HUMAN IGE ANTIBODY RESPONSE TO FILARIAL WORM INFECTION

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DEDICATION

To my husband who put his dreams behind so I could pursue mine

To my parents for their unconditional love and infinite support

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AAMF	Alternatively activated macrophages
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cells
BCR	B cell receptor
Bm	*Brugia malayi*
CAMF	Classically activated macrophages
CDC	U.S. centers for disease control
CDR	Complementarity-determining regions
CSR	Class switch recombination
Di	*Dirofilaria immitis*
EC_{50}	Half-maximal effective concentration
ELISA	Enzyme linked immunosorbent assay
Fab	Fragment-antigen binding
Fc	Fragment crystallizable

FcεR	Fcε receptors
FDA	U.S. food and drug administration
FDC	Follicular dendritic cells
Fv	Fragment variable
GST	Glutathione S-transferase
HC	Heavy chain
HMM	Hidden markov model
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Immunoprecipitation
kDa	Kilodalton
kUA	kilounits of antibody
LC	Light chain
LF	Lymphatic filariasis

mAb	Monoclonal antibody
MCP-1	Macrophage chemoattractant protein-1
MDA	Mass drug administration
MIF	Macrophage migration inhibitory factor
MSA	Multiple sequence alignment
mRNA	Messenger RNA
NCBI	National center for biotechnology information
NK	Natural killer cells
O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PSM	Peptide spectrum matches
RIPA	Radioimmunoprecipitation assay buffer
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutation

TdT	Terminal deoxynucleotidyl transferase
TFH cells	T follicular helper cells
TNF	Tumour necrosis factor
TPE	Tropical pulmonary eosinophilia
TTR	Transthyretin related protein
UCA	Unmutated common ancestor
Wb	*Wuchereria bancrofti*
WHO	World health organization

CHAPTER I
INTRODUCTION

Thesis Overview

This document contains the results and conclusions of my work investigating the human antibody response to filarial worms. It is divided into six chapters; chapter I contains the required background information on human antibodies with special focus on IgE antibody isotype, IgE antibody mechanism of action and its two receptors, Lymphatic Filariasis (LF), helminth-specific immune response, worm evading /regulatory mechanisms, current LF management and treatment strategies, LF vaccine development efforts and potential link between helminth infection and allergy.

My thesis work begins in chapter II, which focuses on the human antibody response to filarial worms. In this chapter, I describe the development of an optimized human hybridoma method to generate human IgE monoclonal antibodies (mAb). I was able to isolate 56 human IgE mAbs from patients with prior history of laboratory-confirmed filarial infection. Frequency of IgE producing B cells was calculated. These IgE mAbs were tested against filarial antigens using three different assays. In addition, the sequence analysis of filarial-specific IgE B cell clonotypes provided valuable information regarding antibody variable region (VH and VL) gene usage, the length distribution of complementarity-determining region 3 (CDR3) and the degree of somatic hypermutation (SHM) in filarial-reactive IgE antibodies.

Chapter III focuses largely on the characterization of filarial-reactive IgE mAbs that exhibited potent reactivity against somatic extracts from *B. malayi* and *D. immitis* worms. Using these IgE mAbs, I was able to discover the important filarial antigens responsible for eliciting human IgE response against filarial infection. While most helminth antibody research has been focused on

allergen like molecules, my studies have shown that IgE antibodies specifically target nematode specific excreted/secretory (E/S) proteins and do not show cross-reactivity with commonly known allergens.

Chapter IV focuses largely on the characterization of transthyretin-related (TTR) family of proteins, a prominent filarial antigen targeted by IgE antibodies. TTR-specific antibodies exhibited a broad TTR response with varying degrees of cross-reactivity across different TTR protein family members. I provide detailed molecular evidence to support that the majority of filarial specific IgE antibodies bind to TTR family of proteins and function in a unique manner by forming dimers and crosslinking IgE mAbs bound to the FcεRIα, a mechanism similar to what is happening during an allergic reaction.

In chapter V, I propose development of a diagnostic test for screening individuals for filariasis using filarial antigens discovered here. I compared the serum antibody responses in patients with filaraisis against different filarial antigens. Sera from patients recognize different combination of filarial antigens, suggesting that antigens identified here are immunodominant filarial antigens and it further confirms the unbiasedness of our study. These filarial antigens can be further pursued to develop novel more sensitive serodiagnostic assays for filariasis.

In chapter VI, I summarize all my findings on antibody-mediated immunity to filarial worms and propose future direction for this work. Majority of my thesis work has been focused on identifying and characterizing the filarial antigens targeted by human IgE antibody response and understanding the molecular mechanisms by which filaria-reactive antibodies can provide protective immunity.

Introduction to Antibodies

Human immune system is divided into two categories -innate and adaptive- with some cells acting as connector in the network. Innate immune system (also known as natural or native immunity) is the first line of defense against invading organisms and pathogens, providing a fast and non-specific response. The principal components of innate immunity are 1) physical and chemical barriers including epithelial cells and their antimicrobial chemical products, 2) innate cells including phagocytic cells (neutrophils, macrophages), dendritic cells, natural killer (NK) cells and innate lymphoid cells and 3) blood proteins such as complement system and other inflammatory mediators [1]

Adaptive immune system (also called specific or acquired immunity) provides the more specialized response to pathogens and its main components are B cells and T cells. The adaptive immune system is unique in the sense that it can provide highly-specific response to a wide range of pathogens, and it builds up a memory to provide a faster and more efficient response to the pathogens seen once in the next exposures [2].

In this thesis, I will primarily focus on the humoral immunity, B cells and the antigen-recognition molecules they produce, known as immunoglobulin (IG) [3]. Two forms of Immunoglobulins exist; membrane bound B cell receptors (BCR) that are responsible for antigen recognition, and the secreted effector molecules known as antibodies [4].Antibodies are extraordinary protein molecules responsible for recognizing their targets with extreme precision (i.e., antigens) and mediating effective neutralization/elimination of the pathogen [1]. In this section, I will briefly review the antibody structure, as well as mechanisms of antibody diversity and antibody function.

Antibody Structure

Antibodies are homodimers of heterodimers, where each heterodimer is made of one heavy and one light chain (Figure 1-1) [5]. An antibody molecule can be divided into two segments; 1) the variable domain (Fv or Fab) that is different from one antibody to another and is responsible for antigen recognition, 2) the constant domain (Fc) that is identical between antibodies of the same isotype and has the same effector function. The mammalian antibody heavy chain consists of either four or five IG domains determined by the antibody isotype, the first two domains make Fab portion and the remaining two or three domains make the Fc part of antibody. The mammalian light chain consists of two IG domains and is a part of antibody's Fab. The light chain and heavy chain interact via non-covalent interactions and one disulfide bond and the heterodimers homo-dimerize via forming one or two disulfide bonds. The IG domains are spatially arranged in a way to bring heavy and light chain close and form the paratope. The very first IG domain on the N-terminal of each chain makes the variable domain (Fv). Variable domains contain three complementarity-determining regions (CDRs), called CDRH1-3 on the heavy chain, and CDRL1-3 on the light chain. The six CDRs on each arm of the Y shaped antibody is responsible for antigen recognition [6].

Human Antibody Isotypes

Human antibodies are categorized into five isotypes (IgM, IgD, IgG, IgA, and IgE) according to their Fc regions. Each antibody isotype has a distinct characteristic and function that is summarized in Table 1-1 [7].

Table 1-1. Biophysical and functional properties of the human immunoglobulin isotypes

Isotype of antibody	Serum level (mean adult mg/mL)	Half-life in serum (days)	Functions
IgA	2.1	6	Mucosal Immunity
IgD	0.04	3	Naive B cell antigen receptor
IgE	$3* 10^{-5}$	2	immunity against helminth parasites, allergic reaction
IgG	13.5	23	Opsonization, complement activation, antibody dependent cell-mediated cytotoxicity, neonatal immunity, feedback inhibition of B cells
IgM	1.5	10	Naive B cell antigen receptor, complement activation

Each antibody isotype serum level, half-life in serum and its proposed function is summarized here.

IgE Antibody Structure

There are some unique features associated with IgE antibody structure. IgE molecule contains four heavy chain constant domain, which is different from Fc structure observed in IgG, IgD and IgA. There is no hinge region present in the ε-chain unlike IgG, IgD and IgA. IgE-Fc resembles the IgM Fc structure in its dimer form [8]. IgE is the most heavily glycosylated [~12% w/w carbohydrate] antibody compared to other antibody isotypes. There are seven N-linked glycosylation sites

distributed across the ε-chain [9]. The Asn394 site in the Cε3 domain of IgE is the structural homologue to Asn297 located in the Cγ2 domain of IgG. The only difference is that the glycosylation at Asn394 in IgE is of the "high-mannose" type (predominantly Man5GlcNAc2) [10], while the glycosylation at Asn297 in IgG is of the "complex-type" [11]. The N-linked glycans significantly reduce the flexibility of IgE and it is shown that the IgE glycosylation, and specifically sialylation affects IgE binding to its receptors [12]. For example, it is shown that glycosilation at Asn-394 is required for both appropriate IgE folding and FcεRI binding [13], [14]

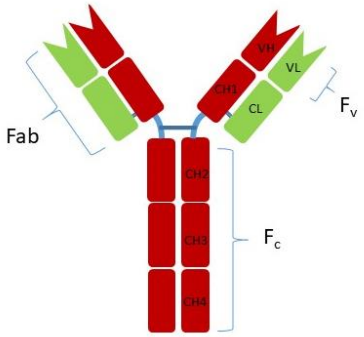


Figure 1-1. Structure of an IgE antibody molecule. Cartoon representation of a human IgE molecule showing the heavy and light chain variable and constant domains. The heavy chain is shown in dark red, the light chain is shown in green. Fc, Fv and Fab regions are marked.

Human Antibody Germline Organization

In human, the genes encoding antibody heavy chain are located on chromosome 14. The heavy chain loci consists of multiple gene segments referred to as the variable (V), diversity (D), and joining (J) genes. Antibody light chains may be produced from either the κ loci located on chromosomes 2 or λ loci located on chromosomes 22 [15]. Antibody light chain is formed by joining V and J gene segments, as no D region is present in the light chain loci. There are 43 V,

23 D and 6 J gene segments present in heavy chain loci [16].There are 33 V and 4 J genes in κ light chain loci and 38 V and 5 J genes in λ light chain loci [1], [15]. The unique organization of the immunoglobulin loci allows generation of a highly diverse pool of antibody proteins, as we will see in the next section.

Mechanisms of Antibody Diversity

Adaptive immunity is responsible for recognition of a virtually unlimited number of foreign pathogens and particles, that is why antibodies must generate enough diversity to be capable of responding to each pathogenic threat with high specificity [17]. Such diversity is achieved through four main mechanisms. The first mechanism is somatic recombination or V(D)J recombination [18], [19] , in which various combination of V, D and J gene segments are used to produce immunoglobulin domains. In the case of light chain, since no D segment is present, it is only V and J recombination.

The second mechanism of diversification is random pairing of heavy and light chain (λ or κ) to form the fully functional antibody. The heavy and light chain pairing together with V(D)J recombination can hypothetically generate a total of 2×10^6 unique potential antibodies.

The third mechanism of diversity known as junctional diversity occurs during V(D)J recombination [20] and is a combination of nucleotides removal at the recombination site and subsequent nucleotide addition to join the segments [21]. Recombinase activating gene (RAG) proteins known as RAG1 and RAG2 enzymes recognize specific sequence motifs adjacent to recombination site called Recombination Signal Sequences, or RSSs. The RAG protein complexes produce nicks in the dsDNA, and remove nucleotides from single-stranded DNA at the junction

[22]–[24] Subsequently, the enzyme terminal deoxyribonucleotidyl transferase (TdT) nonspecifically adds back nucleotides to repair and join the segments [25]–[27] Finally, DNA ligase IV rejoins double strand breaks into a single coding strand [28]–[30]. The junctional diversity takes place in the third CDR loop of both heavy and light chains; that is why this loop is the most diverse of the CDR loops, and is majorly involved in antigen recognition [15]. Junctional diversity can theoretically result in 10^{11} different antibody specificities.

The fourth mechanism of diversity is somatic hypermutation (SHM), also known as affinity maturation [18], [31]. Affinity maturation is a process in which the most useful, high-affinity B cells are selected from the initial pool of antigen-specific B cells produced in response to a particular antigen, a process similar to Darwinian natural selection ensuring survival of the best antigen binding B cells. Affinity maturation occurs in germinal centers after naïve B cells are activated via antigen recognition and T cell interaction, which induce somatic hypermutation of Ig genes. In proliferating germinal center, B cells in the dark zone will go through rounds of mutations and divisions and the rate of point mutation is 1 in 103 V gene base pairs per cell division; this implies that an average one mutation occurs per cell division [32]. The enzyme activation-induced cytidine deaminase (AID) causes point mutations in the antibody sequence via its DNA deaminase activity [33]. These mutations will accumulate in expressed V regions in the progeny of individual B cells. Mutations primarily occur in the antigen-binding complementarity-determining regions [34].

As these mutations occur randomly, a huge proportion of the mutations have declining effect on the antigen binding and may even result in a complete loss of binding. That is why, the subsequent selection step is crucial, in which B cells with the highest affinity binding for antigens in germinal centers are selected for survival. After undergoing rounds of somatic mutation B cells will migrate

from dark zone of germinal center into the light zone of the germinal center, which is full of follicular DC (FDC) and T cells presenting antigens to B cells [35]. . Only those B cells that are capable of antigen recognition survive, the rest of B cells die by apoptosis. In addition, those B cells that are high-affinity binders to the antigen will survive preferentially because they are able to bind to the antigen even at low concentrations [36]. Finally, those B cells that were selected in this process will either differentiate into memory B cells or into precursors of plasma cells, which will then exit the germinal center to produce antibody [37].

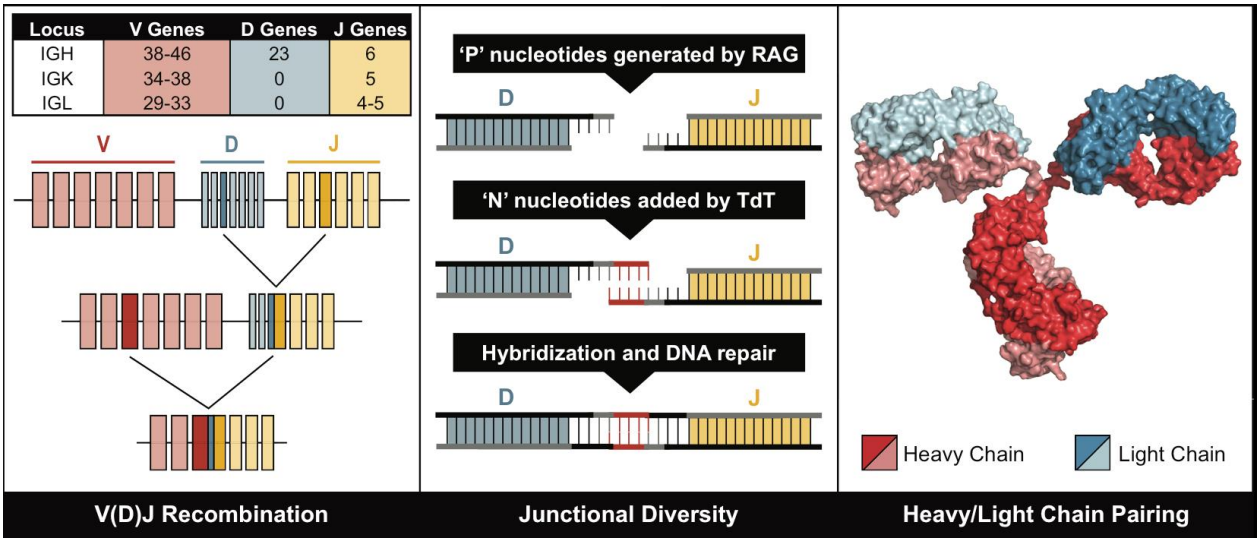


Figure 1-2. Diversity in the antibody repertoire is mediated by four principal mechanisms: V(D)J Recombination, Junctional Diversity, Heavy/Light Chain Pairing and somatic hypermutation (SHM) (Not shown). Figure obtained from Finn and Crowe, 2013 and used with permission from Elsevier, license number 3866260348974.

Antibody Class Switch Recombination (CSR)

All B cells initially produce IgM but different antibody isotypes are required to perform distinct effector functions against various types of infectious agents. As much as the variable region of antibody is important for bringing in the antigen-recognition specificity, the C region of an immunoglobulin is equally important as it determines the effector function of the antibody by interacting with various Fc receptors [38]. Isotype switching is regulated by the signals from T cells (CD40L) and the cytokines produced in response to pathogen.

Isotype class switching is a process in which the the heavy chains constant regions are changed without altering the specificity of the antibodies (variable region) [39]. In B cells, the Ig heavy chain DNA is cut and recombined in a way that a previously rearranged V region is brought next to a new downstream C region, and the DNA in between is deleted. The key enzyme required for isotype switching is activation induced deaminase (AID), similar to SHM [40]. Signals from cytokines and ligands from TFH induce AID expression [41]. AID generates nicks in the nucleotide sequences called switch regions that is located right next to each constant region [33]. These breaks are then joined together through non-homologous end joining mechanism [42]. The end result is generation of class-switched antibody with the same VDJ recombination of the original IgM produced by that B cell. Germinal centers are the preferential site of antibody class switching, but there is evidence of isotype switching in B cells outside the germinal centers, driven by extrafollicular helper T cells [43].

Antibody Functions

The main job of antibodies is to neutralize and eliminate invading organisms and pathogens. There are various effector mechanisms utilized by antibodies to eliminate antigens such as neutralization

of microbes and toxins, antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), phagocytosis of microbes opsonized with complement fragments, inflammation and lysis of microbes that is accomplished by different antibody isotypes [1].

IgE Antibody, IgE B Cells and Humoral IgE Memory

Immunoglobulin E (IgE) was first observed in 1966 but it wasn't named IgE until in 1968 (Hermans and Vaerman 1962). In 1921, Prausnitz and K€ustner described an experiment, so-called Prausnitz K€ustner (PK) reaction, which showed that injecting the serum of an allergic patient to a nonallergic patient, followed by allergen injection into the same skin site the caused a wheal and erythema reaction. This antibody-like component in the serum of allergic patient that caused erythema-wheal reactions was named "reagin" and 47 years later it was identified as the last class of human antibodies [45]. IgE was the last of the five human antibody isotypes to be discovered partially because it is the least abundant Ig isotype. Nowadays IgE is commonly associated with various allergic diseases, but evolutionarily it has been associated with the host immunity against helminth infection [46].

There are two proposed mechanisms for the origin of IgE producing B cells: 1) direct class-switching from IgM B cells and 2) sequential class switching from antigen-specific IgG producing B cells. There is a body of literature supporting IgE sequential class switching. For example, it is shown that allergen-induced class switching experiments *in vivo* not only increases allergen-specific IgE level but also boosts IgG1, IgG4 levels, while leaving IgG2 and IgG3 levels unchanged, indicating that production of IgG1, IgG4 and IgE isotypes moves in the same direction [47]. In addition, high-throughput immunoglobulin repertoire analysis of both allergic and healthy individuals showed that IgG1 and IgE producing B cells are arising from same B cell clonal

lineages [48]. Finally, remnants of Sγ switch region repeat was found in the Sμ-Sε switch regions of IgE producing B cells, which indicates that an IgM to IgG class switch has occurred prior to IgG to IgE class switch [49]–[51].

Although the mechanism by which naive B cells differentiate into IgE^+ B cells and form humoral IgE memory is not fully characterized, there was a study using mice infected with *Nippostrongylus brasiliensis* helminth showing that IgE^+ cells have plasma cells characteristics and are mainly residing outside of germinal centers. So, they proposed that $IgG1^+$ B cell generated in germinal centers will differentiate into IgE^+ plasma cells outside germinal centers [47]. Another study also shows that in germinal center, IgG1 memory B cells that were subjected to antigen selection and affinity maturation can act as humoral IgE memory [52]. A recent study shows that IgE response memory is kept inside long-lived IgE^+ plasma cells residing in the bone marrow of allergic individuals subsequent to chronic exposure to allergen, however they acknowledge that these long-lived IgE+ plasma cells are developed from germinal-center IgG1+ intermediates [53].

IgE's function by binding to Fc receptors is unique. Two types of Fcε receptors exist, FcεRI, which is the high-affinity IgE receptor, and FcεRII, also known as CD23, which is the low-affinity IgE receptor [54]. IgE is commonly found bound to its receptors, which explains its long immune surveillance in tissues. IgE half-life is about two weeks in tissues such as skin. In addition, there are no known inhibitory Fc receptors reported for IgE antibodies [55].

FcεRl

FcεRI is a multimeric cell-surface receptor binding IgE with the affinity 100 to 100,000 times IgG affinity for its corresponding Fc receptors [56]. The main cells targeted by IgE are mast cells and basophils, as they express high levels of FcεRI on their surface. These high-affinity IgE receptors

are also found on antigen-presenting dendritic cells, eosinophils, monocytes, macrophages, platelets and even epithelial cells in humans [57]–[60].

FcεRI exists in two forms as a tetrameric and trimeric complex. The tetrameric form of FcεRI consists of four polypeptide chains an α-chain (FcεRIα), a β-chain (FcεRIβ) and two γ-chains linked by disulfide bonds (FcεRIγ; αβγ2). The extracellular region of the α-chain is the only component responsible for binding to the IgE molecule. (The FcεRI β- and γ-chains play no parts in ligand binding). Upon antigen exposure and subsequent crosslinking of receptor-bound IgE molecules, the immunoreceptor tyrosine-based activation motifs (ITAM) on FcεRI β- and γ-chains become tyrosine phosphorylated. The role of human β-chain is to intensify the FcεRI signaling and to increase its cell-surface expression that is why FcεRI can also be made in trimer form without β-chain being present. The γ-chain is the main and indispensable FcεRI signaling unit. The amount of FcεRI expressed on the surface of cells is upregulated by its ligand, IgE, because mechanistically ligand-bound receptor is more stable and is resistant to degradation [61]–[63]. In addition, interleukin-4 (IL-4) cytokine derived from T helper 2 (TH2) upregulates FcεRI expression on the surface of cells [64]–[66].

