytoskeletal 9	K1C9_HUMAN	288.92	14.77	6
4 PNM super-natant	H6VRG2	Keratin 1	H6VRG2_HUMAN	1224.46	45.34	27
	P35527	Keratin, type I cytoskeletal 9	K1C9_HUMAN	1111.79	48.96	23
	H2R1Z0	Uncharacterized protein	H2R1Z0_PANTR	1043.18	31.38	22
	P35908	Keratin, type II cytoskeletal 2	K22E_HUMAN	884.80	37.87	18
	H2QCX3	Uncharacterized protein	H2QCX3_PANTR	824.64	33.97	19
	B7Z4X2	Lactoferroxin-C	B7Z4X2_HUMAN	574.03	21.77	11
	P02769	Serum albumin	ALBU_BOVIN	470.60	18.29	9
	Q16552	Interleukin-17A	IL17_HUMAN	465.80	30.97	8
	P06702	Protein S100-A9	S10A9_HUMAN	417.47	44.74	8
	P02533	Keratin, type I cytoskeletal 14	K1C14_HUMAN	369.84	19.92	8
	E7EUE8	Keratin, type II cytoskeletal 6A	E7EUE8_HUMAN	310.57	10.19	5
	P05164-2	Myeloperoxidase Isoform H14	PERM_HUMAN	299.78	10.31	6
	P00761	Trypsin	TRYP_PIG	296.09	31.60	8
	H2Q5Z8	Uncharacterized protein	H2Q5Z8_PANTR	253.08	8.83	5
	P05109	Protein S100-A8	S10A8_HUMAN	187.32	23.66	2
	Q8IUK7	ALB protein	Q8IUK7_HUMAN	179.26	10.86	4
	P02671-2	Fibrinogen alpha chain Isoform 2	FIBA_HUMAN	169.93	4.50	2
	Q99LC4	Igh protein	Q99LC4_MOUSE	158.96	7.13	7.03
	P81605	Dermcidin	DCD_HUMAN	94.02	20.00	2
	P02675	Fibrinogen beta chain	FIBB_HUMAN	81.70	5.70	2

Table 5-11. Identification of proteins precipitated by mouse anti-human IL-17A IgG_1 using MASCOT search engine against the Uniprot database. Proteins with high coverage and at least 2 peptides per proteins were considered identifications. * Samples were processed through the antibody-coupled column in the stated order. PMN were stimulated for 4 hours at 5 x 10^6 cells/ml with WoLP 1 µg/ml; PBMC were stimulated for 12 hours with PMA (50 ng/ml)/ionomycin (10 µg/ml) at 5 x 10^6 cells/ml and used as positive control after testing positive for IL-17A in ELISA (2 µg/ml); recombinant human IL-17A (rhIL-17A, R&D, 200 µg) was used as positive control.

5.4 DISCUSSION

The *Wolbachia*-induced features of *O. volvulus* infection, such as neutrophil recruitment in the cornea and in onchocercomas, and the cytokine environment promoted by the *Wolbachia*-filarial worm system suggest the possible involvement of Type-17 immune responses in onchocerciasis. Although very limited, some evidence exists of the development of Th17 cells in lymphatic filariasis, and the possible involvement of IL-17 in lymphatic pathology in this infection, but no studies have so far addressed the specific question of the presence and induction of Type-17 immune responses in onchocerciasis.

The work presented here first approached this question by investigating IL-17A (from now on referred to as IL-17) expression in *O. volvulus* nodules by IHC. The results showed that an abundant IL-17$^+$ cell infiltrate is present in onchocercomas around and in contact with *Wolbachia*-containing worms. Its presence depends on that of *Wolbachia*, as the IL-17$^+$ infiltrate dramatically decreased in nodules from patients treated with doxycycline, containing worms depleted from the endosymbiont. Doxycycline treatment induces worm sterility and death, but the dynamic of the IL-17$^+$ cell infiltrate in onchocercomas does not appear to be related to adult viability and microfilarial production. Indeed, nodules from doxycycline treated patients contained both viable and not viable adult worms of both sexes (analysis conducted by Dr K Johnston, LSTM -data not shown), around which almost no IL-17$^+$ cells were found. Moreover, IL-17$^+$ cells were found also surrounding *Wolbachia*-containing adult males, not only fertile females. Of particular note, IL-17$^+$ cells were found around the only *Wolbachia*-positive worm in a doxycycline-treated nodule.

Investigation of the cell source(s) of IL-17 revealed that both mononucleated and polymorphonucleated cells in onchocercomas were IL-17$^+$. Th17 cells are the most studied and best known source of IL-17. In the investigated nodules, the CD4$^+$ cell infiltrate localization around adult worms was similar to that of IL-17$^+$ cells. Also, CD4$^+$ T cells were found to dramatically decrease in doxycycline-treated nodules, suggesting that the IL-17$^+$ cell infiltrate might be composed by this cell population. However, by double-staining for IL-17 and CD4 it was shown that, although present, Th17 cells did not represent the majority of the IL-17$^+$ cell infiltrate in nodules. 3.72% of all CD4$^+$ T cells in placebo-treated nodules were IL-17$^+$. This is a high percentage when compared to that found in other conditions characterized by the presence Th17 cells. For example, Th17

cells were reported to be 0.5% to 3% of total $CD4^+$ cells in rheumatoid arthritis, spondiloarthritis or psoriatic lesions [568, 570, 571, 631, 632]. Th17 cells in onchocercomas could therefore be of biological importance, even though they represented a small proportion (5%) of all IL-17^+ cells in nodules.

Approximately one third of IL-17^+ cells in nodules were mononucleated cells. Besides $CD4^+$ T cells, it is known that the mononucleated inflammatory infiltrate in onchocercomas includes $CD8^+$ T cells, Tregs, γδ T cells, macrophages, DCs and mast cells [87, 627, 633, 634]. With the exception of DCs, all these cells have been reported to be possible sources of IL-17 (Table 5.2). Macrophages are particularly abundant in onchocercomas, but these cells were excluded as the source of IL-17 in nodules by previous work carried out in our lab (G. Smith MSc thesis [628]). Unfortunately, it was not possible to carry out any further identification of IL-17^+ mononucleated cells in onchocercomas due to the scarcity of the available material.