FcεRII

FcεRII or CD23 is a homo-trimeric single-spanning membrane protein. The extracellular portion of receptor forms a lectin-like domain, which is the IgE binding site. Mature B cells and a range of other cell types such as macrophages, eosinophils, platelets, and some T cells are known to express a low-affinity IgE receptor (FcεRII or CD23) on their surfaces. Membrane IgE and soluble IgE cross-linking by CD23 is required for signaling in the context of B cell or APC activation [67].

There are also soluble versions of CD23 molecules. IgE binding to CD23 expressed on the surface of B cells might be also a mechanism for IgE homeostasis [68].

IgE Antibody Mechanism of Action

Receptor-bound antigen-specific IgE cross-linking on mast cells and basophils initiates a complex intracellular signaling cascade, which results in effector functions including: 1) release of pre-formed mediators of inflammation such as histamine, heparin, proteases, etc. 2) generation of leukotrienes and prostaglandins and 3) de novo synthesis of type 2 cytokines like interleukin-4 (IL-4) and interleukin-13 (IL-13), chemokines and other inflammatory mediators. Mast cell and basophil degranulation recruits and activates immune cells to the site of antigen challenge [1].

In addition, IgE can elicit other effector functions via binding to FcεRI and CD23. IgE antibodies can induce antibody-dependent cell-mediated cytotoxicity (ADCC) by engaging FcεRI on monocytes, macrophages, eosinophils and mast cells [69], [70]. These cells are able to release toxic mediators (e.g., nitric oxide), proteases, cytokines and chemokines (e.g., tumour necrosis factor, TNF , macrophage chemoattractant protein-1, MCP-1) that have cell lysis function [71]. Also, IgE antibodies can induce antibody-dependent cell-mediated phagocytosis (ADCP) by activating macrophages and monocytes [71], . In addition, IgE antibodies can increase the antigen uptake and presentation by APC [72]. It should be noted that IgE is incapable of binding to both Fcε receptors simultaneously [73]. Finally, inducing a potent IgE response requires sufficient epitope specificity, affinity and particular combination of antibodies [74].

It is worth mentioning that there is a type of IgE antibody known as highly cytokinergic IgE, which induces much larger aggregates of FcεRI compared to the poorly cytokinergic IgE. It is shown that highly cytokinergic IgEs alone are capable of inducing antigen independent mast cell activation.

Some studies have proved the effects of FcεRI-bound IgE on mast cells survival regardless of antigen binding status. [75], [76].

Overview of Lymphatic Filariasis Disease

According to WHO estimates, more than 1.5 billion people (>24% worldwide) are living with some type of soil-transmitted helminth infection. Helminths are multi cellular organisms with their own organs and complex life cycles that involve multiple hosts. Although helminth infection is considered a low mortality disease, it has a high rate of morbidity. Helminth infection is listed as neglected tropical disease and is creating significant social and economic burdens for developing countries where helminth infections are endemic [77].

Lymphatic filariasis (LF) is a mosquito-borne infection caused by three closely related filarial worms: *Wuchereria bancrofti, Brugia malayi and Brugia timori*. Lymphatic filariasis is the second leading cause of permanent long-term disability globally. Currently more than 50 million people are suffering from LF, a third of whom show disfigurement. Additionally, over 800 million people are at risk for LF development [78]. Infection with *Wuchereria bancrofti* accounts for 90% of lymphatic filariasis, *Brugia malayi* is responsible for 9% of cases and *Brugia timori* accounts for the remaining 1%.

Currently, LF is endemic in 72 countries, mainly in subtropical and tropical regions of the world. While brugian filariasis is common in Southeast Asia, *W. bancrofti* is geographically widespread in tropical regions of Asia, Africa, the Americas and the Pacific. Although LF is eradicated in North America, Australia, Japan, Korea, China and most of the developed countries, it remains a major public health problem in Southern and Southeast Asian countries [79]–[82].

LF is not the only disease caused by filarial worms. For example, onchocerciasis (river blindness), which affects about 20.9 million individuals with more than 99% of all cases occurring in sub-saharan Africa, is caused by *Onchocerca volvulus* worm [83]. Onchocerciasis is the world's second leading cause of infectious blindness. Loiasis is another disease caused by the parasitic worm *Loa loa*, which is endemic in West and Central Africa. An estimated 14.4 million people live in these areas with high rates of *Loa loa* infection [84].

Filarial Worm Life Cycle

Filarial worms are difficult to study partly due to their complex life cycles composed of arthropod intermediate host and mammalian definitive host. Filarial worms have five developmental stages inducing various immune responses that results in a range of clinical manifestations of filariasis. Infection begins with infective larvae (L3) entering the skin when bitten by a mosquito. The typical vector for filarial worms are mosquito species such as *Anopheles, Culex, Aedes and Mansonia spp.* After passing through the primary line of defense, L3 larvae find their way to the afferent lymphatics where they molt and develop into the L4 stage and adult male and female worms in approximately 3–12 months time period. Adult worms can live in the lymph nodes/ lymphatics for as long as 20 years, but their average lifespan is much shorter. After mating, female worms produce live progeny called first-stage larvae microfilariae (Mf), which migrate into lymph and enters the blood stream circulation. Adult worms can produce up to 50,000 microfilaria per day. The microfilaria in the peripheral blood will be picked up by the mosquito during a subsequent blood meal. Mf develops into first-stage larvae (L1) then migrates to thoracic muscles where it undergoes several rounds of molting to form L3 larvae. The infective third-stage larvae reside in mosquito's proboscis and is ready for transmission to a new final host [85]. Since all filarial worms reproduce

sexually, any increase in adult worm burden is an indication of re-infection by new larvae. Human immune system responds to different worm life stages differently. For example, it is shown that larval and adult stages induce a heavily biased Th2 response, while the microfilariae induces Th1 immune response [86].

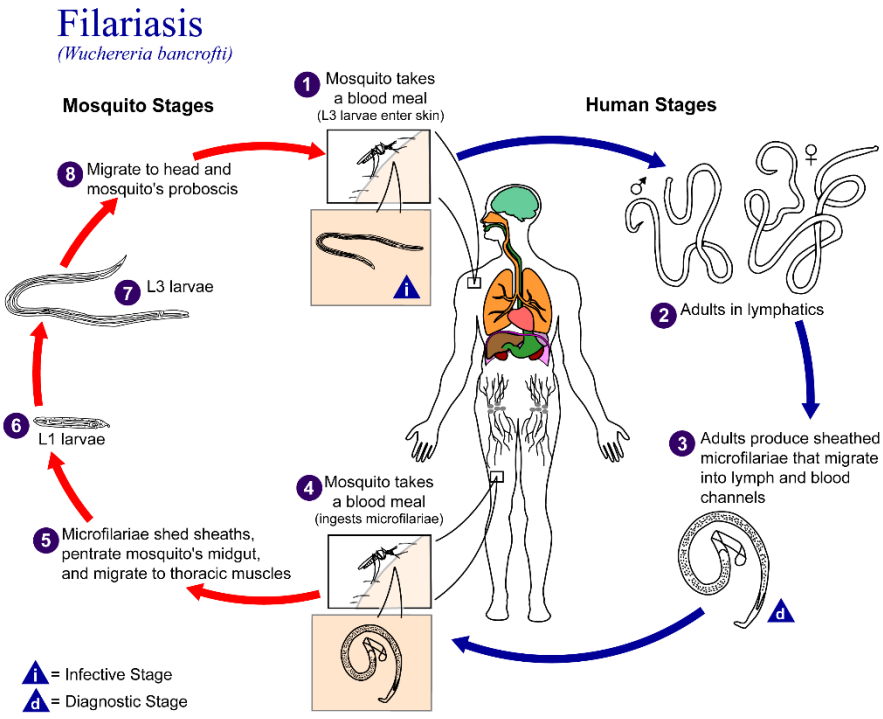


Figure 1-3. General life cycle of a filarial worm, *Wuchereria bancrofti*. (1) Third-stage filarial larvae enters the skin of the human host when bitten by an infected mosquito. (2) They molt and develop into adults residing in the lymphatics. (3) microfilariae produced by adult worms are sheated into blood. (4) Microfilariae enters mosquito when it bites an infected individual, (5-7) The microfilariae develop into first-stage larvae and subsequently into third-stage infective larvae within the mosquito. (8) Another human can get infected by L3 larvae when bitten by an infected mosquito. Figure obtained from Global Health, Division of Parasitic Diseases and Malaria on CDC website (https://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html), accessed in August 2021.

Lymphatic Filariasis Clinical Manifestations

Although filarial worms are easily transmitted by mosquitos, a patent LF infection is only established after exposure to intense transmission over long periods. That is why microfilaraemia is rarely observed in short-term visitors to endemic [87]. There is evidence of a threshold effect as most hosts can tolerate low levels of helminth infection without ill effects because host immune response does not reach the level required to trigger the effector mechanisms [88], [89].

The key factors that have direct impact on the severity of the LF disease observed among individuals are as followed: 1) The intensity, and type of immune response stimulated by parasite [90], 2) Infection rates and the intensity of transmission, which determines the infection load [91], 3) Parasite and host genetic polymorphisms [92]–[95]. 4) Secondary infection of already damaged lymphatics by bacteria or fungus [96], [97]. 5) Prenatal exposure to parasite antigens. It is shown that children born to microfilaraemic mothers are more likely to develop microfilaraemia in later life compared to children born to amicrofilaraemic mothers [95].

LF shows a broad range of clinical manifestations, listed in below.

1. Endemic Normal (EN) are individuals in endemic communities that are amicrofilaraemic and show no obvious signs of filariasis infection. These individuals either were not sufficiently exposed to become infected, or they have had prior infections that have been cleared, or they might still have adult worm infection but without circulating microfilaraemia. This group shows most marked T-cell responses to filarial antigens.[98]–[100]
2. Asymptomatic Microfilaeremics (MF) are individuals in endemic communities that have circulating microfilariae but show no symptoms or signs of disease also known as

'asymptomatic microfilaria carriers'[101], [102]. MF individuals show tolerance towards filarial worms and most of them will remain microfilaraemic and asymptomatic for many years. This group is immunologically hyporesponsive to the parasite and is unable to clear their microfilariae. MF patients have significantly lower number of filarial-specific Th1- and Th2 lymphocytes [103], [104], lower eosinophilia, decreased IFN- γ production [105], [106] and lower parasite-specific IgE and IgG levels compared to AMF, CP and TPE patients [107]–[110].

3. Acute LF Manifestations occur in a group of people in endemic areas. They suffer from episodic filarial fever event (acute manifestation of filariasis) presented in two forms acute dermatolymphangioadenitis (ADLA) and acute filarial lymphangitis (AFL). ADLA attack starts with fever and chills and results in tender, enlarged and swollen lymph nodes in affected part. AFL episodes occur only after the parasite death, either spontaneously or due to treatment. In the course of AFL an inflammatory nodule is formed around degenerating adult worms and results in a mild clinical course[100], [111]. A subset of these people subsequently develop chronic lymphatic pathology.
4. Symptomatic Chronic Pathology (CP) occurs in people who mount excessive inflammatory responses to filarial worms. The powerful immune response kills the parasites and causes unintended injury to lymphatic vessels. That is why patients with chronic lymphoedema are amicrofilaraemic and are tested negative for filarial antigen. Chronic LF can progress to develop clinical manifestations such as chronic lymphedema, elephantiasis and hydrocoele (in men), which result in severe disabilities[100].
5. Tropical pulmonary eosinophilia (TPE) is an uncommon clinical syndrome [112] that is caused by highly aggravated immune responses to microfilariae with immune clearance of

the parasites in the lung [113]. Although there is no microfilaria in the blood of these patients, it can be seen in lung biopsies surrounded by inflammatory cells. These patients suffer from paroxysmal coughing and wheezing, impaired lung function and reduced vital and lung capacity. Individuals with TPE show elevated eosinophil counts (>3,000 cells/mm^3 of blood) and elevated levels of both total IgE and filaria-specific antibodies [114], [115].

Laboratory Diagnosis of LF

Traditional laboratory diagnosis for LF, loiasis and onchocerciasis relied on microfilaria detection. Therefore, several laboratory diagnostic tests have been developed with the objective of microfilariae detection in the blood of patients including microscopic examination of stained thick blood films [116] and venous blood membrane filtration [117]. These methods have major drawbacks including lack of correlation between blood microfilaria counts and disease severity, inconvenience of nighttime blood sample collection, limited sensitivity, lack of consistency, false positive test reactions, etc. However, these techniques are still used for hospital diagnosis and field surveys in endemic areas due to the simplicity of use and its low cost.

Detection of live adult filarial worms and lymphatic vessels dilation by Ultrasonography (USG) is another LF diagnostic method. Adult *W. bancrofti* can be spotted in the majority of infected males by ultrasonography of the scrotum [118], [119]. However, this method fails to detect any adult worms of *B. malayi* [120].

Circulating Filarial Antigens (CFA) tests detect circulating antigens of *W. bancrofti* in human blood (either night or day). Two forms of antigen tests are commercially available including a

rapid format card test (ICT)[121] and an ELISA test [122]. These tests are designed to detect circulating antigens of adult *W. bancrofti* worms. In these tests an IgG mAb, Og4C3, which is generated in response to Onchocerca gibsoni antigens is used to capture circulating antigen of *W. bancrofti*. CFA tests have higher sensitivity and specificity for *W. bancrofti* infection compared to previous methods. No antigen detection tests are commercially available for diagnosis of infections with the Brugia species [123].

Antifilarial antibody tests detect IgG4 subclass antibodies directed against recombinant filarial antigens in an ELISA. For example, an ELISA test is commercially available that detects IgG4 antibodies against recombinant antigen Bm14 [124], [125], recombinant antigen BmR1[126] and recombinant antigen Wb123 [127]. The main drawback of antibody tests is that they cannot differentiate between active infection and past infection, while CFA can accurately detect active infections.

Filarial DNA detection [128] using polymerase chain reaction (PCR) is another sensitive diagnostic method. Filarial DNA sequences are amplified and detected by fluorometry or agarose gel electrophoresis[129]. The main disadvantage of this method is the need for nighttime blood samples from filarial patients.

LF Management and Current Treatments

Current filariasis control measures include disease control through improved sanitation and hygiene, chemotherapy or Mass Drug Administration (MDA), vector control [130], health education, and case management for patients with clinically evident disease [131].

In 2000, Global program to Eliminate Lymphatic Filariasis (GPELF) was launched by WHO. The goal of this program was to eliminate lymphatic filariasis as a public health problem by year 2020 through administration of antifilarial medicine to entire populations that live in filariasis-endemic areas an approach known as mass drug administrations (MDA). Currently, MDA is being provided in 53 of 72 filariasis-endemic countries. The GPELF focuses on both transmission interruption and morbidity control. According to WHO guidelines 4–6 rounds of MDA should be delivered until community microfilaria rates fall below 1%.

Helminth-specific Immune Response

Human immune response is broadly categorized as type 1, type 2, and type 3 immunity. Type 1 immunity elicits more cytotoxic functions via enhanced natural killer (NK), TH1, and CD8+ T cell activities. Type 1 immunity is mainly involved in human defense against intracellular pathogens and in combat against cancer cells. While, type 2 immunity elicits a combination of an inflammatory environment dominated by cytokines IL-4, IL-5 and IL-13 and a more immunosuppressive environment dominated by cytokines TGF-β and IL-10. Type 2 immunity is involved in defense against parasites through activation of mast cells and eosinophils, and increased production of IgE by B cells. This type of defense is important for the eradication of helminthic infections because helminths cannot be phagocytosed by neutrophils and macrophages due to their size and they also show resistance to the microbicidal activities of these phagocytes. In addition, type 2 immunity is infamous for its pathological effect in allergy diseases, where mast cells and eosinophils activation is triggered by innocuous allergen molecules. Generally, type 2 immunity is more protective of tissues, that is why it plays a role in wound healing as well [132],

[133]. Finally, Type 3 immunity majorly involves Th17/Th22, neutrophils, and ILC3 and is directed against extracellular microorganisms.

The coevolution of human immunity and helminthic parasites for so long has shaped the immune response to act in an efficient way to limit worm burden, while not inducing tissue damage. It is a nice balance between inflammatory and immunosuppressive response that is provided as part of controlled type 2 immunity. Although there are still a lot unknown about helminth infection, it is well characterized that the increased level of IgE antibody, the T helper 2 (Th2) cells, eosinophils, mast cells and basophils, alternatively activated macrophages, cytokines – IL-4, IL-5, IL-9, IL-10 and IL-13 are the hallmark of helminth infections, including lymphatic filariasis, loiasis and onchocerciasis.

Innate Immune Response in Helminth Infection

The type 2 immunity activates the phagocyte-independent defense via exploiting eosinophils and mast cells. Here, I will briefly touch on the main functions of innate immune cells that are important in human response against parasitic worms.

Mast cells and basophils are probably the most important effector cells in host defense against parasitic worms. Activated mast cells and basophiles are major sources of IL-4, IL-13, and IL-33 cytokines [134] and they also express CD40L [135], which together amplify Th2 response and induce B cell class switching to express IgE antibodies. FcεRI is highly expressed on the surface of these cells, which binds to the IgE with high affinity. Subsequently antigen binding to FcεRI-bound IgE triggers cell activation and mediator release. A wide variety of biologically active proteins and chemical mediators are released by activated mast cells including: 1) histamine and

heparin, which are toxic to parasite and increase vascular permeability for recruiting inflammatory cells to the site of infection, 2) TNF-α, which promotes inflammation and stimulates many cell types to produce Th2 cytokines 3) lipid mediators like prostaglandins, leukotrienes and platelet-activating factor which activate neutrophils, eosinophils, and platelets and stimulate smooth muscle contraction, etc.

Eosinophils are the first responders brought to the site of helminth infection. Th2 cells and activated mast cells are the primary sources of IL-5, which is shown to stimulate production, maturation and activation of eosinophils [136]. Other cytokines such as IL-4, IL-3 and GM-CSF show stimulatory impact on eosinophils as well [137]. Eosinophils express both FcεRI and FcεRII on their surfaces. Eosinophil-mediated protection against helminth occurs through antibody-induced release of toxic granule proteins including eosinophil derived neurotoxin (EDN), eosinophilic cationic protein (ECP) and major basic proteins (MBP), eosinophil peroxidase, etc [138]–[140].

Type 2 innate lymphoid cells (ILC2), which are located in dermal tissues, where infective larva is inoculated, play a major role in initiating Th2 responses against helminth infections via producing large amounts of IL-4, IL-5 and IL-13 cytokines [141], [142]. ILCs are likely the first and most important cells for detecting initial tissue injury and initiating Th2 activation. In addition, damaged tissue signals for the initiation of the Th2 responses via secreting IL-33 [132].

Antigen presenting cells (APCs) express FcεRI and CD23 on their surfaces. Although APCs do not have any granule or preformed toxic mediator to kill parasites, receptors cross linking triggers the synthesis and release of mediators which shape an inflammatory microenvironment. In

addition, receptors promote more efficient antigen uptake, processing and presentation in APC [143].

Dendritic cells (DCs) uptake and present filarial antigens and initiate Th2 actiation. Cytokines and surface ligands expressed by DCs further stimulate Th2 differentiation. It is shown that exposing DCs to helminth products (containing ES-62 antigen) in vitro followed by passive transfer into live recipients reproduces Th2 response [144]. However, there is evidence that *Brugia* microfilaria is impairing DCs antigen presentation function by inducing DC apoptosis via TRAIL pathway and by downregulating Toll-like receptors (TLR) expression and signaling [145], which can explain the T cell hyporesponsiveness seen in patients with high level of microfilaria.

Two major macrophage populations exist [146]:

1) Type I or classically activated macrophages (CAMFs) that exhibit a proinflammatory profile. These IFN-γ–induced macrophages have antimicrobial and cytotoxic functions against intracellular pathogens. They will regulate other cells to produce cytokines and chemokines required for pathogen destruction. IL-4 and IL-13 cytokines have an inhibitory effect on classical macrophage activation.

2) Type II or alternatively activated macrophages (AAMFs) that are known for their anti-inflammatory activities and tissue-repair function. AAMFs are activated in responses to helminth infection (extracellular pathogens) and in wound repair [147]. There is in vitro evidence showing that alternatively activated macrophages are generated in response to Th2 cytokines such as IL-4 and IL-13 [148]. IL-4 can affect macrophage polarization to produce AAMF phenotype even before it is fully differentiated from monocyte stage to macrophage [149]. Further *in vivo* studies using knockout mice also confirmed the fact that Th2 cytokines are required for production of

AAMFs [150]. Alternatively activated macrophages secrets cytokines such as IL-10 and TGF-β that inhibit Th1 development and function including suppression of classical macrophage activation. The AAMFs show impaired phagocytic ability too [149]. The TLR profile in AAMFs is different from CAMFs, as they show down regulated expression of TLR2, TLR3, TLR5, TLR7, and TLR8. That is why, in response to TLR engagement, AAMFs express much less cytokine [149], [151].

Human B Cell Response in Helminth Infection

Elevated IgE level and B cell class switching to produce IgE antibodies is a main characteristic of helminth infection and its mechanism of action was previously explained in detail. IgE production is highly dependent on IL-4, as it was shown by an *in vitro* experiment that addition of anti-IL-4 antibody to the culture of peripheral blood mononuclear cells (PBMCs) from patients with filariasis (primarily induced by parasite antigens) completely inhibited lgE production. The profound IgE class switch bias induced by parasite antigens can result in the production of IgE antibodies against bystander antigens as well, as it was shown in a study in which animals received ovalbumin and *Ascaris* secreted antigens and they produced profound IgE response to ovalbumin as well as *Ascaris* antigens [152]. However, IgE antibody is not the only antibody isotype produced in response to helminth infection, there is evidence of abundant IgG antibodies in the serum of filarial infected patients, which show the same antigen recognition pattern as IgE [153]. These blocking antibodies, which are primarily of the IgG4 isotype can compete with IgE and inhibit IgE-mediated activation of mast-cells and basophils [154]. Another overlooked fact is the presence of polyclonal nonspecific IgE in the blood of filarial infected individuals [107] competing with filarial specific IgE for binding to the FcεRI on effector cells and reduce degranulation and

mediator release. It is shown that the ratio of parasite specific IgE to total IgE differs in various clinical manifestations of LF; patients with TPE have the highest ratio of filarial specific antibody and patients with asymptomatic microfilaremia have the lowest ratio [107].

Human T Cell Response in Helminth Infection

As mentioned before, IL-4 is the signature cytokine of the Th2 cell, which leads into chronic T cell stimulation and CD4+ T cells differentiation into antigen-specific Th2 cells by activating the transcription factors STAT6 and GATA3. After Th2 differentiation, the expression of Th2 cytokine genes IL-4, IL-5, and IL-13 is increased via GATA3 transcription factor. IL-13 is produced by Th2 cells (primary source), basophils, eosinophils, and NKT cells. IL-4 (and IL-13) produced by Th2 and TFh cells stimulates B cell Ig heavy chain class switching to produce polyclonal and antigen-specific IgE [159]. IL-4 also enhances antibody class switching to IgG4, which can act as a blocking antibody [144], [160]. IL-4 provides an autocrine positive feedback loop further stimulating Th2 growth and differentiation. IL-5 produced by Th2 cells promotes survival, differentiation, and chemotactic function of eosinophils. IL-4 and IL-13 recruit leukocytes and specifically eosinophils to the site of infection and contribute to differentiation of alternatively activated macrophages.

Parasitic Worms Immunoregulatory Mechanisms

Although Th2 dominance in filarial infection continues to be the most well characterized model, this model fails to explain why some patients show immune hyporesponsiveness to filarial worms. Helminths are masters of immune regulation and that is why they have such a long life span in

their hosts. For example, adult filarial worms can live in their human host for up to 20 years. Immunological tolerance to parasite antigens is beneficial to the parasite as it lets microfilaremia to persist and maximizes the chance of parasite transmission. The mechanisms underlying this immunological hyporeactivity is not completely understood but it is possible that they are taking advantage of the self-imposed immunoregulatory system that protects us from lethal autoimmune diseases.

The immune regulation mechanisms proposed include 1) Development of peripheral immune tolerance to the parasite as a result of persistent or intermittent exposure of host to developing larvae and high amounts of parasite antigen [115], 2) Active suppression of monocytes [102], 3) Upregulation of regulatory T cells [161] or 4) Soluble suppressive parasite products [102] as there is evidence of impaired APC function by exposure to larvae or their excretory/secretory products, which then nonspecifically suppresses lymphocyte proliferation. Helminths produce a variety of immunomodulators including cytokine homologs, protease inhibitors, and a group of novel immune suppressive proteins.

LF Vaccine Development

Although drug treatments for LF exist, rapid re-infection in parasite endemic areas and dramatic rise in drug resistance is raising serious concerns. That is why MDA is not feasible as a long term control strategy and development of effective vaccines against LF becomes the top priority. In order to do so we must first fully understand the pathogen-specific immune response, so we can specifically target the main components of immune protection.

In general, vaccine development against a pathogen is only possible if protective immunity is naturally developed soon after early exposure(s) to it, and if a high antibody titer is produced during that exposure. As there is evidence that acquired immunity is developed naturally against helminth parasites [162], vaccination could be the solution to this public health issue. Natural protective immunity is normally observed against third-stage larvae (L3) while MDA treatments are primarily directed at the microfilariae and adult worms. Thus, vaccines and anti-helminth medicines can be integrated in a complementary treatment approach in a way that infectious larvae are targeted by vaccination and existing adult helminthes are killed by anti-helminth drugs [163].

There are major difficulties in developing an efficient vaccine against filarial worms including: 1) Lack of enough understanding of the pathogen-specific immune response, 2) High degree of antigenic polymorphism and lack of an efficient program to identify immunodominant antigens, 3) Optimization of adjuvant formulations, 4) Difficulty with delivery to developing world countries 5) Understanding the impact of pre-existing immunity or maternal antibodies on vaccination efficacy because there is a chance of activating allergic reactions. For example, a human hookworm vaccine, developed based on Na-ASP-2 antigen, entered clinical trials but due to severe adverse events (generalized urticarial) the study was terminated. The reason for vaccine failure was the pre-existing IgE antibodies against Na-ASP-2 in patients sera because of their prior infection with hookworms [164]. 6) Understanding the impact of underlying infections with other pathogens on helminth vaccine efficacy. 7) Identifying suitable animal models of human LF disease for vaccine testing, 8) Understanding that helminth vaccines might not be sterilizing vaccine. Therefore, drug treatment might still be required.

With all these hurdles, there have been various types of vaccines that were attempted for human and veterinary use that I am describing in below. Again, no FDA approved vaccine is available for human helminths yet.

1. Live attenuated, inactivated or killed larvae. Although this method provides the highest level of protection, there are some serious issues with this method including, the fear of infectivity, and lack of proper technology to grow parasite in industry scale for live parasite vaccine development. Currently, there are a handful of veterinary vaccines commercially available, which are derived from live-attenuated parasites, such as a canine hookworm vaccine derived from radiation-attenuated infective larvae [165]. Regarding filarial vaccines, mice vaccinated with *B. malayi* irradiated L3 larvae showed 56% to 91% protection against L3 antigenic challenge administered subcutaneously (SC) or intraperitoneally (IP) [166]

2. Recombinant anti-helminth vaccines. A few vaccines are produced from purified parasitic components and even a smaller number of vaccines are produced by recombinant technology. The biggest hurdle here is the rational selection of antigens. If the antigens are cloned, the existing technology for recombinant protein production provides an efficient and cost-effective approach for large scale vaccine manufacturing. For example, soluble extracts of microfilariae and adult worms, Excretory-secretory (ES) products of filarial nematodes (majorly containing proteases, protease inhibitors, venom allergen homologues, glycolytic enzymes and lectins) [167], and some recombinant protein antigens including *B. malayi* abundant larval transcript I, among which (Bm-Alt-1) when tested in rodent animal models showed 70% protection against L3 challenge. The limitation of this method is lack of enough antigenicity compared to live attenuated vaccines.

Filarial Immunogenic Proteins

Although there is still much unknown about the antigenic triggers of humoral response against filarial infection, there are some filarial proteins reported as *B. malayi* allergens including tropomyosin (Bru m 3) [155], glutathione S-transferase (Bru m 13) [156], and aspartic protease inhibitor (Bru m Bm33) [157]. In addition, many filarial antigens targeted by IgG antibodies are identified such as Abundant Larval Transcript-1 and -2 (ALT-1 and ALT-2), which mainly induces IgG1 and IgG3 antibodies in individuals with *B. malayi* infection. Another well-characterized filarial antigen targeted by IgG response is WbSXP-1, which has homologous proteins in other worms including *Ascaris, Anisakis* and even *C-elegans*. There are some filarial antigens with immunomodulatory functions identified such as thioredoxin and macrophage migration inhibitory factor in *B. malayi.* Finally, high-throughput proteomics analysis of *B. malayi* has introduced other putative antigens that are highly abundant including BmR1, proteases such as Serpin, structural proteins such as major sperm protein, Bm-MIF-1, heat-shock family of proteins, C2H2 type family protein, transthyretin like proteins, etc. [158].

CHAPTER II

DEVELOPMENT AND CHARACTERIZTION OF FIRST PANEL OF FILARIA-SPECIFIC HUMAN IGE MONOCLONAL ANTIBODIES

Introduction

Human lymphatic filariasis is a highly debilitating disease caused by three closely related filarial worms *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori.* It affects 859 million people in 50 countries worldwide with 90% of the infections being caused by *W. bancrofti* worm [168]. The Global Program to Eliminate Lymphatic Filariasis (GPELF), which was initiated by World Health Organization (WHO) is far from reaching its goal of eliminating LF by year 2020 [169]. Massive epidemiologic studies have conclusively demonstrated a clear link between helminth infection and eosinophilia and elevated levels of IgE. Thus it is clear that this branch of human immunity is induced, but the role of IgE in protecting against helminth parasites or controlling worm burden is unclear [170], [171]. While elevated levels of IgE antibodies is the hallmark of the humoral response to helminth infections, including lymphatic filariasis [172], knowledge about the underlying antigenic triggers of this response and their protective function remains limited. Early serologic studies introduce IgE antibodies as being 'nonspecific'[173] and others have focused on the allergen-like proteins such as tropomyosin, to find a link between helminthic disease and allergy [174].

Immunoglobulin E (IgE) was the last human antibody isotype to be discovered partially because it is the least abundant antibody isotype in serum[175], [176]. The story of the discovery of IgE is beautifully described in a recent paper by Ishizaka and Ishizaka [177]. Despite the importance of IgE antibodies in parasite immunity and allergic diseases, few studies have directly examined the B cells that encode IgE antibodies in humans [178], [179], that is why this class of human antibody remains poorly characterized. The main obstacle in studying human IgE antibodies at the

molecular level is the inability to generate naturally occurring human IgE monoclonal antibodies. Prior studies on IgE were limited to studying serum IgE from patients with parasitic infection and allergies, IgE antibodies developed using mouse hybridoma technology, and artificially class-switched polyclonal IgE antibodies produced by using the cytokine IL-4 in B-cell culture [180], [181]. U266, or its derivatives (SKO-007), are the only IgE secreting human cell lines available to study, which were originally isolated from a patient with multiple myeloma [182]. This cell line was made HAT-sensitive and was used to generate fully human hybridomas [183]. There are still a lot of unanswered questions regarding the nature of antigens targeted by IgE antibodies, the functional properties of such antibodies, the degree to which these features are shared across individuals, etc.

in 1975, G. Kohler and C. Milstein described hybridoma technology for the first time[184]. In the early 1970s mouse myeloma cells were used as fusion partners for primary human B cells [185]. Antibodies have progressed from mouse to fully human mAbs and their immunogenicity has progressively decreased. In 1980, the first successful human monoclonal antibody (mAb) was produced [185]. In addition, various structural modifications of antibodies led to their improved specificity and selective cytotoxicity [186]. The main advantage of using human hybridoma technology to produce mAbs instead of newer high-throughput technologies is that the pairing and authentic sequence of full-length antibody DNA from a naturally occurring B cell is preserved in hybridoma cell line. So, all the information regarding natural B cell selection, class switching, and affinity maturation is imprinted in the mAb sequence. On the other hand, the major drawbacks of using hybridoma technology is the low fusion efficiency to isolate hybridoma cell line and the lengthy process of hybridoma development taking on average between 3 to 4 months to obtain a hybridoma clone. To overcome this obstacle the antibody variable genes of desired antibodies can

easily be amplified for recombinant mAb production. Currently, thanks to the improved fusion partner, HMMA2.5 nonsecreting myeloma cell line, human hybridoma technology is still commonly used to develop panels of antigen-specific antibodies.

In this chapter, I have described the isolation and characterization of human IgE mAbs to filarial worms isolated from individuals with prior history of laboratory-confirmed filarial infection. Here I present a detailed protocol for B cell culture, hybridoma formation, selection for IgE producing B cells, single cell indexing, large scale production, and antibody purification. Then, all IgE mAbs were tested for binding to filarial antigens. My results indicate that contrary to previous studies reporting the dominance of inflammation-driven 'nonspecific' IgEs [173], about 46% of the successfully generated IgE mAbs were positive in at least one of the screenings. Furthermore, my results reveal that IgE antibodies obtained from filarial patients do not cross-react with common allergens. In addition, the sequence analysis of filaria-specific human IgE mAbs show that there is not an overrepresentation of any specific antibody heavy chain variable region (VH) gene. In addition, IgE antibodies show similar CDR3 lengths distribution when compared to other antibody isotypes.

I acknowledge Dr. Thomas Nutman, NIH, Bethesda, for processing and cryopreserving PBMCs used in my study, Dr. Heather Stockdale Walden; University of Florida College of Veterinary Medicine for providing adult male and female *D. immitis* worms, NIAID/NIH Filariasis Research Reagent Repository Center (FR3; Athens, GA; www.filariasiscenter.org) for providing *B. malayi* worms as research material for this study, Dr. DeWitt's group, Thermofisher scientific, for developing *B.malayi* immunoCAP assays and performing binding assays, and past members of the Smith laboratory for developing and optimizing the hybridoma technique that allowed me to isolate large panels of human IgE mAbs.

Primary B Cell Culture

We have used PBMC as the source of B cells, so the next step is identifying antigen-specific B cells via fluorescence activated cell sorting (FACS) or magnetic enrichment method and expanding them in culture. However, identification of IgE^+ B cells by using flow cytometry has been controversial due to the following reasons: 1) IgE producing B cells are present at a very low frequency in the blood of patients with helminth infection or allergy, 2) IgE BCRs are poorly expressed on the surface of IgE producing B cells, 3) Other immune cells including B cells producing other antibody isotypes can capture IgE with their Fcε receptors [187]. So, it makes it almost impossible to sort for IgE producing B cells via flow cytometry.

In order to overcome this obstacle we have developed an optimized B cell culture media to expand B cell populations regardless of their antibody isotype. In order to stimulate B cells, we used a genetically engineered cell line that produces signals required for B cell growth and stimulation. The NIH3T3 fibroblast line genetically engineered to constitutively express 1) CD40-ligand (CD40L or CD154) -which supports primary human B cell growth, 2) human interleukin-21, and 3) B-cell activating factor (BAFF) - a member of the tumor necrosis factor family, which provides costimulatory signal and acts as a potent B cell growth factor [188]. In addition, CpG oligonucleotide a toll-like receptor 9 (TLR9) agonist was added, which was previously shown to stimulate B cells proliferation and differentiation[189]. Although Epstein-Barr virus (EBV) transformation is commonly used to immortalize B cells and amplify their population [190], we did not use this method primarily because EBV transformation generally causes B cells to grow poorly, secrete lower amount of antibodies and even undergo chromosomal instability [191], [192]. Frozen PBMC samples were thawed quickly and after viability assessment using Trypan blue

staining, 2 x 10^6 cells were added to prefusion medium along with 1 x 10^6 gamma irradiated NIH3T3 feeder cell line per plate. Plates were incubated at 37°C with 5% CO_2 for 6 days, prior to screening for IgE secretion using an isotype-specific sandwich ELISA.

Fusion of B Cells with Myeloma Fusion Partner

The efficiency limiting step in the hybridoma production is the fusion step. Three main techniques used to generate hybridomas are 1) chemical fusion using polyethylene glycol (PEG) as the fusing agent, 2) viral fusion [193]–[195], and 3) electrical cytofusion.

Chemical fusion using PEG is the most commonly used method in generating hybridomas due to its simplicity and low cost. However, there are some drawbacks associated with this method including its toxicity for cells, its ability to fuse multiple cells together and from polykaryons, and its capability to fuse intracellular membrane structures and form nonfunctional cells. Electrical cytofusion is another efficient method used for cell fusion. In this method an alternating current electrical field is applied to cell suspension to bring cells into contact. Afterwards, multiple high-intensity electric field pulses are introduced which form pores in the membranes and fuse adjacent cells together. Finally, alternating current fields are used to keep cells in contact for a longer period of time until the fusion process is complete. This process has been optimized in order to maximize the number of B cell myeloma hybridomas generated. Electrofusion cycles were as followed: 40 seconds of 70 V AC current, DC current pulses of 360 V for 0.04 ms and finally 9 seconds of 40 V AC current. After 30 minutes incubation of cuvettes at 37°C, hybridoma cells are selected from unfused cells and products of like cells fusions. HAT (hypoxanthine-aminopterin-thymidine)

medium containing ouabain is used in selection process. Only fusion products containing both B cell and myeloma cell nuclei can survive and grow in HAT selection medium.

In addition to optimizing the fusion process, selecting the right fusion partners is critical. Two major types of fusion partner cell lines are fully human and heterohybridomas, which are the fusion products of murine myeloma cells and human cells. The HMMA2.5 is a heterohybridoma fusion partner [196] used to generate large panels of fully human mAbs with specificity against many viruses, such as influenza and dengue viruses [197], [198]. So, I used HMMA2.5 cell line as fusion partner for hybridoma generation.

Based on the ELISA screening, cells from the wells containing IgE producing B cells were transferred to cytofusion medium and were then subjected to electrocytofusion with HMMA2.5 cells. Each cuvette was examined under microscope to ensure the formation of pearl and string construct. Then fusion products were added to the hypoxanthine-aminopterin-thymidine (HAT) medium containing ouabain, in 384 well plates and were incubated at 37°C for 14 days before screening hybridomas for IgE antibody production by isotype-specific ELISA.

Biological Cloning

Biological cloning is an essential step in generating monoclonal antibodies. I used a combination of limiting dilution plating and flow cytometric automated single-cell index sorting to ensure that clonal mAb-producing hybridoma is separated from the mixed population of cells generated in the fusion process. I repeated limiting dilution plating several times to maximize the chance of isolating a stable clone that secrete sufficient amounts of IgE mAb. After each round of limiting dilution a sandwich isotype-specific ELISA was performed to screen for the presence of IgE

antibody in the supernatant. As limiting dilution cloning is a low-throughput technique, and it is never guaranteed to obtain clonality, the final step was to use single-cell index sorting, which if set up correctly will place only one cell in each well.

MAb Production and Purification

After single clones were recovered, they were expanded in postfusion medium to reach 50% confluency. MAbs were expressed by large-scale growth of the hybridomas in serum free medium in 225-cm^2 flasks. Immunoaffinity chromatography (Omalizumab covalently coupled to GE Healthcare NHS activated HiTRAP; 17-0717-01) was used to purify IgE mAbs. The mAb concentration was measured by absorbance at UV 280 nm using a NanoDrop spectrophotometer and setting the extinction coefficient for IgE antibody. In addition, IgE mAbs were run on SDS PAGE to estimate the purity.

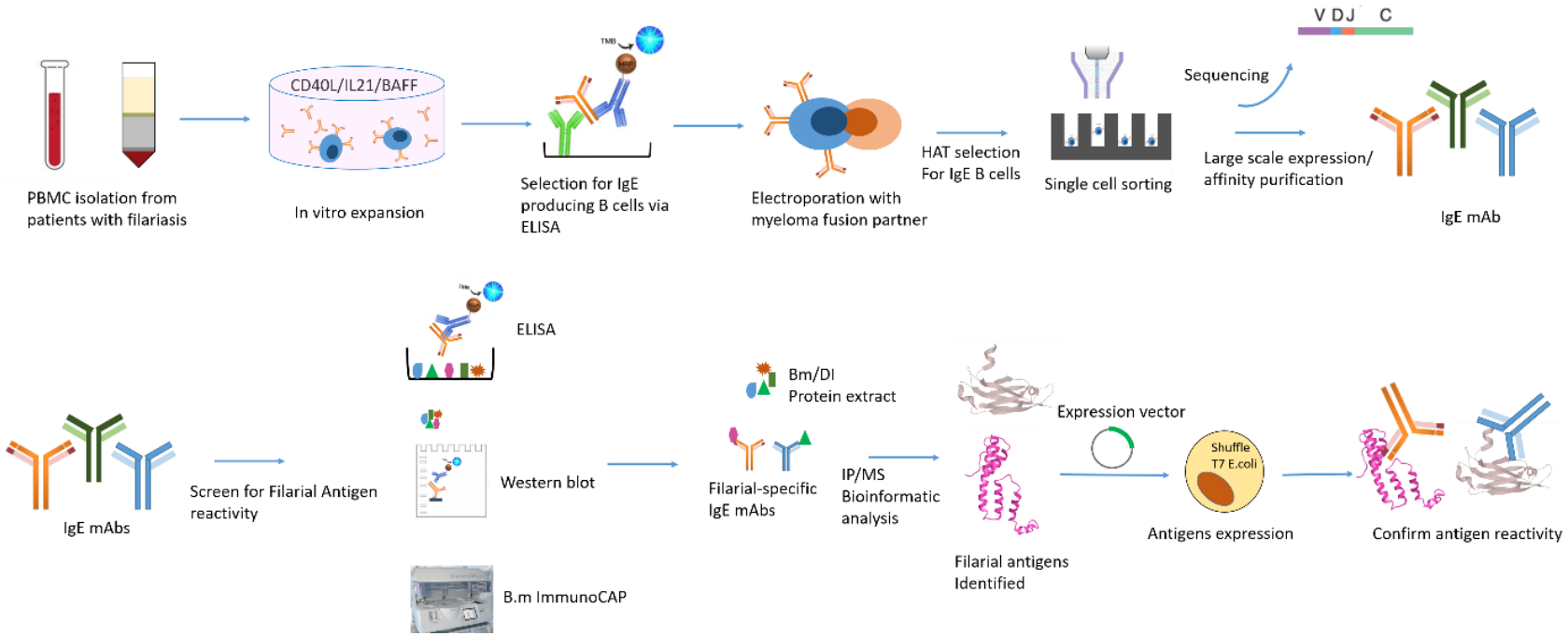


Figure 2-1. Schematic showing human IgE antibody generation and workflow of filarial antigen discovery. a, PBMCs were isolated from blood and processed for in vitro expansion of B cells prior to screening for IgE production by ELISA. IgE-producing B cells were fused with a myeloma fusion partner followed by HAT selection and single cell sorting. Each hybridoma was expanded in serum-free media for large-scale mAb production and affinity purification. b, IgE mAbs were tested for reactivity to *B. malayi* and *D. immitis* using ELISA, immunoblot, and *B. malayi* ImmunoCAP. IgE mAbs showing reactivity in multiple assays were subjected to IP and mass spectrometry analysis for antigen discovery. Filarial antigens were expressed in E. coli as their homolog *W. bancrofti* antigens. IgE mAbs were validated against recombinant antigens by ELISA and immunoblot analysis.