The finding that more than 70% of IL-17^+ cells in onchocercomas were polymorphonucleated was unexpected. Of all possible polymorphonucleated cells, neutrophils were the most likely candidate cells. In the investigated samples, eosinophils were virtually absent while neutrophils were an abundant population, found around and in contact with adult worms in placebo-treated nodules. Moreover, neutrophils almost disappeared in *Wolbachia*-depleted doxycycline treated nodules. Also, IL-17^+ polymorphonucleated cells were often found in a $DAPI^+$ net-like structure, highly suggestive of the recently described neutrophil NETs (reviewed in [284]). The results of H&E plus IL-17 sequential staining confirmed that IL-17^+ polymorphonucleated cells in nodules were neutrophils. In the study presented here, it was not possible to use neutrophil or eosinophil-specific markers, and one could argue that on H&E neutrophils and degranulated eosinophils may have the same appearance. However eosinophils do not seem to be likely candidate sources of IL-17 in the investigated material because: i) no extra-cellular eosinophilic granules, identifying degranulated eosinophils, were observed in the vicinity of polymorphonucleated cells, ii) eosinophils were much less abundant than neutrophils in the investigated material and also in other investigations of onchocercomas [376], and iii) the distribution and dynamics of neutrophils and not eosinophils mirrored that of polymorphonucleated cells in the nodules investigated in this work and in other studies [44, 376].

Neutrophils have been reported to be a source of IL-17 in several studies (Table

5.2), but this finding, at least for human neutrophils, remains controversial [263, 519]. In *Wolbachia*-containing nodules, virtually all neutrophils were IL-17$^+$, as well as the very rare neutrophils in doxycycline treated nodules. However, due to the scarcity of these cells in *Wolbachia*-depleted nodules, it was not possible to investigate the correlation between presence of *Wolbachia* and IL-17 positivity of neutrophils. To do so, *in vitro* experiments with human neutrophils from at healthy volunteers were performed. It was unexpectedly found that all circulating neutrophils, in the absence of any stimulation, were IL-17$^+$ by IHC. Equal results obtained with formalin-fixed and ethanol-fixed neutrophil preparations exclude this being due to fixation artifacts. Absence of staining when secondary antibody only or isotype control primary antibodies were used also demonstrate that this was not due to non-specific binding of primary or secondary antibody, or to antibody binding to Fc receptors on neutrophils. Also, staining was not due to non-specific detection of leukocytes, as in PBMC preparations only contaminating neutrophils were IL-17$^+$. HEK cells, used as bona fide negative control cells, were IL-17$^-$. The pattern of staining appeared granular and intracellular, as confirmed by immune transmission electron microscopy. Neutrophils contain granules with molecules pre-synthesized in the bone marrow before mature cells are released in the blood stream. It was therefore possible that IL-17 was detectable in unstimulated circulating neutrophils because of its pre-synthesis in the bone marrow. To test this hypothesis, presence of IL-17 in neutrophil-precursor HL60 cells was assessed along their differentiation to neutrophil-like cells. HL60 cells are widely used as a cell culture model of human neutrophils and upon differentiation they share several functional and biochemical features of mature neutrophils such as chemotaxis in response to fMLP or IL-8 [635], apoptosis gene expression profile (e.g. caspase 1-4 and 7-10) [629], respiratory burst after priming with IL-8 and stimulation with fMLP [636, 637], and cytokine (IL-8) production upon TLR ligand stimulation [638]. No IL-17 staining was detected by IHC at any time point. Moreover, upon stimulation by WoLP and LPS (even if used in serum-free conditions), staining intensity was noticeably increased. Taken together, these finding suggested that IL-17 may be synthetized only by mature neutrophils.

To investigate this result further, a Western Blot analysis of cell lysates was performed. To test the IL-17 specificity of the antibody used in IHC (goat anti-human IL-17A from R&D, referred to as goat R&D), three other anti-human antibodies were used in parallel on the same samples. It was expected that IL-17 specific bands would be consistently detected while non-specific ones would differ between antibodies. This approach gave results difficult to interpret. The

antibody from Santa Cruz was discarded because it was difficult to optimise and gave inconsistent results. The goat R&D antibody and the antibody from Sigma gave overlapping results, with multiple bands detected in all neutrophil samples but not PBMC samples, while the mouse anti-human IL-17 monoclonal antibody from R&D consistently found one band in all samples tested, including in putatively negative control samples. No difference in band intensity was found upon neutrophil stimulation with any of the antibodies used, in contrast with what was found by IHC. The result that goat R&D antibody was able to detect an IL-17 compatible band in positive control PBMC samples (stimulated with PMA/ionomycin) only using a high sensitivity technique suggested that goat R&D polyclonal antibody contained a low concentration of anti-IL-17 antibodies specific for IL-17 in reducing conditions, and raised some concerns about the IL-17 specificity of this antibody when used on neutrophil samples.

To circumvent the possible problems constituted by the reducing conditions used, detection of IL-17 in supernatants and cell lysates in native conditions was carried out by ELISA and Luminex. None of the four assays used detected IL-17 in any sample with the exception of positive control PBMC samples. This was also the case for the ELISA kit from R&D, which uses as the capture antibody the goat anti-human IL-17 IgG used here for IHC and Western Blot. One possible explanation for the inconsistent results between Western Blot and ELISA/Luminex could be that IL-17 may be present on the cell surface of neutrophils, bound to the IL-17RA subunit of the IL-17 receptor, and therefore not detectable in the cell preparations used for ELISA/Luminex. However, this does not seem the case as: i) IHC and ITEM results showed an intracellular rather than membrane-bound IL-17 signal; ii) membrane-bound proteins are meant to be solubilised by the cell lysis reagents used, thus surface-bound IL-17 should have been detected if present; and iii) preliminary results of IL-17 detection by Flow Cytometry using PE-conjugated mouse anti-human IL-17 IgG_1 (BD Pharmigen) using a protocol that would stain both intra- and extra-cellular IL-17 did not detect any IL-17 in isolated neutrophils (data not shown). Moreover, Garley *et al* [586] reported IL-17 detection by Western Blot in native conditions only in the cytosolic fractions of neutrophil cell lysates.