Study of B Cell Response in Patients with Filarial Infection

All samples used for this study were obtained from patients with prior history of laboratory-confirmed filarial infection. Our first question, therefore, was whether IgE-producing B cells are present in the blood of patients infected with filarial worms and and, if so, could they be harnessed to generate human IgE mAbs. To do so, we obtained cryopreserved peripheral blood mononuclear cells (PBMC) collected from 7 subjects with lymphatic filariasis, TPE, loiasis, or onchocerciasis. I plated the PBMCs on 384 well plates, in the presence of feeder layers expressing CD40L and secreting IL-21 and BAFF, but no IL-4 cytokine was added to avoid artificial class switching. After 6 days in culture, supernatants were screened by ELISA for presence of secreted IgE antibodies. Our data demonstrates that IgE expressing B cells do exist in circulation, with a frequency ranging from 6 to 14 cells per 10 million PBMCs (Figure 2-2), slightly lower than the number of IgE expressing B cells we previously reported for subjects with allergic bronchopulmonary aspergillosis [178]. Subject demographics, IgE encoding B cell frequencies and hybridomas yield is shown in Table 2-1.

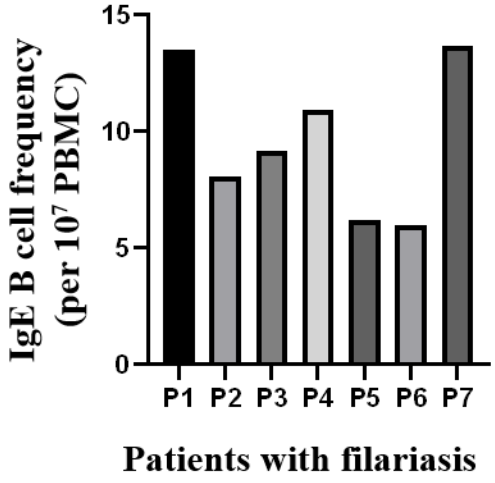


Figure 2-2. Frequency of IgE producing B cells in patients with filarial infection. PBMCs from patients were plated in 384-well plates and supernatants were screened by ELISA for the presence of IgE antibodies. IgE B cell frequencies are expressed as the number of IgE-positive cells per 10 million PBMCs.

Table 2-1. Subject disease status and hybridoma yield

Subject code	Age	Sex	Diagnosis	Serum IgE IU/ml	IgE B-cell frequency (per 10^7 PBMCs)	IgE hybridomas generated
P1	61	M	TPE	4260	13.5	22
P2	29	M	TPE	9810	8.1	1
P3	21	F	Loiasis	20290	9.2	9
P4	40	M	Loiasis	8520	10.9	12
P5	61	F	Loiasis	11362	6.2	5
P6	29	M	Loiasis	7628	6.0	1
P7	42	M	Onchocerciasis	8680	13.7	6
P8	49	F	TPE	6140	~~Serum only~~	~~Serum only~~
P9	27	M	TPE	7440	~~Serum only~~	~~Serum only~~

Subject helminth disease, IgE B-cell frequency and IgE hybridomas yield are shown. IgE B cell frequencies are expressed as the number of IgE-positive cells per 10 million PBMCs. The total IgE-expressing human hybridomas generated for each subject is listed. TPE, Tropical pulmonary eosinophilia.

Filarial Antigens

Because *W. bancrofti* (which is responsible for 90% of LF infections), *Loa loa* and *Onchocerca volvulus* cannot be maintained in conventional mouse strains, it has been a common practice to use *Brugia malayi* worms the closest strain to *W. bancrofti* as the filarial antigen source. Studies have shown that at the protein level there is a high (>90%) sequence identity across the filarial species that are pathogenic for humans [199].

In this study, I used *B. malayi* adult worms as well as *D. immitis* adult worms (dog heart worm) as surrogate systems for filarial antigen discovery. *B. malayi* worms were grown in Mongolian gerbil and because these worms are not fully grown they are as thin as hair and they are about 12

millimeters long. The main reason that I used canine filarial worm, *D. immitis*, as a source of antigen was because these worms are fully grown and large amounts of filarial proteins can be extracted from them. *D. immitis* adult female worms are 100 to 170 long and 4.6 to 6.3 mm thick, and *D. immitis* adult male worms are 50 to 70 mm long and 3.7 to 4.5 mm thick [200]. The adult male and female *D. immitis* parasites were obtained by surgical removal in severe cases of heartworm infection in canines (Figure 2-3)

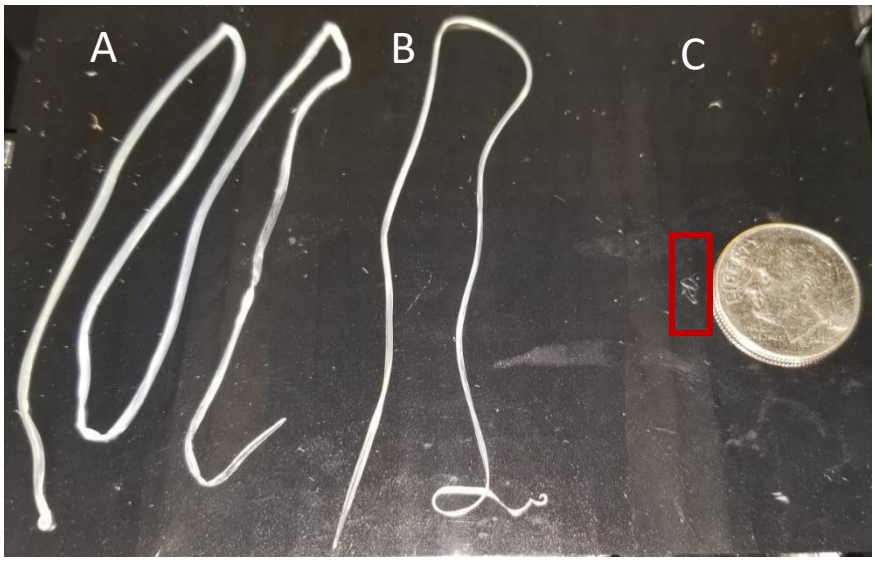


Figure 2-3. Male and female adult worms of *D. immitis* and female adult worm of *B.malayi*. A Dime is shown as a scale reference point. **A.** Adult female *D. immitis* (250 mm in length and 7.1 mm in diameter), **B.** Adult male *D. immitis* (136 mm in length and 5.1 mm in diameter) and **C.** Adult female *B.malayi*.

Various protein extraction methods were attempted to maximize the protein yield. Using Radioimmunoprecipitation assay buffer (RIPA buffer) in conjunction with sonication gave a high yield of protein but presence of RIPA buffer was interfering with the subsequent immunoprecipitation (IP) assays. Buffer exchange attempts using Zeba™ Spin Desalting Columns and Slide-A-Lyzer Dialysis Cassettes were not successful in removing RIPA buffer from antigen solution. In addition, I attempted using gentleMACS™ Tissue Dissociators but the protein yield

was insufficient. Finally, the method of choice was cryogenic grinding of filarial worms with liquid Nitrogen using a mortar and pestle and dissolving the fine powder in PBS buffer supplemented with protease and phosphatase inhibitors.

Filarial-binding Characterization of IgE MAbs from Patients with Filarial Infection

B cell lines that were positive in isotype-specific ELISA were selected for fusion with HMMA2.5 myeloma cells and IgE secreting hybridoma cell lines were generated. I utilized PBMCs collected from a total of 7 subjects to isolate a panel of 56 cloned hybridomas secreting IgE mAbs. I used two different sources of filarial antigens, *B.malayi* and *D.immitis* to make whole-worm somatic extracts. I tested the reactivity of IgE mAbs against these extracts using three complimentary assays immunoblotting, ELISA and *B. malayi* ImmunoCAP, to compensate for shortcomings of each screening methodology. Having IgE mAbs allows for far greater sensitivity than one would have using polyclonal serum.

A prototype *B.malayi* immunoCAP research use only (RUO) test was developed from the extract of *B. malayi* in collaboration with Thermo Fisher Scientific group. Its analytical characteristics were determined and an accelerated stability study was performed. ImmunoCAP platform is a highly sensitive gold standard method used by allergists for detection of IgE in serum of patients suspected to have allergy. The developed *B.malayi* immunoCAP test was used to screen the reactivity of human monoclonal IgE mAbs (with approximate concentration of 1-10ug/mL) against *B. malayi*. An antibody was considered positive if it bound to *B. malayi* in ImmunoCAP with signal >1.0 kUA/L. It should be noted that the assigned cutoff for ImmunoCAP positivity is much higher

than the standard cutoff for serum analysis because excess amount of IgE antibody is used and antigen concentration is the limiting factor.

Nine IgE mAbs (2E6, 4E9, 10D5, 7G12, 10H9, 14B2, 1A5, 11H12, and 5D2) showed very potent binding against filarial antigens, with immunoCAP values above 10 kUA/L. 9 IgE mAbs (1A5, 18H7, 14B2, 12D4, 11H12, 2E6, 5H1, 9C1 and 12C2) showed binding in Western blot against *B. malayi*, of which 5 mAbs 5H1, 9C1, 12C2, 18H7 and 12D4 were positive in Western blot analysis against *D.immitis* as well (Figure 2-4 A-B). As a point of reference some of the Western blot-negative antibodies are shown in Figure 2-4C. For IgE mAb binding to *B. malayi* and *D. immitis* extracts in ELISA, – indicates no binding detected, and + indicates binding between 2-10 times background, ++ is binding between 10-100 times background, +++ shows binding >100 times background. For mAb binding to filarial antigens in WB, – indicates no binding, and +++ indicates presence of a clear band on WB with minimal background.

In contrast to previous studies reporting the predominance of inflammation-driven 'nonspecific' IgE antibodies [173], 26 of the 56 human mAbs we generated were positive in at least in one of these screens, when tested at approximate concentrations of 1-10 μg/mL (Table 2-2 and Figure 2-5). Taken together this suggests that the IgE antibody response is highly specific to antigens of the infecting filarial worm, though, at the protein level there is a high (>90%) sequence identity across the filarial species that are pathogenic for humans. In addition, the percentage of filaria-specific IgE mAbs was highly variable among different patients probably due to the difference in their disease status as shown in Figure 2-6.

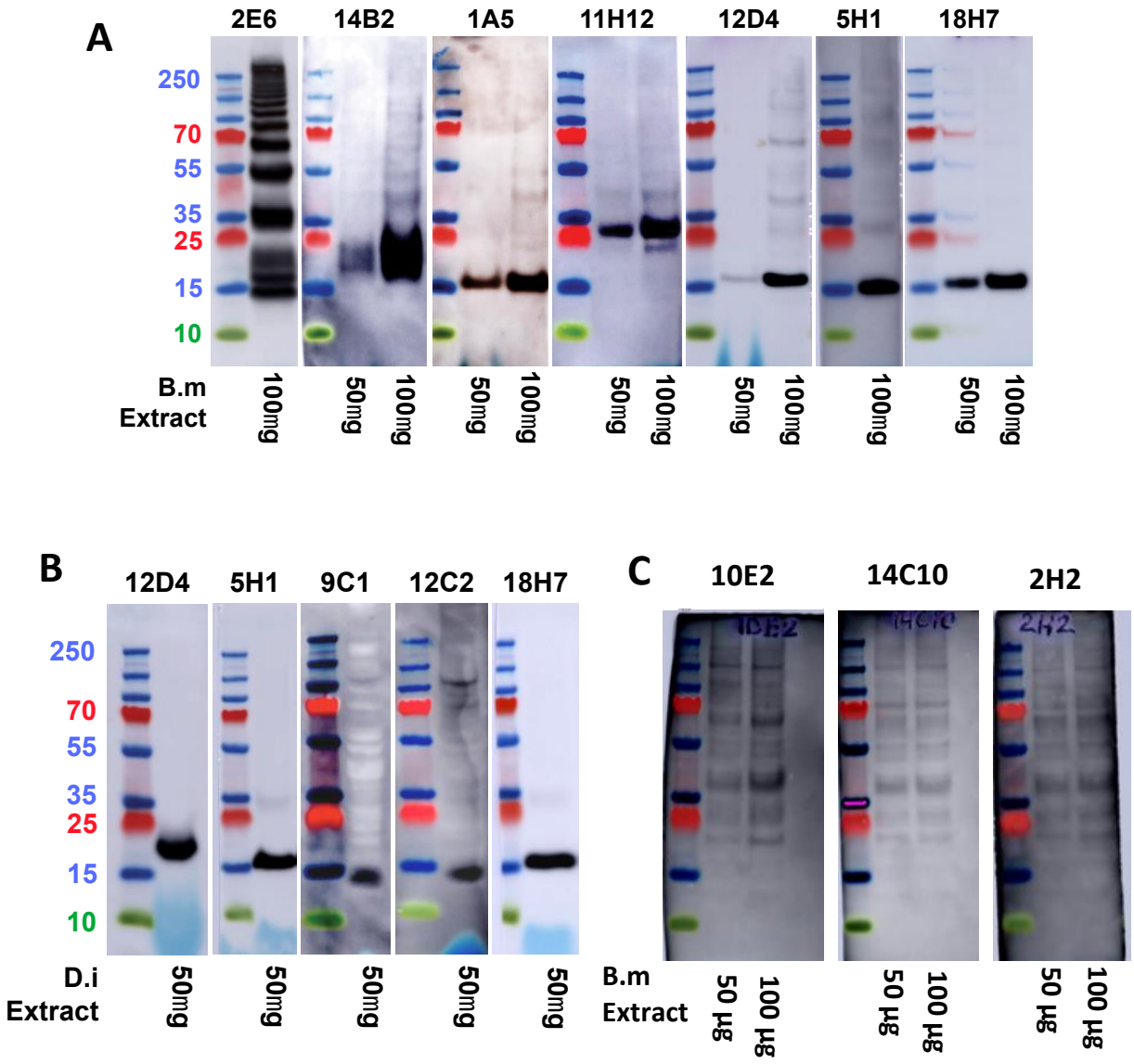


Figure 2-4. Western blot analysis of IgE mAbs binding whole-worm somatic extracts of *B. malayi* and *D. immitis*. A. Immunoblot analysis of IgE mAb binding to protein in somatic extracts from *B. malayi*. **B**, Analysis as in (B) using *D. immitis* extracts. **C,** Three IgE mAbs with no bindings to filarial antigens in Western blot analysis .Western blot background shows a ladder like pattern but no clear band is observed.

Table 2-2. Reactivity of IgE mAbs against *B. malayi* and *D. immitis*

Subject code	IgE hybridoma	*B. malayi* ImmunoCAP reactivity (kUA/L)	Binding to *B. malayi* extract		Binding to *D. immitis* extract		IP
			ELISA	Western blot	ELISA	Western blot	
P1	1A5	14.2	++	+++	+	-	+
	10H9	28.6	++	-	++	-	N/A
	11H12	12	++	+++	-	-	+
	14B2	23.9	+	+++	-	-	+
	7G12	29.3	+++	-	+	-	ND
	19E9	0.2	-	-	-	-	N/A
	23F10	0.5	+	-	-	-	N/A
	18H7	0.0	+	+++	+	+++	N/A
	4G8	2.2	++	-	-	-	N/A
	18D4	3.5	+	-	+	-	N/A
	17G5	0.1	+	-	-	-	N/A
	14C10	0.5	++	-	-	-	N/A
	22F5	0.0	-	-	-	-	N/A
	2E6	465.7	+++	+++	-	-	+
	15C1	0.1	-	-	-	-	N/A
	8A7	0.2	-	-	-	-	N/A
	9G6	2.9	+	-	-	-	N/A
	6D9	0.2	-	-	-	-	N/A
	15C10	0.2	-	-	-	-	N/A
	14F5	0.2	-	-	-	-	N/A
	5H1	1.4	++	+++	++	+++	+
	17G1	0.2	-	-	-	-	N/A
P2	11G1	4.8	+	-	+	-	+

P3	9C1	1.0	+	+++	+	+++	+
	5D2	11.9	+	-	-	-	ND
	2H2	0.1	+	-	-	-	N/A
	12D4	5.3	+	+++	+	+++	+
	4E1	0.5	-	+++	-	-	N/A
	10E2	0.1	-	-	-	-	N/A
	4E9	57.5	+++	-	+	-	+
	12C2	0.3	+	+++	+	+++	N/A
P4	16A4	0.1	-	-	-	-	N/A
	63C6	0.3	-	-	-	-	N/A
	30A10	2.5	+	-	+	-	+
	10E7	0.3	-	-	+	-	N/A
	47D1	0.1	-	-	-	-	N/A
	54C5	0.1	-	-	-	-	N/A
	20B12	0.1	-	-	-	-	N/A
	38C2	0.2	-	-	-	-	N/A
	40F1	0.1	-	-	-	-	N/A
	58E8	0.2	-	-	-	-	N/A
	38D12	0.1	-	-	-	-	N/A
	5A8	0.1	-	-	+	-	N/A
P5	13A11	0.1	-	-	-	-	N/A
	5C1	0.4	-	-	-	-	N/A
	16B5	0.4	-	-	-	-	N/A
	11D5	0.1	-	-	-	-	N/A
	10D5	31.0	++	-	-	-	+
P6	18A3	0.1	-	-	-	-	N/A
P7	1G1	0.2	-	-	-	-	N/A

	2C7	0.3	-	-	-	-	N/A
	4E3	0.6	-	-	-	-	N/A
	2G4	0.3	-	-	-	-	N/A
	3A7	0.3	-	-	-	-	N/A
	4H3	0.2	-	-	-	-	N/A

All IgE mAbs were tested against B. malayi and D. immitis in ELISA and WB as well as B. malayi ImmunoCAP. IgE mAb binding to B. malayi and D. immitis extracts in ELISA (– no binding detected; + binding 2-10 times background; ++ binding 10-100 times background; +++ binding >100 times background). IgE mAb binding to B. malayi and D. immitis extracts in Western blot (– no binding detected; +++ presence of a clear band). The B. malayi ImmunoCAP was considered positive if > 1 kUA/L. If binding was detected to lysate by any assay, IP of the target antigen was attempted from crude worm lysate using mass spectrometry. A target antigen was considered positive if representing >10 times that precipitated by a control IgE mAb. N/A indicates that IP was not attempted. ND, not determined; kUA, allergen-specific kilo-units of antibody.

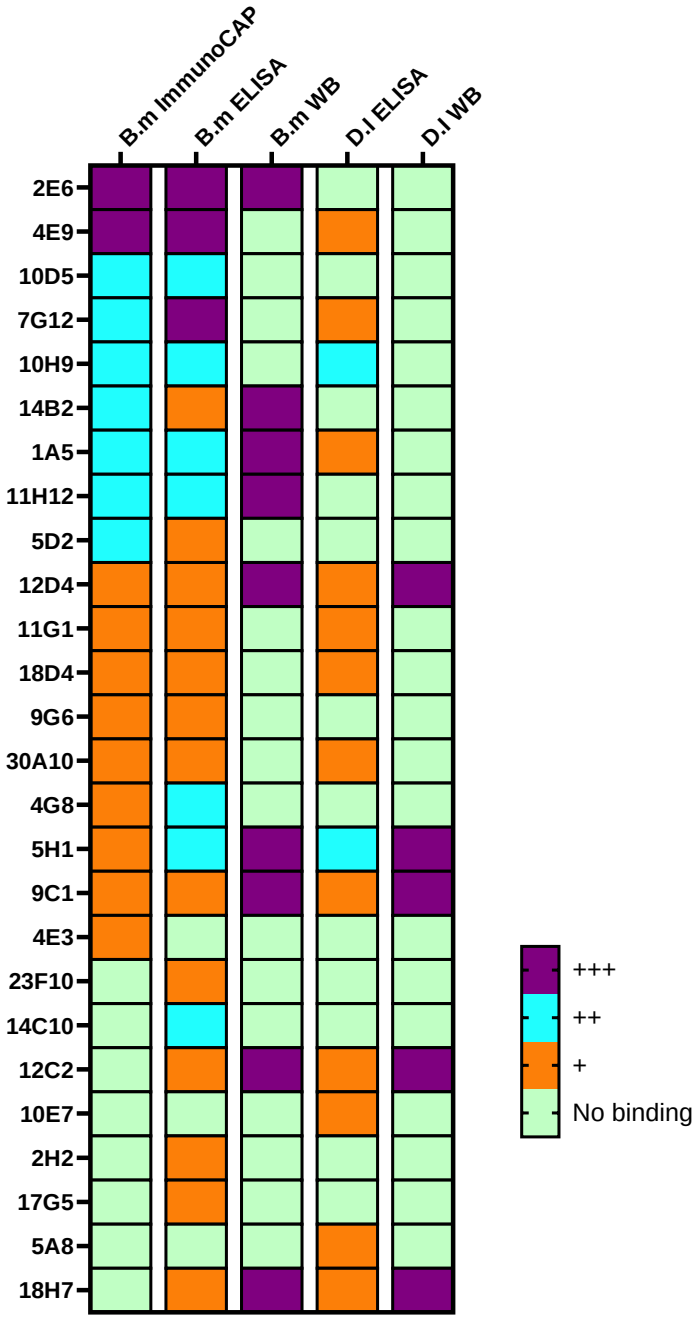


Figure 2-5. Heatmap representing filaria-specific IgE mAbs binding score in different assays. The antibodies are arranged in the order of their binding score in *B. malayi* ImmunoCAP assay with the most potent antibodies on the top. The scoring criteria is the same as Table 2-2.

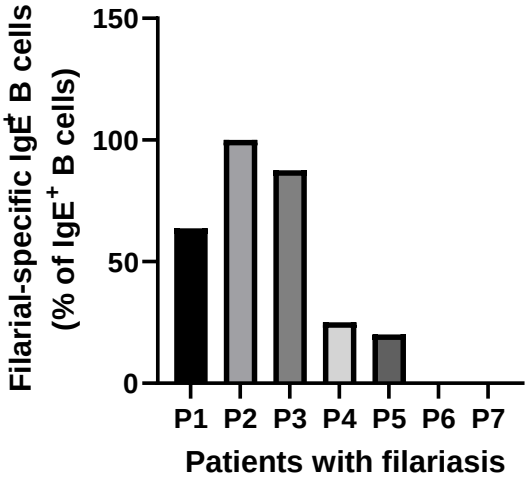


Figure 2-6. Percentage of filaria-specific IgE+ B cell in patients with filariasis. Filarial-specific IgE+ B cell frequencies are expressed as the number of IgE mAbs showing filarial binding divided by the total number of IgE mAbs obtained from that patient.

Of note, of the 26 filaria-specific IgE mAbs, 15 were obtained from the two subjects with TPE (15 of their 23 mAbs, 65%). Because *W. bancrofti* (which is responsible for 90% of LF infections), *Loa loa* and *Onchocerca volvulus* cannot be maintained in conventional mouse strains, we could not screen our IgE mAbs against these filarial somatic extracts and thus could not identify Wuchereria-specific, Onchocerca-specific, or Loa-specific IgE mAbs in our panel. Of the 26 filaria-specific IgE mAbs, only 13 showed cross-reactivity towards dog heartworm. Taken together this suggests that the IgE antibody response is highly specific to antigens of the infecting filarial worm, though, at the protein level there is a high (>90%) sequence identity across the filarial species that are pathogenic for humans. The co-endemicity of the filarial worms with other parasitic worms can also be an explanation for presence of IgE mAbs not reactive to filarial worms. To further investigate the specificity of the filariasis-associated antibody response, we focused subsequent efforts on antibodies which showed reactivity to filarial worm lysate in multiple assays (Figure 2-5).

Cross-reactivity Analysis of IgE MAbs from Patients with Filarial Infection against Common Allergens

As there are a lot of studies reporting cross-reactivity of IgE from patients with parasitic infection with allergens, I sought to address whether the IgE antibodies obtained from filaria-infected patients cross-react with common allergen proteins [201]. To examine the cross-reactive nature of antibodies secreted by B cells, IgE mAbs were tested using ISAC allergen microarray, containing 112 purified allergens, which suggests that the IgE antibody response induced during filarial infection is highly filarial-specific (Figure 2-7). Only two IgE mAbs, 5D2 and 4G8 showed weak binding to several allergen proteins in the immunoCAP ISAC assay, suggesting poly-reactivity to a carbohydrate epitope.

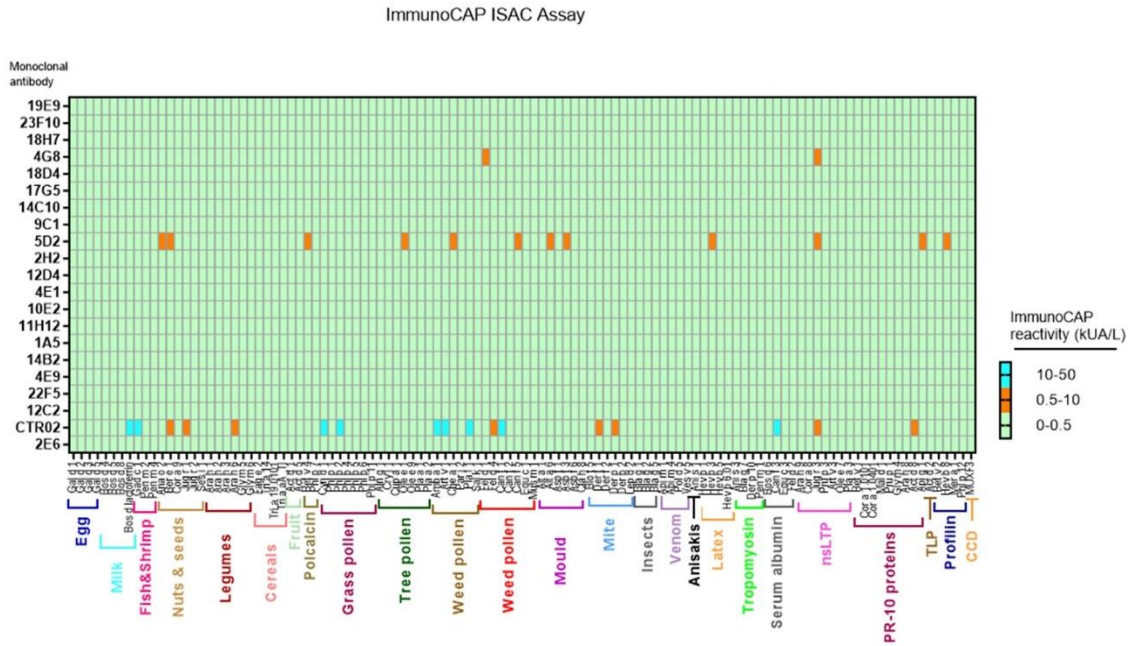


Figure 2-7 Cross-reactivity analysis of IgE mAbs from patients with filarial infection against common allergens. Heatmap of IgE binding in ISAC allergen array. IgE mAbs were used at estimated concentration of 1-10 ug/mL against 112 common allergens. CTR02 is a positive control used for immunoCAP ISAC assay.

Genetic Features of Filaria-Specific Human IgE MAbs

To evaluate sequence diversity and degree of somatic hypermutation, we performed sequence analysis of 16 filaria-specific IgE hybridoma clones (Fig 2-8). That analysis revealed 16 unique Vh–Jh–CDRH3–Vl–Jl–CDRL3 clonotypes (Table 2-3). Filaria-specific IgE antibodies showed a wide range of antibody variable region (VH and VL) gene usage, with no statistically significant over-representation of any germline gene (Figure 2-8A). IgE B cells had a similar use of λ versus κ light chains when compared to other antibody isotypes, with 8 antibodies using κ light chains and 8 antibodies using λ light chain. The length distribution of VH and VL complementarity-determining region 3 (CDR3) amino acids in IgE B cells is similar to other antibody isotypes[202] (Figure 2-8B). Filaria-specific IgE mAbs exhibited a high degree of somatic hypermutation (SHM) in the VH and VL region, and no correlation was observed between the VH and VL mutation frequency of an antibody (Figure 2-8C). The VH and VL of filaria-specific monoclonal antibodies contained on average 23 and 19 SHMs, respectively.

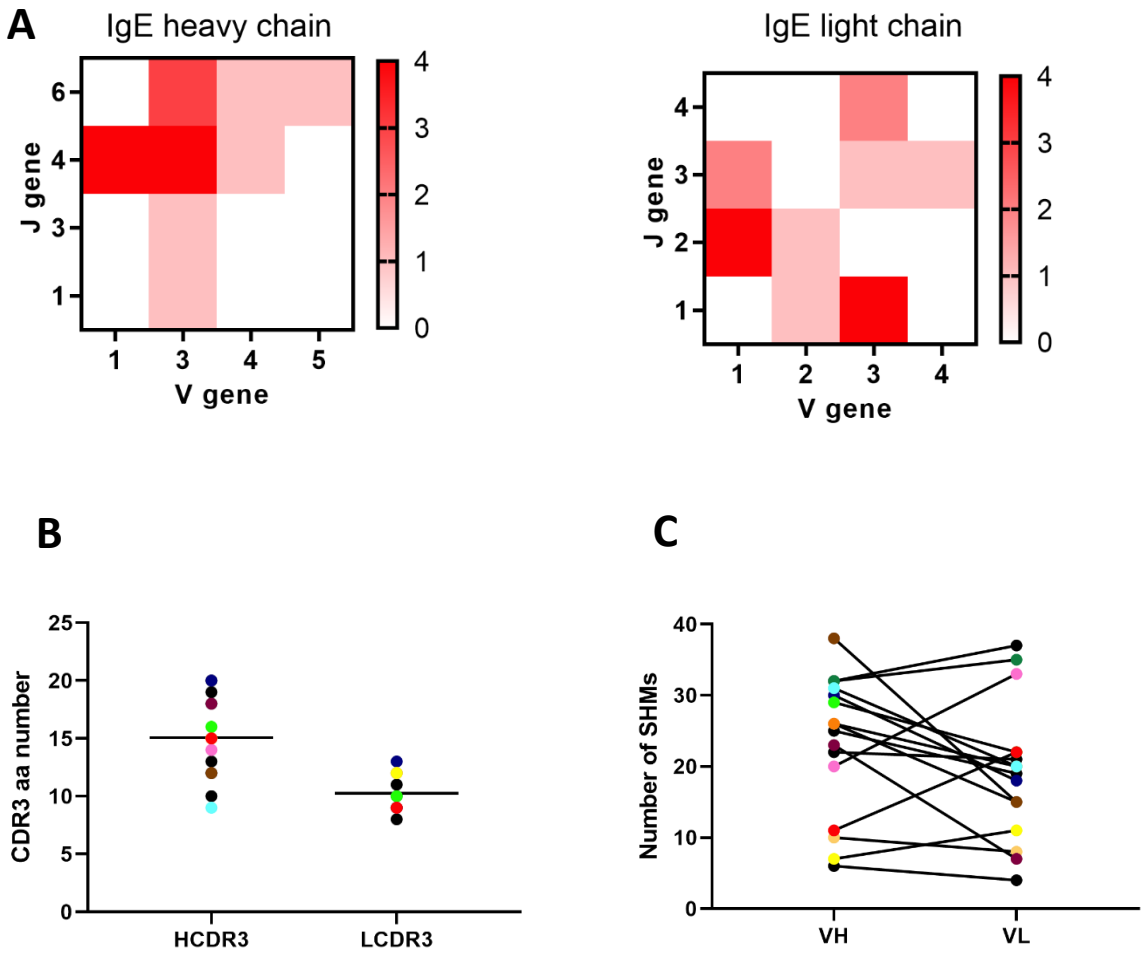


Figure 2-8. The IgH and IgL gene sequence analysis of the filaria-specific IgE antibodies. Genetic features of 16 filaria-specific IgE antibodies with unique sequences are reported. **A.** Heatmap showing the total number of IgE antibodies with the corresponding V and J genes. **B.** CDR3 amino acid length distribution for heavy and light chains with indicated mean (n = 16). IgH and IgL CDR3 amino acid number of the antibodies were determined using ImMunoGeneTics (IMGT) database. **C.** Number of SHMs in the VH and VL chain of filaria-specific IgE mAbs.

Table 2-3. Human IgE variable gene sequence characteristics. Antibody germline gene segment usages and their percent identity are shown for variable (V), diverse (D), and joining (J) regions of both light and heavy chains based on the ImMunoGeneTics (IMGT) database. The number of amino acids that make up the CDRs (HCDR & LCDR length) and the amino acids making up the junction is shown.

		Heavy chain							Light chain					
IgE MAb	Light	V gene	V gene % identity	J gene	J gene % identity	D gene	Junction	HCDR length	V gene	V gene % identity	J gene	J gene % identity	Junction	LCDR length
4E1	κ	*V1-46*01 F, or V1-46*03 F*	96.18	*J4*02 F*	93.75	*D3-22*01 F*	CATLYYYDDSGYYTDYW	8.8.15	*V3-20*01 F*	92.2	*J1*01 F*	100	CQQYGNSPWTF	7.3.9
12D4	λ	*V1-45*02 F*	97.57	*J4*02 F*	93.75	*D3-22*01 F*	CAVAYHYDSSGPYFDHW	8.8.15	*V1-40*01 F*	96.18	*J2*01 F, or J3*01 F*	94.74	CQSYDSSLSGPVVF	9.3.12
5H1	κ	*V3-30*03 F, or V3-30*18 F or V3-30-5*01 F*	90.28	*J4*02 F*	85.11	*D3-16*01 F*	CAKDGPYAYRWGGFLDDW	8.8.16	*V3-15*01 F*	92.47	*J4*01 F*	100	CQQYHSWPPLTF	6.3.10
9C1	κ	*V3-23*01 F, or V3-23D*01 F*	92.01	*J6*02 F*	79.03	*D6-19*01 F*	CAKQKRECSGWCKGWGLDVW	8.8.18	*V2-28*01 F, or V2D-28*01 F*	97.62	*J2*01 F*	97.3	CMQALQTPYTF	11.3.9
14C10	λ	*V3-21*01 F*	93.06	*J3*02 F*	96	*D4-17*01 F*	CARDTYGDTDDAFDIW	8.8.14	*V3-25*03 F*	89.25	*J3*02 F*	89.47	CHVVDRTETYRSVF	6.3.12
7G12	κ	*V1-18*01 F*	89.24	*J4*02 F*	82.98	*D5-12*01 F*	CARGRDSPDHW	8.8.9	*V1-5*03 F*	92.83	*J2*01 F*	86.84	CQQYDHFPHTF	6.3.9
1A5	λ	*V3-30*03 F, or V3-30*18 F or V3-30-5*01 F*	96.88	*J4*02 F*	56	*D3-22*01 F*	CAKAMDDSSGYYCPDYW	8.8.15	*V3-19*01 F*	97.13	*J1*01 F*	100	CNSRDSSGNHLYVF	6.3.12
10H9	λ	*V3-11*06 F*	89.93	*J1*01 F*	66.67	*D2-8*02 F*	CAKDYCGSGACYTADPGFFHQW	8.8.20	*V3-21*01 F*	93.91	*J1*01 F*	94.74	CQVWDNTNDHPSYVF	6.3.13
11G1	λ	*V5-51*03 F*	88.89	*J6*02 F, or J6*04 F*	58.14	*ND*	CAREVYVASTDSDYYGMDV	8.8.18	*V2-23*01 F, or V2-23*02 F or V2-23*03 F*	88.19	*J1*01 F*	86.11	CCSYAGGNTYVF	9.3.10
14B2	κ	*V3-53*02 F*	87.37	*J4*02 F*	74.07	*D5-24*01*	CVRHGDGWNYVDSW	8.7.12	*V3-15*01 F*	94.62	*J4*01 F*	71.05	CQQYNNWPLTF	6.3.9
10D5	κ	*V3-21*01 F*	90.97	*J6*03 F*	83.87	*D3-22*01 F*	CARVMVVGLYYYYMDIW	8.8.15	*V3-20*01 F*	94.68	*J1*01 F*	97.37	CQLYGTSPTWTF	7.3.10
18D4	κ	*V4-34*02 F, or V4-34*08 F*	91.23	*J4*02 F*	77.08	*D1-1*01 F*	CGRGRGYTWNDW	8.7.10	*V1-39*01 F, or V1D-39*01 F*	93.55	*J2*01 F, or J2*02 (F)*	88.57	CQQSYSGPPTF	6.3.9
30A10	κ	*V3-21*01 F*	97.92	*J6*03 F*	87.1	*D6-13*01 F*	CARDGPPIVAAGLIYYYYMGVW	8.8.20	*V1-9*01 F*	98.57	*J2*02 (F)*	97.22	CQHLNSYRTF	6.3.8
4E9	λ	*V4-34*02 F*	92.28	*J6*02 F*	93.55	*D6-13*01 F*	CASSGESSRRGYFYYYGMDVW	8.7.19	*V1-47*01 F, or V1-47*02 F*	92.6	*J3*02 F*	89.47	CAAWDDILSGPRVF	8.3.12
2E6	λ	*V1-69*01 F*	91.32	*J4*02 F*	100	*D5-18*01 F*	CASYPYGKYYFDYW	8.8.12	*V1-51*01 F*	94.39	*J3*02 F*	97.14	CGAWDLGLNAGVF	8.3.11
11H12	λ	*V3-11*01 F*	89.24	*J4*02 F*	85.11	ND	CARDWGTTLVTFDLW	8.8.13	*V4-60*03 F*	86.74	*J3*02 F*	75.68	CFTWDSDSRVF	7.7.9

Discussion

Human hybridoma technology is a versatile method extensively used to study human antibody repertoire. Although high throughput mAb generation technologies have improved the antibody production yield, hybridomas are still one of the most favored method as it preserves the native pairing and antibody sequence information in the B cells. Reliable protocols have long been available for using human hybridoma technology for mAb generation against various antigens. Yet hybridoma method applicability in producing full-length human IgE mAb has never been reported. The IgE production methods that are currently available fail to represent naturally occurring human response against parasites or allergens, as they are produced using mouse hybridoma cell lines, or are artificially class switched by adding IL-4 to the culture system. One of the main obstacles to generate IgE producing human hybridomas was the technical difficulty to isolate and expand desired populations of IgE^+ B cells. Isolation of IgE^+ B cells using flow cytometry sorting is not feasible due to the low level of IgE expression on the surface of IgE^+ B cells and presence of low affinity FcεRII on the surface of other B cells and basophiles. It is shown that there is larger amount of IgE on the surface of basophils ($CD19^- IgE^+$) compared to IgE^+ B cells, which are producers of IgE antibodies [203]. A recent study using flow cytometry to gate for IgE^+ B cells shows that only 29.5% of cells in the $CD19^+IgM^-IgG^-IgE^+$ gate were actually IgE producing B cells as determined by antibody heavy chain transcript assembly [203]. I have modified existing hybridoma protocols to develop a reliable method to generate human IgE-producing hybridomas, from this very rare population of B cells in peripheral blood of infected subjects. The procedure consists of IgE B cell isolation, unbiased B cell culture, hybridoma formation, rounds of selection, single cell indexing, large scale production, and antibody purification. The method takes about 2-3 months, depending on the growth rate of hybridomas.

The ability to produce IgE mAbs using hybridoma method allows for better characterization of human antibody response to parasitic worms, antigen discovery, therapeutic purposes, etc.

The IgE^+ B cell frequency among $CD19^+$ B cells was previously reported to be in the range of 1 per 10^6 to 5×10^5 B cells in patients with peanut allergy [179]. Our data demonstrates that IgE expressing B cells are present with a frequency ranging from 6 to 14 cells per 10 million PBMCs in patients with filarial infection. As 5-10% of PBMC is composed of B cells, the numbers would translate into one IgE^+ B cell per 10^5 B cells. This number is slightly lower than the number of IgE expressing B cells we previously reported for subjects with allergic bronchopulmonary aspergillosis, 13 to 16 IgE^+ B cells per 10 million PBMCs [178].

I report here the characterization of first panel of human IgE mAbs obtained from patients with filariasis. 26 of the 56 human IgE mAbs we generated exhibited binding to filarial antigens in at least one of the ELISA, western blot and *B.malayi* ImmunoCAP assays, when tested at approximate concentrations of 1-10 μg/mL. Nine IgE mAbs showed very potent binding against *B.malayi* antigens in immunoCAP assay with values above 10 kUA/L. Nine IgE mAbs showed binding in Western blot against *B. malayi* antigens and 23 IgE mAbs were positive in ELISA. The variability observed in binding results from different assays further confirms the importance of performing multiple binding assays and using different protein extraction methods to compensate for the flaws of each methodology. Of note, because *W. bancrofti* (which is responsible for 90% of LF infections), *Loa loa* and *Onchocerca volvulus* cannot be maintained in conventional mouse strains, we could not screen our IgE mAbs against these filarial somatic extracts and thus could not identify *Wuchereria*-specific, *Onchocerca*-specific, or *Loa*-specific IgE mAbs in our panel. Of the 26 filaria-specific IgE mAbs only 13 showed cross-reactivity towards dog heartworm, which further confirms that despite high level of sequence identity (>90%) across the filarial species that

are pathogenic for humans, IgE antibody response is highly specific to antigens of the infecting filarial worm. The co-endemicity of the filarial worms with other parasitic worms can also be an explanation for presence of IgE mAbs that are not reactive to filarial worms. Collectively, these results suggest that the majority of IgE antibodies obtained from patients with filarial infection were targeting specific filarial antigens. So, prior studies reporting IgE antibodies as being 'off-target' or 'nonspecific' were probably limited by the quantity of IgE antibodies present in the serum of patients [173].

It has long been noted that there are similarities between allergy and the immune response to parasitic worms (helminths). The immune response induced in both cases is strongly Th2-skewed with elevated levels of IgE antibodies and eosinophilia [204]. An important question is how does helminth induced IgE response relate to allergy? Recent research on immunology of helminth infection has been focused on establishing the link between helminthic disease and allergy. Their main argument is that there are homologues of nearly all known allergens in metazoan parasites, so theoretically they could be bound by the same parasite-specific IgE molecule. A study has shown that allergic patient serum bound to the components in Ascaris worm extract, confirming that cross-reactivity exists between Ascaris worm and mites. The observed cross-reactivity was associated primarily with tropomyosin and glutathione-S-transferase.[205]. Another study has shown cross-reactivity between a major glutathione-S transferase allergen of cockroach and the protein homolog found in *Wuchereria bancrofti* using polyclonal serum from patients with filariasis [206]–[208]. However, our results does not show any cross-reactivity between filarial-specific IgE mAbs and common allergens as tested in an ISAC allergen microarray, containing 112 purified allergens. Only two IgE mAbs, 5D2 and 4G8 showed weak binding across several

allergen proteins in the ISAC assay, which can be attributed to their carbohydrate binding capability.

Finally, analysis of antibody heavy chain and light chain sequences of filaria-specific IgE antibodies did not show statistically significant over-representation of any germline gene, similar to the results from prior study showing that 89 IgE antibodies obtained from peanut allergic patients varied widely in antibody heavy chain germline [179]. The length distributions of CDR3 amino acids in the heavy and light chains of filaria-specific IgE mAbs were similar to what is reported for other isotypes [202]. The fact that filaria-specific IgE antibodies are highly mutated suggests a prolonged and repeated antigen exposure in patients during infection.

In summary, my data shows that most of the IgE antibody produced in filaria-infected hosts are targeting filarial immunogenes, despite prior research reporting these antibodies as nonspecific polyclonal IgE with no reactivity towards parasitic antigens [209].