Besides the possibility of IL-17 being bound to surface receptors, another possibility is that intracellular IL-17 in neutrophils derives from its uptake from the surrounding tissutal environment. This possibility was not addressed in this work for time constraint. However, whatever the origin of IL-17, endogenously synthetized or acquired exogenously, the impossibility to detect IL-17 in

neutrophil samples by techniques other than IHC raised concerns about the specificity of the goat anti-IL-17A antibody used in IHC.

The vast majority of the reports of IL-17 production by human neutrophils have relied on IHC, using the same single goat anti-human IL-17A antibody source (R&D Systems) used in the work presented here. In publications where this data is reported, IHC detected IL-17A only in a fraction of circulating neutrophils, ranging from 20% to 79% [568, 571, 585], in contrast to what was found in this work, where virtually all neutrophils were IL-17$^+$ by IHC in *Onchocerca* nodules and from healthy volunteers. A possible explanation could be that directly-conjugated secondary antibodies were used in all the three mentioned published works, while here a fluorescent amplification system was employed, which may have increased the sensitivity of the assay.
PBMC contamination of neutrophil cultures needs to be taken into account when interpreting published results and the results of this work. Among the reports that detected IL-17A production by human neutrophils by Western Blot, Flow Cytometry or in culture supernatants by ELISA-based assays, only that of Lin and colleagues [571] used highly pure neutrophil populations sorted by flow cytometry. In their work, IL-17A was also detected in unstimulated circulating neutrophils by Western Blot. So far, only one study reported IL-17 expression by human neutrophils at the mRNA level, but these data were not presented [585]. However, the purity of neutrophils in that work, as in the work presented here, was not enough to exclude that IL-17 expression by contaminating PBMC could have accounted for the result. Indeed, the results shown here of IL-17A expression by RT-PCR in neutrophils supplemented with 5% PBMC argue in favour of this possibility.

As expression of IL-17 in neutrophils both at the protein and at the mRNA level could not be convincingly confirmed, the doubt over the specificity of the antibodies used remained. One possible way of testing the antigen-specificity of an antibody is by pre-absorbing it with the antigen and then assessing the negative staining of an antigen-positive sample in IHC when the pre-absorbed antibody is used. In the case of goat anti-human IL-17A (R&D Sytems), this approach was successful in the work of Appel *et al* [570], but in the experimental work presented here it resulted in non-specific background staining in all conditions tested (data not shown). An immunoprecipitation approach followed by mass spectrometric analysis of precipitated proteins was therefore used to address this issue. Unfortunately only one sample from one donor and one technical replicate could be processed due to technical problems and time con-

straint, therefore the results presented here should only be considered as preliminary. Both anti-human IL-17A antibodies (goat and mouse raised, R&D System) used for immunoprecipitation were able to bind recombinant human IL-17A, confirming their IL-17A specificity. No IL-17 was detected in the positive control PBMC supernatant, despite the presence of an estimated 5 ng in each of the 2.5 ml sample used for immunoprecipitation based on ELISA results, and notwithstanding the extremely high sensitivity of the mass spectrometric technique used. This suggests that the IL-17 present in the sample may have been lost during processing and/or degraded in the time elapsed between processing and mass spectrometric analysis. It could, therefore not be completely excluded that IL-17 might have been present in the PMN cell lysate, but not detected. IL-17 was detected in the PMN supernatant. It must be highlighted that this sample was processed in the antibody-coupled columns after rhIL-17A. It is therefore likely that this result would come from sample contamination rather than being a true identification of IL-17A produced by PMN, in the light of the procedure used (absence of clean-up elution with glycine pH 3.0 after rhIL-17A processing) and of evidence of possible contamination of samples during the elution step with proteins that had remained in the column from the preceding sample elution.

To conclude, the work presented here confirmed the association between presence of *Wolbachia* in onchocercomas and recruitment of neutrophils. Most importantly, it described for the first time the presence of Th17 cells in *Onchocerca* nodules, in a percentage higher than what reported in other pathologic conditions, and in association with the presence of *Wolbachia*. Further studies should address the role of *Wolbachia* and in particular *Wolbachia* lipoproteins in the differentiation of Th17 cells, and the role of IL-17 in the pathologic manifestations of onchocerciasis.

The result that neutrophils were the most abundant IL-17$^+$ cells in onchocercomas was surprising. However, the work presented here could not confirm the association between IL-17 positivity of neutrophils and presence of *Wolbachia*. Further, it appears that IL-17 production by human neutrophils is still not supported by enough evidence. Almost all reports relied on one technique (IHC) and one antibody (goat anti-human IL-17A, R&D Systems). When different techniques were used, including in this work, contaminating PBMC may have been a problem. Further studies should investigate the expression of IL-17 by these cells at the mRNA level only in highly pure neutrophil populations, for example using flow cytometric cell sorting or magnetic beads-based techniques, and address the possibility that neutrophils amy acquire IL-17A from the tissu-

tal environment, for example using labelled rhIL-17A. Immunoprecipitation followed by mass spectrometry is a very highly sensitive tool to investigate antibody specificity and presence of an investigated protein in a specimen. Although the results presented here using this approach could be considered only preliminary, they could confirm that the commercially available anti-IL-17A antibody used actually binds rhIL-17A in its native form, and could validate the protocol used in terms of column antibody binding and stability of coupled antibodies. However, the amount of native IL-17 in the starting samples may be a critical issue and the evidence of contaminants carried over from one elution to the other highlights the absolute need for an elution step with glycine pH 3.0 only between each sample, together with the assessment of residual proteins in each wash elution. The results presented here could not exclude that the commercially available anti-IL-17A antibodies used might also bind molecules different from IL-17A. Both antibodies were able to consistently precipitate myeloperoxidase in the neutrophil cell lysate. Although this does not constitute evidence of myeloperoxidase specificity of these antibodies, nevertheless the fact that IL-17 staining had a granule-like pattern and was present also in non-stimulated granulocytes would be consistent, if indeed cross-reactivity exists, with that being with a granular component. Further studies should address this by testing anti-IL-17 antibodies against major granular component of neutrophils. Considering that neutrophils stained consistently with this commercial antibody, once addressed the issue of its specificity, this may be useful as a marker for human neutrophils.

Although not fully characterised in this study, the DAPI-stained net-like structures present around adult worms in onchocercomas where also neutrophils are localised is highly suggestive of the recently described Neutrophil Extracellular Traps (NETs). These arise from the release of granulocyte nuclear content extracellularily, decorated with granular and cytoplasmic protein, and act by limiting both microbial spread and possibly collateral damage from granular enzymes [284], but the molecular regulation of their formation and thir biological functions have not been fully elucidated yet. NETs are known to play a role in killing bacteria and fungi such as *Staphylococcus aureus, Streptococcus pyogenes, Mycobacterium tuberculosis* and *Candida albicans*, and have been described in protozoan infections such as leishmaniasis, *Plasmodium falciparum* malaria and toxoplasmosis [284, 639-642]. However, no work so far reported the presence of NETs in helminth infections. The filarial and/or *Wolbachia*-derived molecules stimulating NETosis deserve further investigation. In particular, it would be intriguing to assess the relative contribution of filarial- and *Wolbachia*-derived stimuli in neutrophil recruitment and NETosis. Indeed,

WoLP was shown here to induce neutrophil recruitment, but it has been reported that NET formation in response to *Toxoplasma gondii* tachizoites was independent from MyD88 pathway activation, as assessd using neutrophils from MyD88$^{-/-}$ mice [639]. Thus, it would be intriguing to speculate that NET formation, by trapping neutrophil enzymes, would protect worms from the potential harm caused by neutrophil activation.

Chapter 6. SUMMARY AND CONCLUSIONS

The discovery of *Wolbachia* bacterial endosymbiont in filarial nematodes of medical importance, including the causative agent of River Blindness, and of its crucial role in worm biology and disease pathogenesis has opened the opportunity for a safe and effective control of onchocerciasis. However, the employment of anti-wolbachial treatment as a public health tool requires further study and optimisation on pharmacology, field implementation strategies and impact on onchocerciasis control. Moreover, this has also raised important questions about the nature of *Wolbachia*-nematode interaction and the possible effects of anti-filarial therapy targeting *Wolbachia* in the context of the host immune responses. The work presented here aimed to explore several of these aspects.

Although extensive research is ongoing to optimise regimes of existing anti-*Wolbachia* drugs and to identify alternative drugs and combinations more suitable for Mass Drug Administration (MDA), it has recently been shown in a pilot implementation trial in Cameroon that the delivery of the "gold-standard" macrofilaricidal 6 week course of doxycycline therapy as a MDA is feasible, safe and well accepted by communities when delivered with a community-directed strategy. The work presented here in chapter 2 evaluated the long-term effectiveness of this intervention followed by standard ivermectin (IVM) MDA treatment by assessing prevalence and burden of infection 4 years after implementation and with ongoing transmission. The results showing a significant reduction in microfilaridermia prevalence and load in people that completed the 6 week course of doxycycline followed by IVM compared to those who received only IVM MDA demonstrated the long term effectiveness of doxycycline MDA and validated the very high compliance rate reported in the implementation trial. These results encourage the introduction of anti-wolbachial therapy in onchocerciasis control programmes where needed and provide important data to address the practical aspects of the implementation of macrofilaricidal drugs.

The host inflammatory response to *Onchocerca volvulus* microfilariae (mf) and their endosymbiont *Wolbachia* is at the basis of onchocercal pathology. Through TLR2/6 activation by *Wolbachia* peptidoglycan-associated lipoprotein, *Wolbachia* interacts with the host's immune cells inducing the production of pro-inflammatory cytokines. This may create, in concert with the anti-inflammatory cytokine environment promoted by the filarial worm, a suitable milieu for the development of a Type-17 immune response. This is characterized by the production of the signature pro-inflammatory cytokine IL-

17 and the recruitment of neutrophils into tissues, this latter being a feature of onchocerciasis strictly dependent on the presence of *Wolbachia*. Although some data exists on the development of a Type-17 immune response in lymphatic filariasis and its possible role in the pathogenesis of lymphatic disease, so far no investigation was carried out in onchocerciasis or on the role of *Wolbachia* in this context. Moreover, no human study so far investigated the relative role of *Wolbachia* and the filarial nematode in the induction of pro- and anti-inflammatory cytokines in onchocerciasis infected humans *ex vivo*. In the work presented here in chapter 3, peripheral blood mononuclear cells (PBMC) from patients with onchocerciasis and control subjects from Ghana were stimulated with filarial extracts and the cytokine response was analysed in the light of presence or absence of *Wolbachia* in the filarial extracts and patients' microfilaridermia loads. The results showed a predominant Th2 response in patients compared to controls, while Th1 and Th17 responses were minimal in both groups. Thus, the filarial-specific Type-17 immune response appears to be regulated in the same way of Th1 and Th2 responses, which are heightened in pathology but down-regulated by active infection.
Interestingly, parasite loads showed a different influence on Th2 compared to pro- and anti-inflammatory responses with patients with higher mf loads showing depressed Th2 responses but heightened spontaneous production of both pro- and anti-inflammatory cytokines. These results suggest that filarial parasites are able to promote both pro- and anti-inflammatory responses at the same time. This preliminary study did not show any difference in the ability of *Wolbachia*-containing and *Wolbachia*-depleted filarial extracts to stimulate PBMC. However, practical and time constraints prevented a rigorous optimization and control of reagents and culture conditions, which should be carried out in further studies of this type. These should take advantage of the availability of adulticidal therapy with doxycycline to investigate the relative role of *Wolbachia* and of different parasite life stages in shaping the immune profile in onchocercal infection and disease using a rigorous classification of patients' infection and disease status.