Methods

Data reporting. The sample size was not predetermined. Experiments were performed in unblinded fashion and were not randomized. Data analysis and outcome assessment were performed in unblinded fashion as well.

Study approval. PBMCs were collected at the NIH facility under supervision of Dr. Thomas Nutman after written informed consent from patients with prior filarial worm infection. The study and protocols were approved by the Institutional Review Board of Vanderbilt University Medical Center (IRB 170308).

Research subjects. We analyzed seven subjects with a clinical history of filarial worm infection. Two patients were diagnosed with tropical pulmonary eosinophilia (TPE), four patients with loiasis and one patient with onchocerciasis. Relevant clinical information is summarized in Table 2-1.

PBMC isolation and hybridoma generation. PBMCs were obtained by leukapheresis from patients and were cryopreserved and transferred to Vanderbilt University Medical Center by our collaborator, Dr. Thomas Nutman at NIH facility. Cells remained stored in liquid nitrogen until use. For hybridoma generation cryopreserved samples were thawed rapidly in a 37°C water bath and washed in 10 ml of prefusion medium (ClonaCell-HY 03801; Stemcell Technologies). Trypan blue staining (Gibco 15250-061) was used to determine cell viability for PBMC. Cells were counted and plated at the approximate density of 2 million viable cells / plate into a 96-well flat bottom plate (Falcon, 353072) in the presence of 1 million gamma-irradiated NIH3T3 feeder cells genetically engineered to constitutively express human CD40L, IL-21 and BAFF (kindly provided by Dr. Deepta Bhattacharya; University of Arizona). Cells were incubated at 37°C with 5% CO_2 for 6 days and then supernatants were screened for the presence of IgE antibodies using an

optimized isotype-specific sandwich enzyme-linked immunosorbent assay (ELISA). Next, the positive wells containing IgE secreting B cells were immortalized through electrical cytofusion (BTX electrofusion device) with a non-secreting myeloma partner, HMMA2.5 myeloma cells (kindly provided by Marshall Posner), to produce an IgE-secreting hybridoma cell [210]. To select for IgE-secreting hybridoma cells, fusion products were plated at 50 μl/well into 384-well plates (Nunc, 164688) containing HAT selection medium. After incubating plates at 37°C for 14 days they were screened for IgE antibody production by ELISA. After several rounds of limiting dilution plating indexed single-cell flow cytometric sorting was employed to obtain a single clone of hybridoma. Once clonality was achieved, IgE mAbs were expressed in serum-free medium (Gibco Hybridoma-SFM; Invitrogen, 12045084). Immunoaffinity chromatography using the therapeutic monoclonal antibody Omalizumab (Xolair) covalently coupled to GE Healthcare NHS activated HiTRAP (17-0717-01) was employed for the purification of IgE antibodies. Purified mAbs were quantified using UV 280 nm spectrophotometry ona NanoDrop spectrophotometer and setting the extinction coefficient for IgE antibody.

IgE screening ELISA. IgE producing B cells were detected using an optimized isotype-specific sandwich enzyme-linked immunosorbent assay (ELISA). Wells of a 384-well black ELISA plates (Greiner, 781076) were coated with Omalizumab, as a capture antibody, at a concentration of 10 μg/ml in carbonate binding buffer overnight at 4°C. 100 μL of blocking solution (5% nonfat dry milk plus 2% goat serum in PBS) was added to each well and plates were incubated at room temperature for 1 h. After 5 washes with PBS, 100 μL of supernatant was transferred from each well of the 96-well plate containing B-cell lines and plates were incubated for 1h incubation at room temperature. After 5 washes with PBS, Secondary antibody (mouse anti-human IgE Fc; Southern biotech, 9160-05) was added at a 1:1,000 dilution in blocking solution using 25 μl/well,

for 1h incubation at room temperature. After 10 washes with PBS, fluorogenic peroxidase substrate solution (QuantaBlu; Thermo Scientific 15162) was added at 25 μL/well, as per manufacturer instructions, and the plates were incubated at room temperature for 30 min before addition of stop solution. Relative fluorescence intensity was determination on a Molecular Devices plate reader set for excitation and emission maxima of 325 nm and 420 nm, respectively.

Parasite materials. Adult parasites, infective larvae (L3), L4 larvae and microfilariae of *B. malayi* were obtained from the NIAID/NIH Filariasis Research Reagent Repository Center (FR3; Athens, GA; www.filariasiscenter.org). Additional adult *B. malayi* were purchased from TRS Labs, Athens, GA. *D. immitis* worms were also used as a source of filarial antigens. The *D. immitis* parasites were obtained from surgical removal in severe cases of heartworm infestation in canines. Adult male and female *D. immitis* worms were kindly provided by Dr. Heather Stockdale Walden; University of Florida College of Veterinary Medicine. Soluble antigen from adult *B. malayi* and *D. immitis* was prepared by grinding frozen worms with liquid Nitrogen to a fine powder using a mortar and pestle (cryogenic grinding). The homogenized powder was suspended in phosphate buffer saline (PBS 0.05 M, pH 7.2) containing Halt protease inhibitor cocktail (Thermo Scientific, 78429) rocking for 1 hour at 4°C. Proteins soluble in PBS were recovered by centrifugation (15,000×g) at 4°C for 30 min. Protein concentration was measured by absorbance at UV280 nm. Filarial worm extracts were aliquoted and stored at -20°C until use.

Western blot analysis. Filarial worm extract was mixed with loading buffer, separated by SDS-PAGE on a 4-12% gradient gel (Thermo Fisher Scientific, NP0322BOX) along with a prestained protein ladder (Thermo Fisher Scientific 26616) under nonreducing and nondenaturing conditions. The antigen components were electrophoretically transferred to PVDF membranes (Thermo Fisher Scientific, LC2005) at 40 V for 150 min. After blocking with 5% nonfat dry milk in PBS overnight

at 4°C, membranes were incubated with IgE mAbs (at a concentration of 1-10 μg/mL) for 1 h at room temperature. Bound antibodies were probed with murine anti–human IgE-peroxidase conjugate secondary antibody (Southern biotech, 9160-05) at 1:1000 dilution in blocking solution and were incubated for 1 h at room temperature. Blots were washed 3 times with PBS between each step. Blots were visualized using chemiluminescent substrate (Supersignal Pico; Thermo Scientific, 34577) on an Amersham™ Imager 600.

ELISA binding assays. Antibody reactivity to filarial antigens was tested using ELISA. Wells of 384-well clear ELISA plates (Greiner, 781061) were coated with filarial worm extract (with estimated concentration of 10 μg/mL) at 4°C overnight. Plates were blocked with 100 μl of blocking solution/well (5% nonfat dry milk and 2% goat serum in PBS) and were incubated at room temperature for 1 h. IgE mAbs (with estimated concentration of 1-10 μg/mL) were added for a 1 h incubation at room temperature. Bound antibodies were detected using an HRP-conjugated mouse anti–human IgE Fc secondary antibody (Southern biotech, 9160-05, 1:1000 dilution) and a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific). Color development was measured at 390 nm after 10 min using a Molecular Devices plate reader. An antibody was considered filaria-reactive if it bound to filarial extracts with an optical density (OD) greater than three times background.

***B. malayi* ImmunoCAP analysis**. Prototype *B. malayi* ImmunoCAP tests were developed for research use from *B. malayi* somatic extract. Analytical characteristics of ImmunoCAP tests were determined and an accelerated stability study was performed. The test was used to screen reactivity of human monoclonal IgE mAbs (with approximate concentration of 1-10ug/mL) against *Brugia malayi*. An antibody was considered positive if it bound to *B. malayi* in ImmunoCAP (>1.0 kUA/L). It should be noted that the assigned cutoff for ImmunoCAP positivity is much higher than

the standard cutoff for serum analysis because excess IgE antibody is used and antigen concentration is the limiting factor.

Antibody heavy and light chain variable gene sequence analysis. The nucleotide sequence for heavy and light chain of each filaria-reactive IgE mAb was determined. After RNA extraction from IgE-expressing hybridoma clones (approximately 1 million cells) using an RNeasy kit (Qiagen: 74104), RT-PCR

amplification of antibody gene cDNAs was done using OneStep RT-PCR kit (Qiagen: 210210).The 5′ primer set used here was described previously [211] and 3′ primer was specific for IgE constant region. The PCR products for antibody heavy or light chain genes were cloned individually into the pCR2.1 using a TA cloning kit (Invitrogen: 45-0046) and were Sanger sequenced. There were 16 filaria-specific IgE mAbs with unique nucleotide sequences that were analyzed using IMGT database for V/D/J gene usage, CDR3 length and somatic mutation.

CHAPTER III

IDENTIFICATION OF MAJOR FILARIAL ANTIGENS TARGETED BY HUMAN IGE ANTIBODY RESPONSE

Introduction

According to WHO estimates, more than 1.5 billion people (>24% worldwide) are living with some type of soil-transmitted helminth infection. This neglected tropical disease creates significant social and economic burdens for developing countries where helminth infections are endemic. Individuals in the endemic area suffer from constant re-infection and there is no FDA-approved anti-helminth vaccine available. In general, vaccine development against a pathogen is only possible if protective immunity is naturally developed soon after early exposure(s) to it, and if a high antibody titer is produced during that exposure. As there is evidence that acquired immunity is developed naturally against helminth parasites [151], vaccination could be the solution to this public health issue. . Natural protective immunity is normally observed against L3, the infective larval stage of filarial worms, while MDA treatments are primarily directed at the microfilariae and adult worms. Thus, in order to effectively control helminth infections a complementary treatment option of vaccines and anti-helminth medicines can target both existing adult helminthes and infective Larvae.

a combined approach of using drugs to kill existing adult helminthes, and vaccination to target newly encountered infectious larvae might offer a viable solution [163]. The main obstacles in developing an efficient vaccine against filarial worms are 1) lack of enough understanding of the filaria-specific immune response, 2) high degree of antigenic polymorphism and limited knowledge about immunodominant antigens in filarial worms, 3) lack of a suitable animal model of human filarial infections for vaccine testing, 4) Uncertainty about the impact of pre-existing immune reactivity on vaccination efficacy as it might induce an allergic reaction.

In this chapter, I present the identification of major filarial antigens, discovered by isolation and study of filaria-reactive naturally occurring human IgE mAbs, designated 12D4, 4E1, 2E6, 4E9, 11H12, 9C1, 5H1 and 1A5, which were isolated from three patients with prior history of laboratory-confirmed filarial infection and two patients with clinical diagnosis of TPE. So, my research aim was to take an unbiased approach to identify the main filarial antigens targeted by human IgE response. IgE antibodies that showed binding to filarial worm extract in more than one assay (ELISA, Western blot or ImmunoCAP) were further pursued for antigen identification. An optimized Immunoprecipitation (IP) protocol was developed to capture antigens specifically, followed by Mass Spectrometry analysis (IP/MS).

MAb 4E1 and 12D4 recognize WbSXP-1 protein, EC_{50} concentrations were calculated for each antibody. Remarkably, binding studies revealed that 12D4 shows broad cross reactivity towards SXP-1 proteins from all filarial worms tested; *W. bancrofti*, *B. malayi* and *D. immitis*, while 4E1 only recognizes WbSXP-1 protein. Despite these two IgE mAbs targeting distinct, noncompeting epitopes on WbSXP-1, they were not able to function together and initiate mast cell degranulation in transgenic mouse model of anaphylaxis, which can be attributed to unfavorable spatial arrangement of antibodies, possibly bound to distant antigenic sites on WbSXP-1.

MAb 2E6 recognizes a ladder like antigen in *W. bancrofti* with high affinity (EC_{50} = 7.7 ng/mL). Interestingly this antigen is a secretory protein detected in the culture media of adult worms. MAb 4E9 recognizes a homologue of human cytokine Macrophage Migration Inhibitory factor (MIF), which has an immunomodulatory function. Another interesting filarial antigen that I discovered is a 24kDa secretory protein with no implied function, targeted by 11H12 IgE mAb. Finally, three IgE mAbs recognize a family of proteins that is introduced here for the first time as major antigen of filarial worm infection. Interestingly, all filarial antigens identified as inducers of human IgE

response are excretory-secretory (ES) products of the filarial parasites. Recognition of these previously unrecognized filarial antigens provides new target proteins for incorporation into rational design of vaccine candidates or to develop diagnostics.

I acknowledge Dr. Thomas Nutman, NIH, Bethesda, for processing and providing *B. malayi* whole-worm extract used for my study, Dr. Heather Stockdale Walden; University of Florida College of Veterinary Medicine for providing adult male and female *D. immitis* worms, NIAID/NIH Filariasis Research Reagent Repository Center (FR3; Athens, GA; www.filariasiscenter.org) for providing *B. malayi* worms, Digital Proteomics LLC for help with mass spectrometry bioinformatics, Dr. Benjamin Spiller; pharmacology department at Vanderbilt University Medical Center for his help with expression of recombinant filarial antigens, Dr. Stokes Peebles and Jian Zhang for performing the *in vivo* experiments, and past members of the Smith laboratory for developing and optimizing the assays that allowed me to characterize large panels of human IgE mAbs.

Optimized IP/MS Procedure

IP is a biochemical technique used to isolate a protein from biological samples based on antibody-antigen interaction. I tested different methods of IP, using magnetic beads that are epoxy coated and magnetic beads that are streptavidin coated. Comparing the results showed that epoxy coated Dynabeads provide fast and efficient covalent coupling of antibodies and result in an ultralow nonspecific binding. Using biotinylated antibodies in combination with streptavidin-coupled Dynabeads did not improve the results, so all future IPs were done using Dynabeads® M-270 Epoxy beads. As mentioned earlier using Radioimmunoprecipitation assay buffer (RIPA buffer)

in preparation of worm protein extract interfered with antibody-antigen interaction during IP process, probably due to the presence of two ionic detergents 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS) in the formulation (Figure 3-1). Therefore, all whole-worm extracts used for IP as antigen source were obtained from cryogenic grinding of *B. malayi* and *D. immitis* worms.

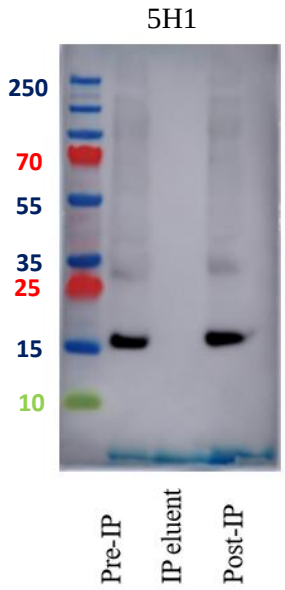


Figure 3-1. RIPA buffer interferes with IP of filarial antigens. *D. immitis* whole-worm extract in RIPA buffer was immunoprecipitated with 5H1 IgE mAb. Whole-worm lysate (Pre-IP), IP product (IP eluent) and lysate after IP (Post-IP) were blotted with 5H1 IgE mAb. Due to the interference of RIPA buffer detergents with antigen-antibody interactions, the target antigen of 5H1 was not enriched in the IP eluent.

In order to account for nonspecific binding of worm proteins to random human IgE antibodies in IP, IgE mAbs specific for irrelevant antigens from mold and peanut (mAbs 21E2, 16A8) were used in parallel with all filaria-specific IgE mAbs to serve as specificity controls. In order to ensure the enrichment of target protein in IP eluent, when possible the presence of target protein was verified via Western blot analysis using IP antibody. A pre-IP whole-worm extract was included in a

parallel SDS-PAGE lane to show the baseline expression level of target protein, post-IP extract was also included to verify depletion of target protein.

One of the main obstacles in obtaining a high quality MS data is lack of a comprehensive protein database to use in MS data analysis due to the poor annotation of filarial worms genomes. An in-house protein database was developed for both *D. immitis* and *B. malayi* to be used for MS data analysis. The RNAseq data for *D. immitis* adult worm and *B. malayi* worms at different life stages were collected [212]–[214] and Python programming was used to translate RNA sequences to protein sequences using each of the six translation frames. Finally, all the protein sequences generated, the majority of which are hypothetical proteins, were used as the database for MS data analysis. The RNA sequences used for *D. immitis* and *B. malayi* databases are available at GenBank under BioProject accession numbers PRJNA80937 and PRJNA344486, respectively.

Identification of Filarial Antigens Targeted by Human IgE MAbs using IP/MS

As explained in chapter II, all hybridoma cell lines secreting IgE mAbs were generated using an unbiased method via screening B cell derived supernatants for the presence of IgE antibodies in an isotype-specific ELISA. The binding properties of each IgE mAb to filarial antigens extracted from *B. malayi and D. immitis* are summarized in Table 2-2 and Figure 2-5. Those IgE mAbs that exhibited binding to filarial worm proteins during the initial screening of B.m immunoCAP, ELISA and western blot analysis were further pursued for antigen identification.

To determine the antigen specificity of each IgE mAb, I immunoprecipitated crude extracts from *D. immitis* worms with IgE mAbs 12D4, 5H1 and 9C1. For IgE mAbs designated 2E6, 4E9, 11H12 and 1A5, which were *Brugia*-specific and did not show strong binding to *D. immitis* worm extract,

I performed immunoprecipitation using *B. malayi* worm extract. As controls for specificity, I analyzed immunoprecipitates with IgE mAbs specific for irrelevant antigens from mold and peanut (mAbs 21E2, 16A8). For western blot positive IgE mAbs (5H1, 9C1, 1A5, 12D4, 2E6 and 11H12) immunoprecipitation was followed by western blot analysis to confirm the enrichment of antigen (Figure 3-2). When possible an additional purification step was taken after IP where the eluent was run on an SDS PAGE and the protein was extracted from the gel and submitted for mass spectrometry proteomics analysis (Fig 3-3). For western blot positive IgE mAbs, we knew what is the approximate molecular weight of antigen that we are looking for, but in the case of 4E9 we did not know what is the size of target, so the prominent band which was around molecular weight of 12 kDa was excised and submitted for mass spectrometry analysis.

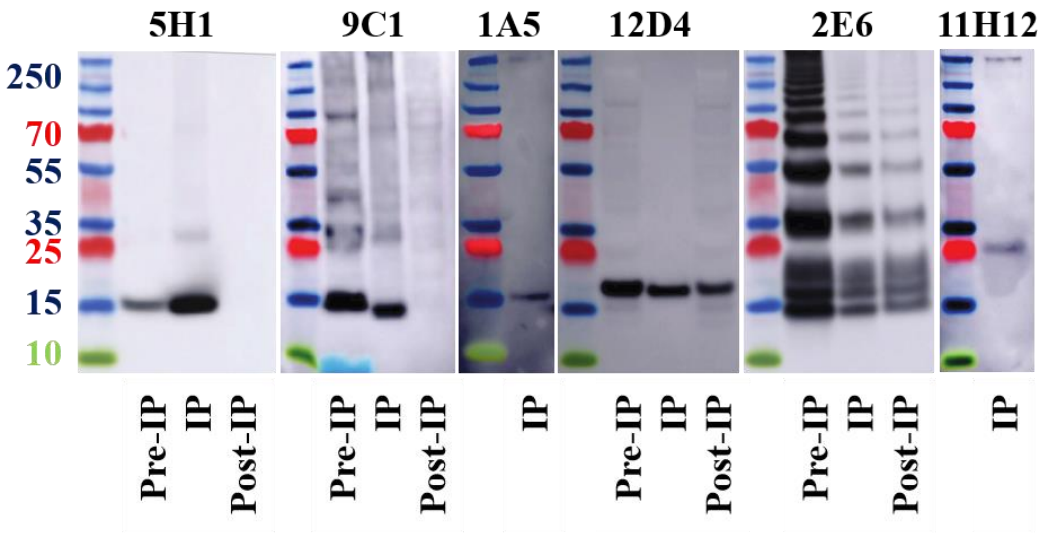


Figure 3-2. Specificity validation of each IgE mAb for its target antigen using western blot analysis following immunoprecipitation. A filarial worm somatic extract was immunoprecipitated with IgE mAbs. Whole somatic extract (Pre-IP), IP product and extract after IP (Post-IP) were then blotted with the same IgE mAb. Only immunoblot-positive antibodies are shown.

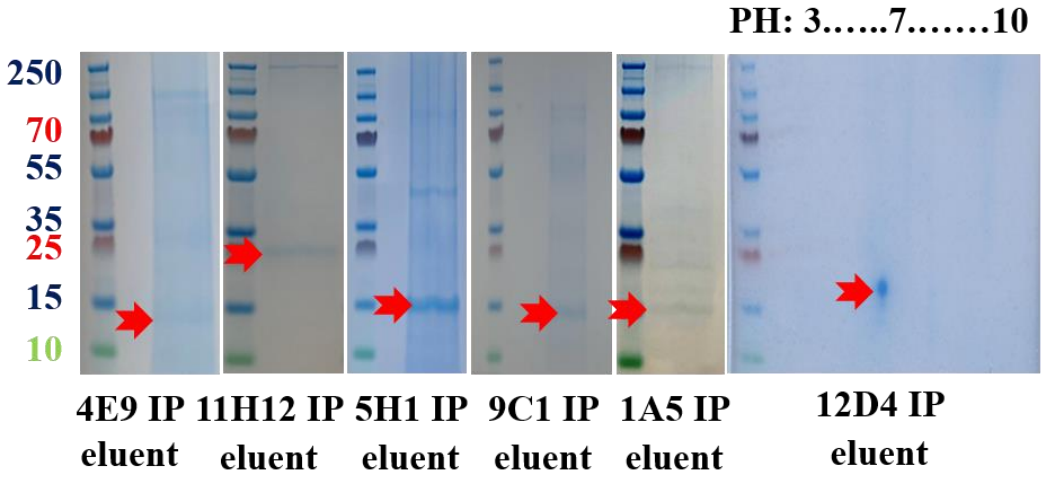


Figure 3-3. Performing an additional purification step for each IP product using SDS PAGE. The IP eluent for each IgE mAb was separated on an SDS-PAGE. After staining gel with SimplyBlue™ SafeStain, the prominent band at correct molecular weight was excised and was submitted to MS for protein identification.