The presence of IL-17 was also investigated *ex vivo* in onchocercomas from patients treated with doxycycline or placebo, as presented in chapter 5. Using immunohistochemistry (IHC), it was found an abundant IL-17^{+} cell infiltrate around and in contact with *Wolbachia*-containing but not *Wolbachia*-depleted *O. volvulus* adults. Interestingly, Th17 cells were present in this infiltrate in a higher percentage compared to other inflammatory diseases characterised by the production of a Type-17 immune response, such as psoriasis and rheuma-

toid arthritis, suggesting that these cells may have a role in the localised immune response elicited by the worm through its endosymbiont in the nodule. However, the vast majority of the IL-17$^+$ cells in onchocercomas were neutrophils, a surprising finding that was investigated in more depth using human neutrophils from healthy donors *in vitro*. Using IHC, unstimulated neutrophils but not neutrophil precursors resulted IL-17$^+$, with an intracellular signal that increased in intensity upon stimulation with LPS or synthetic *Wolbachia* peptydoglycan-associated lipoprotein (WoLP). However, when confirmation of these results was pursued at the protein and the gene transcription level, results were inconclusive. In particular, IL-17 was detected in human neutrophils only by Western Blot, while IL-17 was undetected using ELISA-based assays and different anti-IL-17 antibody sources. Moreover, the small PBMC contamination of purified human neutrophil preparations prevented a definitive conclusion about IL-17 expression by human neutrophils at the mRNA level using RT-PCR. A preliminary experiment using immunoprecipitation followed by mass spectrometric analysis of neutrophil samples failed to detect IL-17 in cell lysates, but this result can not be considered conclusive in the light of the negative result obtained also in one of the positive-control samples, thus requiring further optimization. Although inconclusive on IL-17 expression by human neutrophils and on the role of *Wolbachia* in this response, these results highlight the need for a careful interpretation of published data reporting IL-17 expression by these cells, the vast majority of that being based only on the same IHC technique used in this work. Moreover, they also point out the need for a more stringent neutrophil purification technique in further studies assessing the protein expression by neutrophils using RT-PCR. Finally, IL-17 in neutrophil samples should be found, the source of IL-17 in neutrophils, whether endogenous or exogenous, should be also investigated. However, the characterization of binding specificity of the polyclonal anti-human IL-17A antibody used here deserves further attention in the view of its possible optimization for use as a neutrophil-specific marker.

A typical feature of onchocercomas harbouring *Wolbachia*-containing but not *Wolbachi*a-devoid *Onchocerca* nematodes is the presence of a rich neutrophil infiltrate. Neutrophils are also involved in *Wolbachia*-mediated pathogenesis of onchocercal disease manifestations. However, neutrophils do not appear to be detrimental to living worms, and their role in the filarial nematode parasitism is still not known. The study presented in chapter 4 investigated the interaction between human neutrophils and *Wolbachia in vitro*. The results demonstrate that WoLP activates human neutrophils, as shown by modulation of the surface

expression of adhesion molecules involved in rolling and adhesion and of Fcγ Receptors, chemotaxis, production of IL-8, induction and priming for the production of reactive oxygen species, and enhanced neutrophil survival by delay in cell apoptosis. These results support previous studies demonstrating the link between *Wolbachia* and neutrophils in the host inflammatory responses to *O. volvulus* infection, and also identify *Wolbachia* peptidoglycan-associated lipoprotein as a key molecule driving human neutrophil recruitment and activation. The diverse role of the *Wolbachia*-induced neutrophilia in the contexts of pathology and parasitism without pathology, if ever a difference exists, deserves further attention. When neutrophils were stimulated with *Wolbachia*-containing filarial extract, their phenotype did not match that induced by WoLP. Moreover, it did not differ from that found upon stimulation with the *Wolbachia*-depleted extract. These results are in contrast with what showed with murine DCs and human macrophage cell lines and the reported human neutrophil chemotaxis by *Wolbachia*-containing but not *Wolbachia*-depleted *O. volvulus* soluble extracts. Further study is needed to optimise the conditions for the stimulation of different immune cells by *Wolbachia*-derived molecules and *Wolbachia* contained in filarial extracts.

Finally, the finding in onchocercomas of DAPI-positive structures highly suggestive of Neutrophil Extracellular Traps (NETs), never reported so far in helminth infections, deserves further study. In particular, it would be intriguing to assess the relative contribution of filarial- and *Wolbachia*-derived stimuli in neutrophil recruitment and NETosis to test the hypothesis whether NET formation, by trapping neutrophil enzymes, would protect worms from the potential harm caused by neutrophil activation.

In summary, this study added new insights into the relation *Wolbachia*-filaria-human host and immunopathology mechanisms by describing the development of a high percentage of Th17 cells in *Wolbachia*-containing onchocercomas, while this pro-inflammatory response appears to be down-modulated in active infection. Furthermore, this study showed that *Wolbachia* lipoprotein activates human neutrophils *in vitro*, supporting previous studies demonstrating the link between *Wolbachia* and neutrophils in the host inflammatory responses to *O. volvulus* infection, and identified *Wolbachia* lipoprotein as a key molecule driving human neutrophil recruitment and activation. Of note, neutrophils in onchocercomas were observed within a NET-like structure, never reported previously in a helminth infection, opening new questions on the role of neutrophils in infection control and/or immune evasion. Finally, field work in Cameroon demonstrated the long-term effectiveness of doxycycline MDA

supporting its implementation in existing control strategies and providing important data to inform practical aspects and modelling of the implementation of macrofilaricides.

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PUBLICATIONS AND PRESENTATIONS

Publications

Tamarozzi F, Halliday A, Gentil K, Hoerauf A, Pearlman E, Taylor M J: **Onchocerciasis: the role of Wolbachia bacterial endosymbionts in parasite biology, disease pathogenesis and treatment.** Clinical Microbiology Reviews 2011 24 (3) 459-468

Tamarozzi F, Tendongfor N, Enyong PA, Esum M, Faragher B, Wanji S, Taylor MJ: **Long term impact of large scale community-directed delivery of doxycycline for the treatment of onchocerciasis.** Parasites and Vectors 2012 5(1) 53.

Presentations

Tamarozzi F, Johnston K, Smith G, Taylor MJ: **Wolbachia drives Type 17 immunity in Onchocerca volvulus infection (River blindness).** Molecular Helminthology Conference, 5-10 Sept 2010, Hydra, Greece – Poster; and 6th International Wolbachia Conference, 9-14 June 2010, Asilomar, CA, USA – Poster

Tamarozzi F, Tendongfor N, Enyong PA, Esum M, Faragher B, Wanji S, Taylor MJ. **Long term impact of large scale community-directed delivery of doxycycline for the treatment of onchocerciasis.** British Society for Parasitology, Spring Meeting 2012, 2nd-5th April, Glasgow, UK – Oral presentation

Tamarozzi F, Edwards SW, Taylor MJ: **Wolbachia-neutrophil interaction in onchocerciasis: a key role for Wolbachia peptidoglycan-associated lipoprotein.** British Society for Parasitology, Spring Meeting 2012, 2nd-5th April, Glasgow, UK – Poster

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APPENDIX – MATERIALS

Chemicals, biochemicals and media

Product	Supplier
Lymphoprep	Axis-Shield
Polymorphprep	
DPX Mountant (Low Viscosity)	Bios Europe
Bio-Plex Cell Lysis Kit	Bio-Rad
Annexin-V-FITC	Biosource
Dako Antibody Diluent with Background Reducing Components	Dako
Protogel (30% solution 37.5:1 Acrylamide : Bis-acrylamide)	Geneflow
Hank's Balanced Salt Solution (HBSS)	Gibco
Pen Strep	
RPMI 1640 + 25 mM HEPES + 2 mM L-glutamine	
Trypsin-EDTA 25%	
Rapid Romanowsky staining kit	HD Supplies
ProLong Gold Antifade Reagent	Invitrogen
TRIzol Reagent	
Gentamicin	PAA Labs
L-glutamine	
Penicillin/Streptomycin	
RPMI 1640 + 25 mM HEPES	
Tyramide Signal Amplification (TSA) Plus Cyanine-3 System	Perkin Elmer
Tyramide Signal Amplification (TSA) Plus FITC System	
TNB blocking buffer (reagent FP1020)	
Eosin 1% aqueous	Raymond A Lamb
Harris Haematoxylin	
Ammonium Persulphate (APS)	Sigma Aldrich
Bovine Serum Albumin (BSA)	
Diethylpyrocarbonate (DEPC)	
Dimethil Sulphoxyde (DMSO)	
Eagles Minimum Essential Medium	
Endotoxin-free water	
Ethidium Bromide 10 mg/ml	
Foetal Bovine Serum (FCS)	
Human AB serum	
Hydrogen Peroxide 30%	
Luminol	
N,N,N',N'-Tetramethylethylenediamine (TEMED)	
Nigrosin	
Poly 2-hydroxyethyl methacrylate (Poly-Hema)	
Propidium Iodide (PI)	
Sodium Dodecyl Sulphate (SDS)	
Sodium Pyruvate	
Trypan Blue 0.4%	
Trypsin	
Tris-Borate-EDTA 5x concentrate powder	
Halt Proteases and Phosphatases Inhibitor Cocktail	Thermo Scientific
Pierce IP Lysis Buffer	
Ultra-Vision ONE Detection System AP Polymer & Fast Red Chromogen	
Vectashield Mounting Medium with DAPI	Vector Labs

Other chemicals mentioned in the text but not included in the table were from Sigma Aldrich or BDH Laboratory Supplies or Fluka or Fisher Scientific.

Antibodies

Antibody	Product/Clone	Label*	Supplier
Primary antibodies			
Mouse anti-human β-Actin IgG_1	Clone ab8226	-	Abcam
Mouse anti-human CD16 IgG_1	Clone 3G8	FITC	BD Pharmigen
Mouse anti-human CD32 IgG_{2b}	Clone FLI8.26	FITC	
Mouse anti-human CD68 IgG_1	Clone KP1	-	Dako
Mouse IgG_1 Isotype control	Clone P3.6.2.1	-	eBioscience
Rat anti-human/mouse CD11b IgG_{2b}	Clone M1/70.15.11.5	FITC	Miltenyi Biotec
Mouse anti-human CD4 IgG_1	Clone 1F6	-	Novocastra
Goat anti-human IL-17A IgG	AF-317-NA	-	R&D Systems
Mouse anti-human IL-17A IgG_1	Clone 41802	-	
Normal goat IgG control	AB-108-C	-	
Mouse anti-human CD18 IgG_1	Clone 212701	FITC	
Mouse anti-human L-Selectin IgG_1	Clone 4G8	FITC	
Mouse IgG_1 Isotype control	Clone sc-2855	FITC	Santa Cruz
Goat anti-human IL-17A IgG	Sc-6077	-	Biotechnology
Rabbit anti-human IL-17A IgG	PRS4877	-	Sigma Aldrich
Secondary antibodies			
Donkey anti-goat IgG	OBT1500P	HRP	AbD Serotec
Goat anti-mouse IgG/IgM	M30907	HRP	Caltag
Goat anti-mouse IgG	NEF822	HRP	Perkin Elmer
Goat anti-rabbit IgG	NEF812	HRP	
Donkey anti-goat IgG	Sc-2020	HRP	Santa Cruz
Donkey anti-goat IgG	Sc-2024	FITC	Biotechnology
Sheep anti-mouse IgG	A6782	HRP	Sigma Aldrich
Mouse anti-goat IgG	31400	HRP	Thermo Scientific