The number of peptide spectrum matches (PSMs) were identified for each antigen protein in immunoprecipitation as shown in Figure 3-4. Several filarial antigens were identified in the MS analysis. IgE mAb 12D4 immunoprecipitated a 14 kDa protein, WbSXP-1, also known as WB14 antigen. 11H12 mAb precipitated a 24 kDa secreted protein with unknown function (24 kDA Spro). Interestingly 4E9 mAb immunoprecipitated Macrophage Migration Inhibitory Factor, MIF, which is a multipotent cytokine involved in the regulation of immune and inflammatory responses. 2E6, which showed the strongest binding to *B.malayi* ImmunoCAP and was targeting a ladder like protein with apparent molecular weight 15 kDa to 250 kDa according to western blot analysis, did precipitate a polyprotein called ladder antigen-like protein or gp15/400 protein. Lastly, four IgE mAbs, 1A5, 9C1, 5H1 and 14B2 immunoprecipitated various members of TTR protein family. Interestingly these TTR-specific IgE mAbs showed varied binding patterns to filarial worm extracts in ELISA and western blot analysis, while targeting similar proteins from TTR protein family.

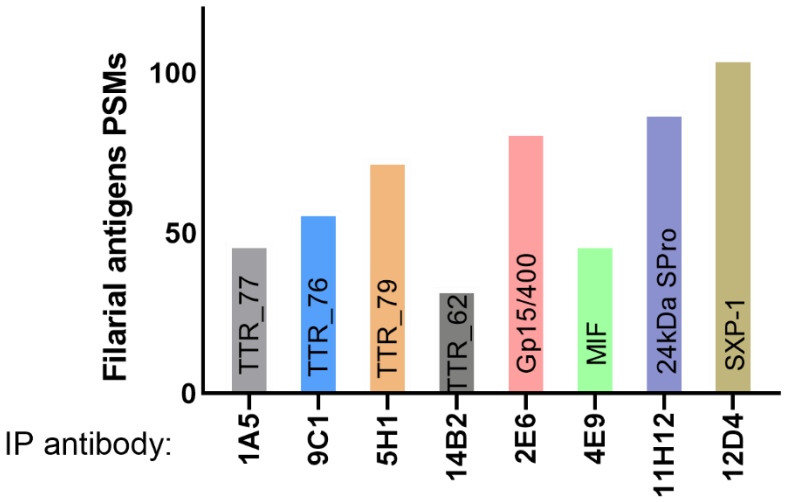


Figure 3-4. Identification of filarial antigens targeted by IgE mAb using MS analysis. Shown is the number of peptide spectrum matches (PSMs) identified for each antigen by mass spectrometry analysis.

To confirm IgE mAb specificity, we expressed recombinant forms of filarial antigens in bacteria using the *W. bancrofti* sequence and tested binding by ELISA (Figure. 3-5). The only exception is 24 kDa secreted protein, which was expressed using *B. malayi* sequence for this protein because there were no sequences reported for *W. bancrofti* 24 kDa secreted protein in NCBI at the time of download (August 2020). All filarial antigens were expressed in SHuffle T7 *E.coli* to ensure the correct formation of disulfide bonds, except for WbSXP-1, which was expressed in One Shot BL21 chemically competent *E. coli* because based on WbSXP-1 amino acid sequence there is no disulfide bond present. In the case of 2E6 IgE mAb that targets gp15/400, a ladder like antigen, its tandem repeat unit containing 132 amino acids was expressed recombinantly. Expression of recombinant proteins were confirmed by western blot assay using an HRP-conjugated anti-His antibody.

When we were able to express and purify antigen protein in sufficient quantities, we calculated the EC_{50} for each antibody. The EC_{50} values and representative binding curves for IgE mAbs are shown in Figure 3-5. If IgE mAbs showed positivity in an immunoblot of somatic extract we also performed immunoblotting (Figure. 3-6). It worth mentioning, when 9C1 was tested against recombinant *W. bancrofti* TTR_76 protein, it showed preferred binding to the dimer form of this TTR protein, which is probably an artifact of concentrating recombinant proteins.

Next, all other IgE mAbs were tested for binding against recombinant filarial antigens in hope of identifying other potential antigen-specific IgE mAbs, which were missed in initial screenings due to the limited amount of that specific antigen in filarial protein extracts or due to their specificity for *W. bancrofti* antigens. I was able to identify two additional filarial-specific IgE mAbs using this approach, 4E1 showed strong binding to recombinant WbSXP-1, while it was clearly negative

in the primary screenings against *B. malayi* and *D. immitis* worm extracts in ELISA and western blot analysis. Also, IgE mAb 12C2 showed strong binding to recombinant TTR_76. When 12C2 reactivity to *B. malayi* and *D. immitis* worm extracts in ELISA and western blot assays was taken into account, it was evident that 12C2 behaves almost the same as 9C1 IgE mAb. So, the nucleotide sequence of the 12C2 mAb variable gene region was determined and it showed the exact same sequence for variable regions as 9C1 mAb, which explains their identical functional activity. Other IgE mAbs with unique sequences that showed various levels of reactivity towards TTR_76 protein will be further discussed in Chapter IV.

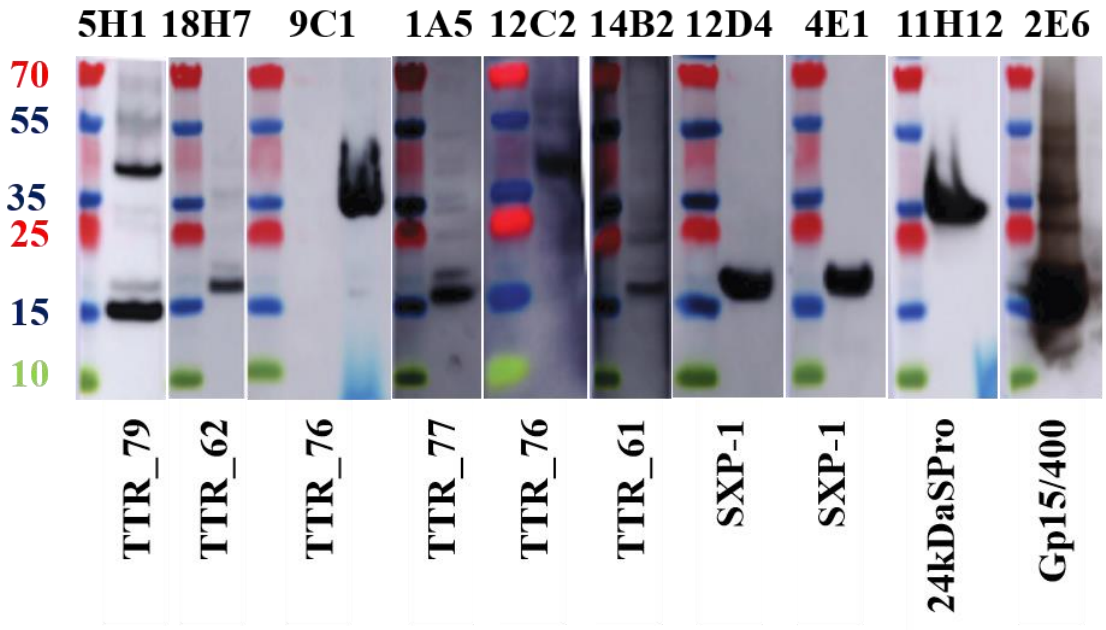


Figure 3-5. Specificity validation of each IgE mAb for its target by immunoblot analysis. Each recombinant filarial antigen was tested against its corresponding IgE mAb using western blot analysis. Only western-blot-positive antibodies are shown.

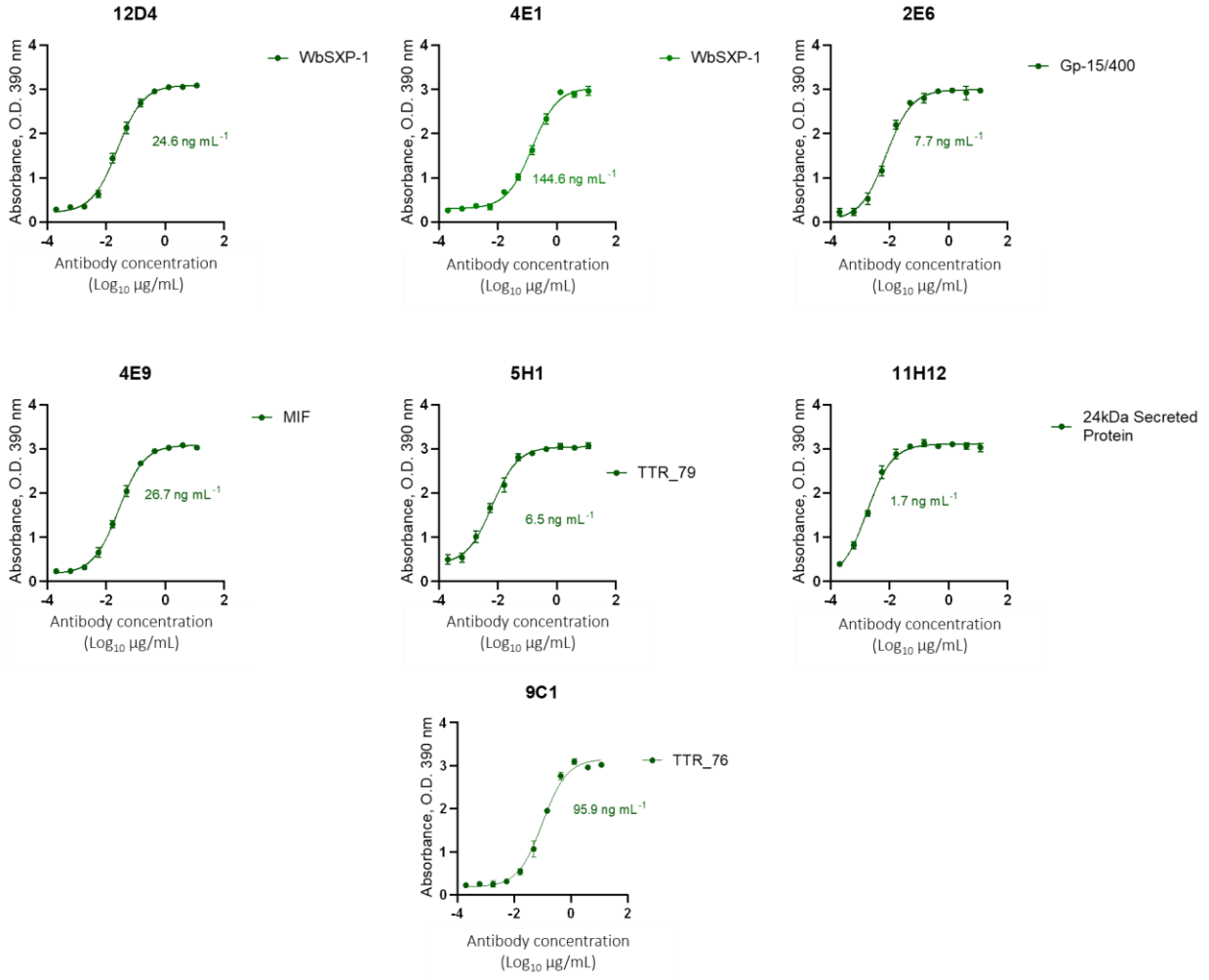


Figure 3-6. Specificity validation of each IgE mAb for its target by ELISA. Data obtained in triplicate are shown as the mean ± SEM and are representative of three experiments. Calculated EC50 values are shown on the graph.

12D4 and 4E1 Show Different Binding Pattern to SXP-1 Proteins from Different Filarial Worms

Although both 12D4 and 4E1 IgE mAbs were obtained from Subject P3, they represented independent clones as evidenced by their unique HCDR3 sequences. 12D4 and 4E1 mAbs show different patterns of binding to SXP-1 proteins. Not only the EC_{50} for 12D4 binding to WbSXP-1 is almost one sixth of the EC_{50} for 4E1 binding to WbSXP-1, but also it shows broader cross-reactivity against WbSXP-1 homologous proteins. As shown in Figure 3-7, while 4E1 is highly specific for binding to WbSXP-1, 12D4 is binding to SXP-1 proteins from *W. bancrofti, B. malayi and D. immitis*. Next we sought to see if there is any conserved region in the SXP-1 proteins that acts as the epitope for 12D4 IgE mAb. The Amino acid sequences for WbSXP-1, BmSXP-1 (AAA27864.1) and DiSXP-1(JR903413.1) were obtained from NCBI and were aligned using Clustal Omega, a multiple sequence alignment (MSA) program that generates alignments between multiple sequences using seeded guide trees and Hidden Markov Models (HMM). Amino acid sequence alignment of WbSXP-1 with homologous proteins in *B. malayi* and *D. immitis* is shown in Figure 3-8. WbSXP-1 was 85% identical to the BmSXP-1 and 65% identical to the DiSXP-1. Although there are some conserved sites across SXP-1 proteins marked with asterisk, identification of the exact antibody epitope requires further alanine mutation studies or X-ray crystallography.

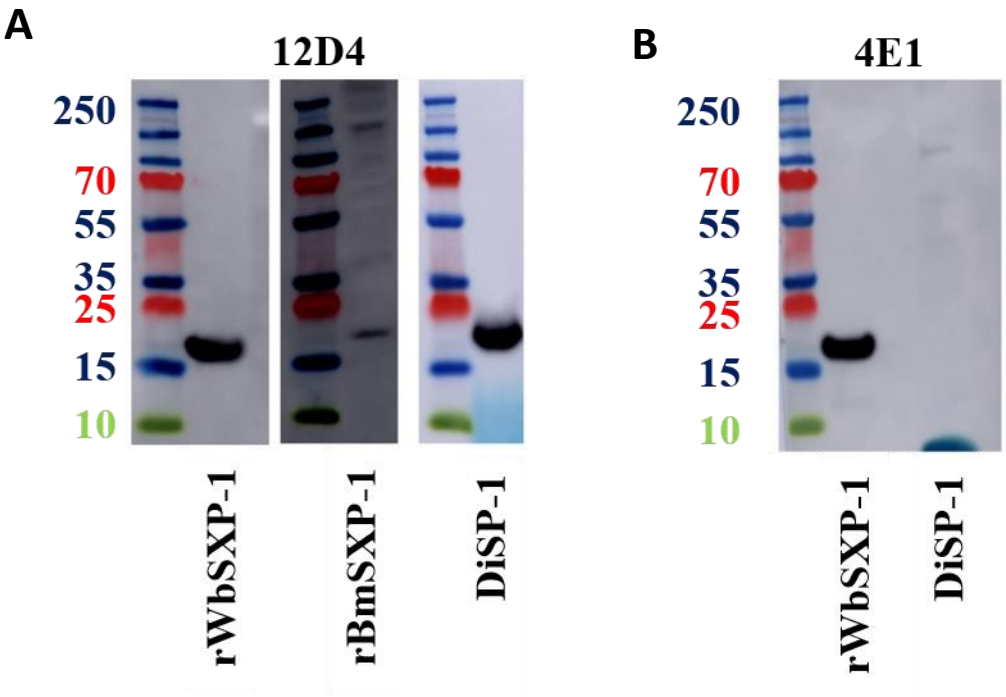


Figure 3-7. 12D4 and 4E1 breadth of cross-reactivity against SXP-1 proteins from *W. bancrofti, B. malayi* and *D. immitis*. **A.** Western blot analysis of 12D4 against recombinant WbSXP-1, BmSXP-1 and against *D. immitis* crude protein extract containing DiSXP-1. **B.** Western blot analysis of 4E1 against recombinant WbSXP-1, 4E1 does not bind *D. immitis* protein extract or BmSXP-1 (data not shown).

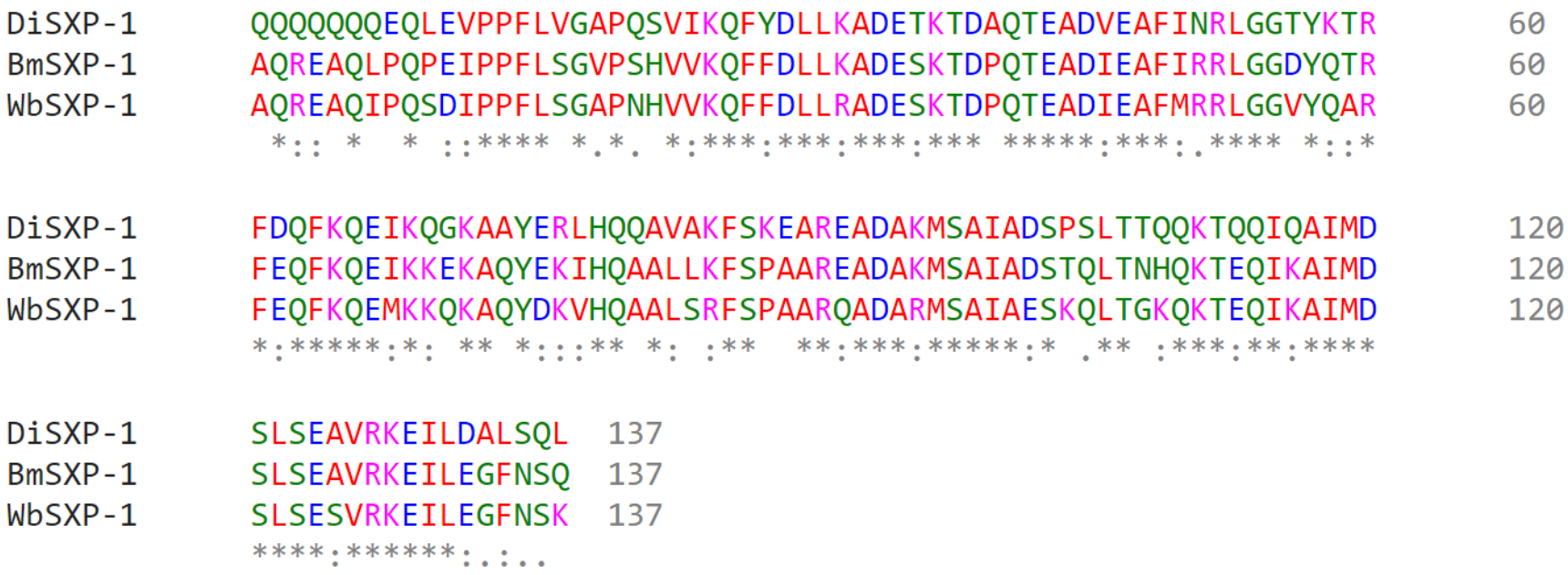


Figure 3-8. Amino acid sequence alignment of filarial SXP-1 proteins bound by 12D4 IgE mAb. Amino acid sequence alignment of WbSXP-1 with homologous proteins in *B. malayi* (AAA27864.1) and *D. immitis* (JR903413.1). Conserved amino acids are marked with asterisk.

Functional Analysis of WbSXP-1-specific MAbs in FcεRIα–Transgenic Mouse Model

The differential binding pattern observed for 12D4 and 4E1 mAbs suggests that they bind to different epitopes on WbSXP-1. Therefore, I attempted to test their ability to induce anaphylaxis in mice. I used a human FcεRIα–transgenic mouse model of passive systemic anaphylaxis (Figure 3-9A). These mice were first sensitized with an injection of 12D4 and 4E1, two WbSXP-1–specific IgE mAbs binding to two different antigenic sites on the protein, or sham sensitized with injection of two isotype control antibodies (Peanut-specific 2F10 and 1B8 IgE mAbs). These two IgE mAbs 12D4 and 4E1 were good candidates for this experiment because of their ability to bind to WbSXP-1 protein at different antigenic sites simultaneously, and therefore could in theory cross-link FcεRI to trigger mast cell degranulation. On day three following sensitization, mice sensitized with a cocktail of 12D4 and 4E1 IgE mAbs or with isotype control antibodies were challenged via an intraperitoneal injection of recombinant WbSXP-1 protein. The timing of the challenge injection was determined by previous observations and relate to the timing of peak FcεRIα induction following sensitization with human IgE. In this model drop in core body temperature is measured as a sign of anaphylaxis. Here, mice that were challenged with injection of recombinant WbSXP-1 did not show a fall in temperature and acted similar to the control animals sensitized with peanut-specific IgE. (Figure 3-9).

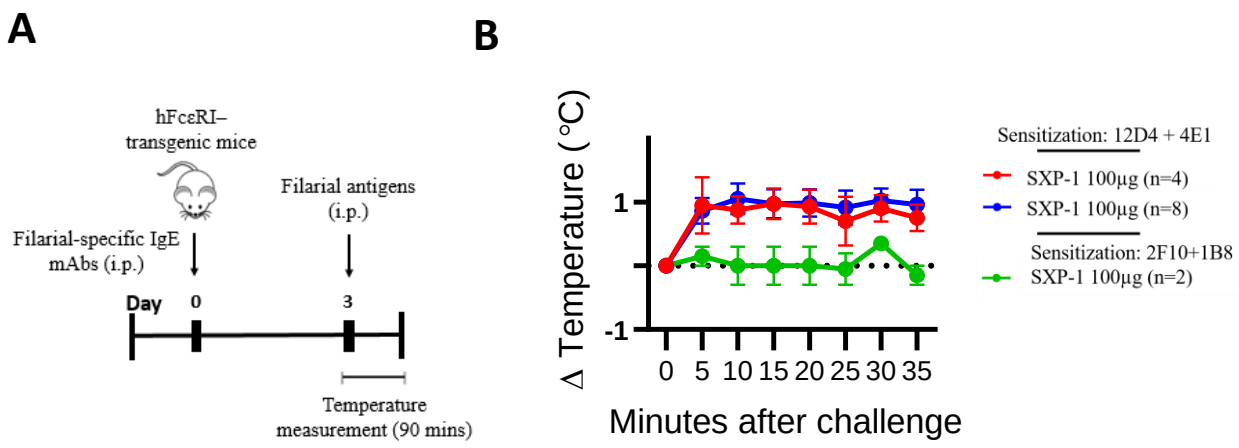


Figure 3-9. Functional analysis of WbSXP-1-specific IgE mAbs in FcεRIα–transgenic mouse model. **A.** hFcεRI transgenic mouse model of anaphylaxis. Mice were sensitized with a 100 µg i.p. injection of human IgE mAbs 4E1 and 12D4. Three days following sensitization, mice were injected i.p. with 100ug or 250 µg of correspondent antigen, WbSXP-1. The change in body temperature was monitored using an implanted temperature probe for 35 minutes. **B.** A paired 2-tailed t test assuming unequal variance was used to compare the change in body temperature of experimental group with the control allergen group at each time point and the result was not significant with calculated P values > 0.05. Data are mean ± SD of each experimental group. There are n mice in each experimental group.