***** FITC = Fluoresceine; HRP = Horse Radish Peroxidase

Stimuli for cell cultures

Filarial soluble extracts

Adult *B. malayi* female worms from Mongolian jirds treated or not with tetracycline in drinking water as described in [47] were used to obtain filarial soluble extracts as described in Turner *et al,* 2006 [152]. Worms were finely chopped with a scalpel and sonicated on ice for 5 cycles of 15 seconds each, in 3 ml sterile endotoxin-free water using a Vibra Cell sonicator (Sonics & Materials Inc.). One minute incubations on ice were included between each sonication pulse to avoid excessive heating of the material. After extraction of soluble components overnight at 4°C in rotation, samples were centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatants (*B. malayi* female extract - BmFE-; extract from tetracycline treated *B. malayi* female worms - BmFEtet-) were filtered-sterilized and tested for protein concentration for their ability to induce TNFα production by mouse macrophages via TLR2/6 stimulation, to assess *Wolbachia* presence and absence in the relevant extracts.

Protein concentration of *B. malayi* soluble extracts was assessed by BCA

Protein Assay Kit (Thermo Scientific) following manufacturer instructions. BSA in a concentration range between 2000 µg/ml and 125 µg/ml was used as the reference protein standard. Samples were diluted 1:8 in supplied Working Reagent in a final volume of 225 µl/well in a 96-well plate. Absorbance was read at 562 nm with a FLUOstar Omega plate reader supporting MARS data analysis software 1.20 (BMG Labtech), and the best-fit curve method was used to calculate the total protein content. Extracts were stored at -20°C until used and at 4°C after being thawed.

O. volvulus adult worm soluble extract (Ov) at 1 mg/ml was kindly provided by Dr Sabine Specht (Institute for Microbiology, Immunology and Parasitology, University Clinics Bonn, Germany).

Synthetic peptides

Synthetic peptides were synthetisized by EMC Microcollections. These were:

- synthetic 20-mers of the N-terminal region of wBmPAL (CSKRGVNAINKMNFVVKQMK), Di-palmitoylated at the N-terminal cysteine residue [47] (Diacyl WoLP);
- synthetic triacylated lipoprotein (Pam_3Cys-SK_4)
- synthetic diacylated lipoprotein (Pam_2Cys-SK_4)

Other stimuli for cell culture

Stimulus	Acronim	Supplier
CpG oligonucleotide (ODN 1826)	CpG	Pfizer (Coley)
Recombinant human Tumor Necrosis Factor α	TNFα	Calbiochem
Mouse anti-human CD3 IgG_{2a}, clone OKT3	αCD3	eBioscience
Mouse anti-human CD28 IgG_{2a}, clone CD28.6	αCD28	
Recombinant human Granulocyte macrophage Colony-Stimulating Factor	GM-CSF	Roche
Ionomycin		Sigma Aldrich
N-formyl-methionine-leucine-phenylalanine	fMLP	
Phorbol 12-myristate 13-acetate	PMA	
Ultra-pure lipopolysaccharide	LPS	

Commercial kits for cytokine assessment

Assay commercial name	Analyte	Format	Supplier
DuoSet ELISA Development Reagent Kit	Human IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, GM-CSF, IFNγ, TNFα	Sandwich ELISA	R&D Systems
Ready-SET-Go! ELISA Kit	Human IL-17A		eBiosciences
Mouse ELISA Kit	Mouse TNFα		Invitrogen
Bio-Plex Pro Reagent Kit	Human IL-17A	Luminex	Bio-Rad
Milliplex MAG Immunoassay Kit	Human IL-17A		Millipore

Materials for RT-PCR

Product	Supplier
Customised oligonucleotides	IDT Integrated DNA Technologies
SuperScript III First-Strand Synthesis System	Invitrogen
Taq 2X Master Mix	New England Biolabs
QuantiTect SYBR Green PCR Kit	Qiagen
QIAEX II Gel Extraction Kit	
RNase-free DNase Set	
RNeasy Mini Kit	

Consumables

Product	Supplier
75 cm² (T-75) tissue culture flasks	Cornig
24-wells tissue culture plates	Costar
96-wells tissue culture plates, flat bottomed	
96-well microplates, white, low adhesion	
Screw-top polypropylene tubes, 1.5 ml	Eppendorf
Cellstar®, 96-well tissue culture plates, U-shape	Greiner bio-one
Leucosep®, Ficoll-Paque containing tubes	
75 cm² (T-75) tissue culture flasks	IWAKI
25 cm² (T-25) tissue culture plates	
Amicon Ultra-4 3K Centrifugal Filter Devices	Millipore
Immobilon-P® 0.45 μm Polyvinylidene Difluoride (PDVF) membrane	
Millicell® 24-wells Cell Culture Hanging Inserts, PET, 3.0 μm	
96-wells tissue culture plates, flat bottomed	Nunc
96-wells tissue culture plates, U-shape	
S-Monovette®, EDTA containing 10 ml tubes	SARSTEDT
Slide-A-Lyzer® Dialysis Cassette, 2000 MWCO	Thermo Scientific

Buffers

Buffer	Components	Storage
Acid Alcool	1% v/v HCl + 70% v/v ethanol	RT*
Ammonium Chloride Lysis Buffer	13.4 mM $KHCO_3$ + 155 mM NH_4Cl + 96.7 µM EDTA	RT
DEPC-treatd water	0.1% w/v (overnight at 37°C then autoclaved)	RT
Enhanced Chemiluminescence Substrate (ECL). Mix the two solutions just before adding to the membrane.	Solution 1 = 2.5 mM Luminol in DMSO + 4.4% v/v para-coumaric acid + 100 mM Tris base pH 8.5 Solution 2 = 0.064% v/v H_2O_2 (30% stock) + 100 mM Tris base pH 8.5	4°C in the dark (max 1 month)
Laemmli buffer	10% v/v Glycerol + 3% w/v SDS + 0.001% w/v Bromophenol Blue + 12.5% v/v 1M Tris pH 6.8 + 10% v/v 1M DTT (to be added before cell lysis)	RT -20°C
Phosphate Buffered Saline (PBS) pH 7.4	137 mM NaCl + 10 mM Na_2HPO_4 + 2.7 mM KCl + 1.8 mM KH_2PO_4	RT
Ponceau S	5% v/v Acetic Acid + 0.1% w/v Ponceau S stain	RT
Scott's Tap Water	238 mM $NaHCO_3$ + 29 mM $MgSO_4$	RT
SDS-PAGE Running Buffer	25 mM Tris base + 192 mM Glycine + 0.1% w/v SDS	RT
SDS-PAGE Transfer Buffer	20% v/v methanol + 95 mM Glycine + 12.5 mM Tris base	4°C
SDS-polyacrylamide Resolving gel (n%)	N% v/v bis-acrylamide + 370 mM Tris base pH 8.8 + 1% w/v SDS + 0.1% w/v APS + 0.1% v/v TEMED (both to be added just before casting)	-
SDS-polyacrylamide Stacking gel (4.5%)	4.5% v/v bis-acrylamide + 122 mM Tris base pH 6.8 + 1% w/v SDS + 0.1% w/v APS + 0.1% v/v TEMED (both to be added just before casting)	-
Tris Buffered Saline (TBS) pH 8.0	1 mM Tris base + 15 mM NaCl	RT

*RT=room temperature

Cell lines

Human Embryonic Kidney 293 (HEK) cell culture

HEK cells were purchased from Invivogen and maintained in culture at 37°C with 5% CO_2 in Eagles Minimum Essential Medium supplemented with 2mM L-glutamine, 10% heat inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (Pen Strep, Gibco), and 1mM Sodium Pyruvate.

Cells were seeded at a density of 3000-5000 cells/cm^2 in 25 cm^2 (T-25) or 75 cm^2 culture flasks (IWAKI) and cultured until confluent (on average 7 days). The culture media was changed when needed, according to its change in color (pH).

For passaging, cells were rinsed with sterile PBS and incubated for ≤ 5 minutes with 0.25% (w/v) trypsin-EDTA. Trypsin activity was then inhibited by the

addition of culture media containing FCS. Cells were detached mechanically, centrifuged at 400 g for 10 minutes at RT, resuspended in culture media, and counted by 0.2% trypan blue using a haemocytometer.
For storage, cells were cryopreserved at -80°C at $3x10^6$ cells/ml in culture media containing 10% DMSO.

Human Caucasian Promyelocytic Leukaemia HL60 cell culture

HL60 cells were purchased from the European Collection of Cell Cultures and maintained in culture at 37°C with 5% CO_2 in RPMI 1640 (Gibco) supplemented with 10% heat inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Pen Strep, Gibco).
For cell culture maintainance, cells were seeded at 1 x 10^5 cells/ml in 75 cm^2 culture flasks (Cornig). Cell count by 0.2% trypan blue, change of culture media and passaging were performed every other day.
For storage, cells were cryopreserved at -80°C at 1.5 x 10^6 cells/ml in culture media containing 10% DMSO.

Mouse peritoneal macrophage cell culture

Peritoneal inflammatory cells from Wild Type (WT) C57BL/6, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ mice, all on a C57BL/6 background. Mice were maintained in the Biomedical Services Unit, University of Liverpool, but originally obtained from Prof S. Akira, Osaka University, Japan. Genotyping of animals by PCR is routinely carried out by Miss A. Halliday (Molecular and Biochemical Parasitology, LSTM) on genomic DNA extracted from tail skin biopsies following the protocol recommended by Prof Akira. This is available at http://hostdefense.ifrec.osaka-u.ac.jp/ja/other/index.html) and summarized below. For the detection of the mutated allele, primers B) and C) are used; for the detection of the wild-type allele, primers B) and A) are used. For all reactions,cycling conditions are 35 cycles of 94ºC for 30 sec, 30 sec to 67ºC, 67ºC for 1 min, 74ºC for 1 min, then 74ºC for 10 min.

A) Primer specific for the targeted gene	B) Primer specific for the gene downstream of the targeted construct	C) Primer specific for the neo resistance gene	Products size (bp)
TLR2$^{-/-}$			
5'-GTT TAG TGC CTG TAT CCA GTC AGT GCG-3'	5'-TTG GAT AAG TCT GAT AGC CTT GCC TCC-3'	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'	900
TLR4$^{-/-}$			
5'-CGT GTA AAC CAG CCA GGT TTT GAA GGC-3'	5'-TGT TGC CCT TCA GTC ACA GAG ACT CTG-3'	5'-TGT TGG GTC GTT TGT TCG GAT CCG TCG-3'	1200
TLR6$^{-/-}$			
5'-GAA ATG TAA ATG AGC TTG GGG ATG GCG-3'	5'-TTA TCA GAA CTC ACC AGA GGT CCA ACC-3'	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'	300

To obtain peritoneal inflammatory cells, mice were intraperitoneally injected with thioglycollate by Dr JD Turner (Molecular and Biochemical Parasitology, LSTM). After 4 days, mice were sacrified and cells harvested by washing the peritoneal cavity with sterile saline.

Cells were incubated overnight at 37°C with 5% CO_2 in 15 ml RPMI 1640 (Gibco) supplemented with 5% heat inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Pen Strep, Gibco) in 75 cm^2 tissue culture flasks (IWAKI). The supernatant containing non-adherent cells was discarded and the macrophages (adherent cells) washed with culture media and collected mechanically from the flask surface.