Filarial Gp15/400 Protein is an Excretory Secretory Protein

As prior studies reported that homologues of gp15/400 protein in *Ascaris suum* is a secretory protein [215], [216], I wanted to test the secretory nature of gp15/400 protein in filarial worms. Therefore, *B.malayi* adult male and female worms were kept in culture media at room temperature for 7 days and then I concentrated the culture media and it was western blotted against 2E6. The presence of gp15/400 was confirmed in the excretory secretory products of of *B. malayi* worms (Figure 3-10).

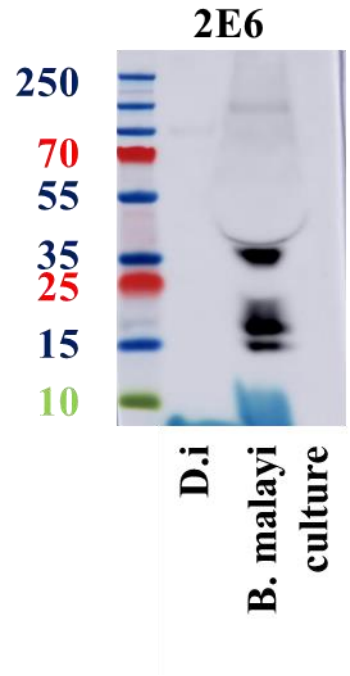


Figure 3-10. Western blot analysis of *D. immitis* extract and *B. malayi* culture using 2E6 IgE mAb. The first lane contains *D. immitis* and second lane contains the concentrated culture media in which *B. malayi* adult male and female worms were kept for 7 days.

Discussion

Filarial worms are difficult to study partly due to their complex life cycles composing of arthropod intermediate host and mammalian definitive host. Helminths are large complicated multicellular parasites encoding more than 11,000 proteins. Many aspects of the human immune response to helminth infection are still unknown, including which immunogenic proteins are responsible for stimulating IgE production. Different parallel approaches have been taken to identify prominent filarial antigens targeted by human antibody response such as: 1) Screening phage display cDNA

expression library of the third stage larvae (L3) of *Brugia malayi* to identify filarial antigens binding the sera from patients with filariasis [217], 2) Biochemical and immunological characterization of major surface or secreted antigens [218], [219], 3) Identifying and characterizing abundant transcripts of multiple stages of *Brugia malayi* using RT-PCR approach[220], [221]. 4) Characterizing parasite proteins that are structurally related to allergens such as tropomyosin [222] and glutathione S-transferases (GST) [206]. 5) Stage-specific secretome analysis of *Brugia malayi* using high-throughput, shot-gun proteomic approach [158], [223]. However, none of these approaches have considered identifying filarial proteins naturally targeted by IgE antibodies during the course of infection.

Here I report the characterization of 8 filaria-specific IgE mAbs, and I have identified their target proteins. Two IgE mAbs 12D4 and 4E1 bound to a filarial protein called SXP-1. This protein was first introduced in 1992 when sera from microfilaria-positive patients were screened against *B. malayi* cDNA library [224]. WbSXP-1 is 14 kDa secreted protein with unknown function. It has been described at the immunodominant target of filaria-specific IgG [225]. WbSXP-1 has been used in commercial diagnostic tests for lymphatic filariasis [226]. WbSXP-1 has been proposed as a vaccine candidate and was tested in animal models[227]. SXP-1 protein has homologues in almost all filarial worms such as *B. malayi*, *D. immitis*, *O. volvulus*, etc. The amino acid similarity between DiSXP-1 and WbSXP-1 is only 65% and yet 12D4 IgE mAb shows binding to both proteins. The closest allergen homologue to WbSXP-1 is Ani s 5, an allergen in *Anisakis* (fish worm) with only 45% amino acid similarity [228].

Three IgE mAbs targeted a single family of proteins, which are secretory proteins with approximate molecular weight of 15 kDa and unknown function. TTR proteins are a family of proteins found only in nematodes and are named after their structural similarity to transthyretin,

which is a transport protein in the blood of vertebrates responsible for carrying thyroid hormone. TTRs are differentially upregulated in the Excretory-secretory products (ESP) of parasitic nematodes including *B. malayi* [229]. In *C. elegans*, TTR-52 is facilitating recognition and engulfment of apoptotic cells by acting as a bridging molecule [230]. It is known that TTR MjTTL5 secreted by plant parasite nematode *Meloidogyne javanica* interferes with host antioxidant system [231]. Regarding the homology between worm antigens and allergens, TTR proteins are nematode-specific and I did not find any reported TTR protein reported as an allergen.

Gpl5/400 is a surface-associated glycoprotein that was originally discovered via molecular cloning and pulse-chase studies in *B. pahangi* [232]. The ladder like antigen, Gpl5/400, is encoded by a single gene as a 400 kDa precursor, which is then cut and processed into making proteins in the size range 15 kDa to 400 kDa. The homologue of gpl5/400 was isolated and sequenced from *Brugia malayi* and *Wuchereria bancrofti* as well [233]. In lymphatic filarial worm species the tandem repeat unit of gpl5/400 is highly conserved but it is quite different from *D. immitis* worm. The gpl5/400 protein is a homologue of allergen ABA-1, which is the most abundant protein in *Ascaris suum* and *A. lumbricoides* body fluid[234], [235]. It is shown that ABA-1 is a secretory protein, which is released when culturing larvae in vitro [215], [216]. Along the same line, my data confirms that gpl5/400 is secreted into the culture media of *B.malayi* adult male and female worms. No putative biological function is evident for this protein based on the sequence similarity. The presence of Gpl5/400-specific immunoglobulin E (IgE) in blood of patients with filariasis was previously reported. Also, individuals with elephantiasis had higher level of Gpl5/400-specific IgE compared to the microfilaremic individuals [233].

MIF is a major immunological mediator of mammalian inflammatory conditions acting through inhibiting monocytes random migration and altering cytokine production [236]. Several functions

have been described for MIF in mammalian immune system, including roles in pathogenesis of septic shock [237], rheumatoid arthritis [238], inflammatory bowel disease and tumor metastasis [239]. In addition, two different catalytic activities have been attributed to mammalian MIF: tautomerase [236] and thiol-protein oxidoreductase activities [240]. MIF proteins from *Brugia malayi* (BmMIF-1 and -2) are biochemically and immunologically well characterized, and their three dimensional crystal structures are resolved and published. BmMIF-1 and BmMIF-2 act similar to human MIF as they induce chemotaxis for human monocytes and prevent the random migration of human monocytes. They also induce IL-8, TNF-α, and endogenous MIF production in monocytes. In addition, BmMIF-1 and BmMIF-2 show tautomerase enzyme activity, similar to hMIF (Zang et al. 2002). Despite considerable phylogenetic gap between human and filarial worms, the crystal structure and biological activity is highly conserved between a host's cytokine and its parasite.

We are mapping the human IgE antibody response to important filarial proteins in helminth infection. Therefore, IgE mAbs can be used for antigen discovery, characterization, standardization, & development of therapeutics. My results greatly extends the number of candidate antigens targeted by human IgE response and introduces some unexpected players, including transthyretin-related proteins, MIF, gp 15/400, and 24 kDa secreted protein, which are all Excretory–secretory products (ESP).

Methods

Parasite material: The original live and frozen *B.malayi* worms were obtained from different sources: Filariasis Research Reagent Repository (FR3) and TRS lab. In addition, ready-to-use

B.malayi whole-worm somatic extract was provided by our collaborator Dr. Thomas Nutman. Frozen adult male and female *D. immitis* worms were provided by our collaborator, Dr. Heather Stockdale Walden. Whole-worm somatic extracts were prepared using cryogenic grinding as reported in previous chapter. In order to test the ESP of *B.malayi*, live *B.malayi* adult male and female worms were kept in culture media at room temperature for 7 days. The concentrated culture media was used in Western blot against 2E6 IgE mAb.

Production of filaria-specific IgE mAbs. Purified IgE mAbs, designated 12D4, 4E1, 2E6, 4E9, 11H12, 5H1, 9C1 and 1A5 were produced by hybridoma cell expression and were used for IP/MS analysis, immunoblotting assay and EC_{50} studies.

Immunoprecipitation and mass spectroscopy. Human IgE mAbs that showed strong binding to *B. malayi* and/or *D. immitis* whole-worm somatic extracts in ELISA, ImmunoCAP and/or immunoblot analysis were used for immunoprecipitation (IP). Covalent coupling of IgE mAbs to magnetic microbeads (100 μg mAb per mg of beads) is achieved by incubation at 37°C overnight on a rotating mixer, following manufacturer's instructions (Invitrogen Dynabeads, 14311D). Antibody-coupled beads were washed several times to remove any non-covalently bound antibody and then quenched. Next, filarial extracts were incubated with antibody-coupled beads for 30 mins at room temperature. Beads were washed five times with PBS prior to elution of target protein using 1M glycine solution pH 3.0. Each IP was performed in parallel with an allergen-specific IgE mAb as a control to account for nonspecific interactions between filarial antigens and IgE antibodies. Eluted target antigen was separated in SDS-PAGE, stained with SimplyBlue SafeStain (Thermo Scientific, LC6060), and the prominent band of the expected size was cut and sent to mass spectrometry (MS) for protein identification. This additional purification step was to improve the signal to noise ratio in mass spectrometry proteomics analysis. Scaffold proteome software

was used to display the MS data and validate the protein identification results with increased accuracy.

Mass spectroscopy data analysis. Due to the poor annotation of the genomes of filarial worms, an in-house protein database was developed for both *D. immitis* and *B. malayi* to be used for MS data analysis. The RNAseq data used in *D. immitis* and *B. malayi* databases are available at GenBank under BioProject accession numbers PRJNA80937 and PRJNA344486, respectively. The Python code for this module is available at GitHub repository under, https://github.com/AzadehHadadianpour.

Recombinant antigens and proteins. Recombinant His-tagged filarial antigens were expressed in bacteria. In brief, the DNA sequences encoding WbSXP-1, Macrophage Migration Inhibitory Factor (MIF), 24 kDa secreted protein, Ladder antigen-like protein (Gp15/400), and Transthyretin-related proteins were amplified from a synthetic gene construct (Genscript), cloned into the expression vector pET-28a (with an N-terminal hexahistidine tag). WbSXP-1 protein was expressed in BL21 (DE3) bacterial expression system, while all other proteins were expressed in SHuffle® T7 Competent *E. coli* (NEB, C3029J) - engineered to correctly fold disulfide bonded proteins in their cytoplasm (Lobstein et al. 2012). Transformed bacteria were grown in 1 L of LB medium containing kanamycin at 50 µg/mL at 37°C shaken at 220 rpm. Cultures were grown to an optical density (at 600 nm) of 0.5-0.8 and protein expression induced by adding 1 mL of 1 M IPTG (isopropyl thio-d-galacto pyranoside) and growing at 16°C and 220 rpm for 20 hrs. The cells were harvested and pelleted by a 20 min centrifugation at 4,000×rpm. Cells were suspended in binding buffer (25 mM NaPi, 150 mM NaCl with pH=8) containing 1 µM PMSF, 0.7 µg/ml pepstatin, 1 µg/mL leupeptin, DNase, Lysozyme. The Avesti EmulsiFlex C3 benchtop homogenizer was used to disrupt the cells and obtain lysate. The lysate was centrifuged at

12,000×rpm for 20 min at room temperature to remove insoluble material. Recombinant protein was purified using TALON metal affinity resin (Takara, 635504) following manufacturer's specifications. Briefly, 5 mL of TALON resin was added to lysate and rocked for 1 h at 4°C. The slurry then was suspended in a glass column for gravity chromatography. The resin was washed with 10 column volumes of washing buffer (25 mM NaPi, 150 mM NaCl + 20 mM imidazole with pH=7) and the protein eluted with 5 column volumes of washing buffer containing imidazole at 500 mM concentration. The Lowry et al method was used to estimate protein concentration (1951). Finally, Zeba Spin Desalting Columns (Thermo Scientific, 89893) were used for imidazole removal and buffer exchange to PBS. Purified proteins were run SDS–PAGE to assess protein purity and correct molecular weight.

Accession codes. The DNA sequences reported for filarial antigens are listed here. WbSXP-1 (Acc number: AAC17637.1) BmSXP-1 (Acc number: AAA27864.1), DiSXP-1 (Acc number: JR903413.1), Macrophage Migration Inhibitory Factor (MIF) (Acc number: EJW88743.1), 24 kDa secreted protein (Acc number: VIO90327.1), Ladder antigen-like protein or gp15/400 (GenBank: AAG31482.1), Transthyretin-related proteins (GenBank: EJW78979.1, VDM13477.1, VDM14676.1).

Western blot analysis. Immunoblot analysis of filarial worm extract and purified recombinant filarial antigens was performed as explained in chapter II. But briefly, antigenic proteins were separated by SDS-PAGE under nonreducing/nondenaturing conditions, were electrophoretically transferred to PVDF membranes. Membranes were blocked overnight at 4°C, and were incubated with IgE mAbs for 1 h at room temperature, followed by probing with murine anti–human IgE-peroxidase conjugate secondary antibody for 1 h at room temperature. Blots were washed 3 times

with PBS between each step. Finally, blots were visualized using chemiluminescent substrate on an Amersham™ Imager 600.

Half maximal effective concentration (EC_{50}) binding analysis. First, 384-well clear ELISA plates (Greiner, 781061) coated with the purified recombinant protein of interest at estimated concentration of 10 μg/mL overnight at 4°C. Block solution (5% non-fat dry milk, 2% goat serum in PBS) was added to the plates for 1 hr incubation at room temperature. For each experiment repeat, three-fold dilutions of IgE mAb was prepared in triplicate starting from 12 μg/mL. MAb dilutions were added to the wells, subjected to a 1 hr incubation, followed by 1 hour incubation of 1:1000 dilution of an HRP-conjugated mouse anti–human IgE Fc secondary antibody (Southern Biotech, 9160-05, 1:1,000 dilution). The plates were washed 3 times between each step with PBS. Substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) was added to the plates. Color development was measured at 390 nm after 10 min using a Molecular Devices plate reader. The EC_{50} values were calculated in Prism software (GraphPad) after log transformation of the mAb concentration using sigmoidal dose–response nonlinear regression analysis.

Multiple protein sequence alignment of SXP-1 proteins. The full-length and non-redundant SXP-1 protein sequences associated with *W. bancrofti*, *B. malayi* and *D. immitis* were downloaded from the NCBI database and MUSCLE was used to align sequences using default parameters.

Human FcεRI–transgenic mouse anaphylaxis. The Guide for the Care and Use of Laboratory Animals of the National Institutes of Health was followed in performing mouse studies. After purchasing human FcεRI–transgenic mice [B6.Cg-Fcεr1α^{tm1Knt} Tg(FCER1A)1Bhk/J] from Jackson Laboratory (stock 010506), they were bred and genotyped. mice are transgenically

modified to express human Fc fragment of IgE receptor α polypeptide (FCER1A) and also to block expression of murine FCER1A. Mice that carry at least one copy of human FCER1A and carry no copy of mouse FcεRI are capable of undergoing anaphylaxis when injected with human IgE followed by antigen challenge. Transgenic mice were sensitized by i.p. injection of 100 μg total IgE and challenged by i.p. injection of 50 μg purified recombinant antigen. Changes in mouse core body temperature were monitored over 90 min using implanted temperature probes. If no drop in temperature was observed the temperature monitoring was stopped at 35 min.

Statistics. For ELISA studies, data from three repeats of assays performed in triplicate were used to calculate mean relative fluorescence units. The descriptive statistics mean ± SEM was determined. To calculate EC_{50}, I log-transformed antibody concentrations and did fit sigmoidal dose-response non-linear model. Statistical analysis of ELISA was performed using Prism v9.0 (GraphPad) software. In mouse studies, a paired 2-tailed t test assuming unequal variance was used to compare the change in body temperature of experimental group with the control allergen group at each time point (Microsoft Excel Office Professional Plus 2016). Time points with calculated P values less than 0.05 were labeled and considered significant.

CHAPTER IV

CHARACTERIZATION OF TTR-SPECIFIC IGE MONOCLONAL ANTIBODIES

Introduction

Transthyretin related proteins (TTR) are one of the nematode-specific protein domain families. TTR proteins in *C. elegans* show weak sequence similarity to transthyretin (SwissProt P49142 and P49143), which transports thyroid hormones. Although tentative, the sequence similarity suggests that TTR proteins in nematodes may be a family of hormone transporters [241]. A more recent study has proposed a new role for TTR-52, one of the members of the TTR family of proteins, serving as a facilitator for recognition of apoptotic cells through cross-linking phosphatidylserine (PS) with the phagocyte receptor CED-1 [230]. Reports of TTR proteins in filarial worms are limited to LC/MS shotgun transcriptomic analysis of adult *B. malayi* excretory secretory products (ESP) identifying 11 different TTR proteins [229].

In this chapter, I will focus mainly on the human IgE antibodies recognizing TTR family of proteins. The reactivity of all IgE mAbs with recombinant TTR proteins was analyzed by western blot and ELISA analysis. The details of the expression of recombinant TTR proteins are reported, and interestingly two of these TTR proteins, TTR_79 and TTR_62, were expressed at high concentrations in *E.coli*, which might be due to their special disulfide bond architecture. TTR-specific antibodies make up 67% of filarial-specific antibodies. Here, I describe the characterization of 10 TTR-specific IgE mAbs starting with identification of their targets, determining their binding strength (EC_{50}) when protein expression was sufficient and finally the functional assay.

Here, I describe the analysis of the epitope breadth and function of the TTR-specific IgE mAbs with special focus on 5H1 and 18H7 IgE mAbs. The ability of 5H1 and 18H7 to recognize 13

different TTR proteins with minimal amino acid sequence similarity suggests that there is a conformational epitope formed by distant amino acids. The ability of IgE mAbs to cross react with different TTR proteins provides insight into previously unrecognized immunogenicity of these proteins. *In vivo* studies show that 5H1 and 18H7 established temperature drop in anaphylaxis mouse model when challenged with TTR_76, TTR_79, and TTR_62 proteins. Further, both these mAbs were used in combination to test for synergy effects, but *in vivo* results were similar to when we used each mAb separately.

Collectively, these findings identify a novel family of proteins/filarial antigens that can aid in development of an anti-filaria vaccine or more sensitive diagnostics. In addition, we have presented the first example showing that parasitic proteins can induce allergy like symptoms *in vivo*, which provides insights into an approach exploited by a TTR-binding antibody.

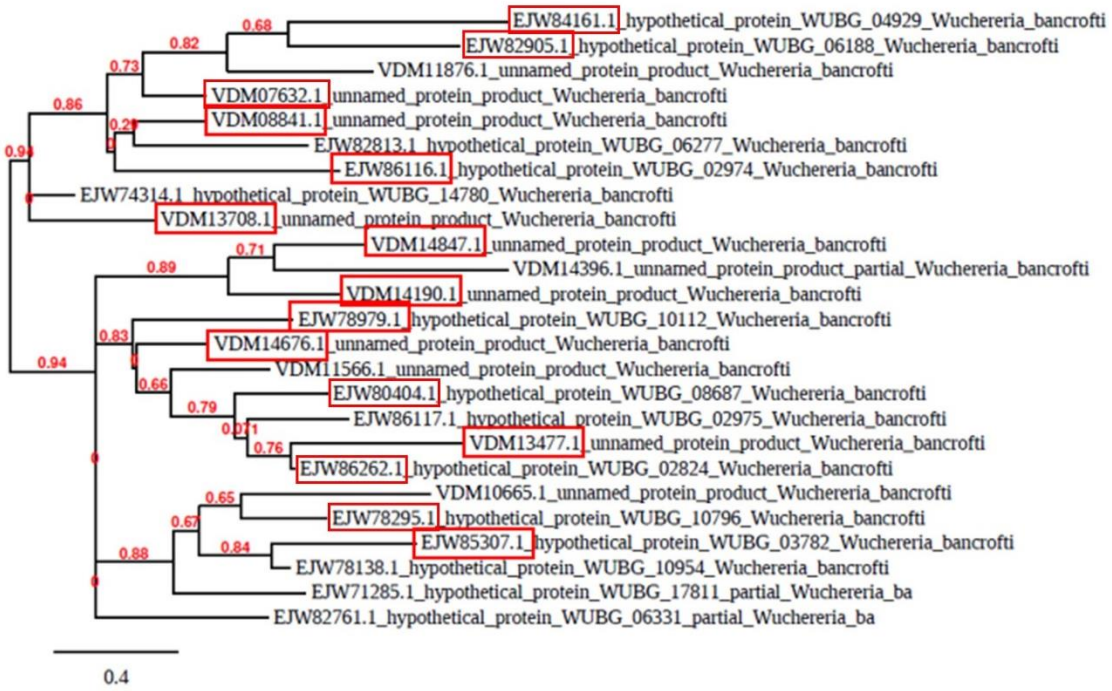
I acknowledge Dr. Benjamin Spiller; pharmacology department at Vanderbilt University Medical Center for his help with expression of recombinant TTR proteins antigens; Dr. Stokes Peebles and Jian Zhang for performing the *in vivo* experiments.

Phylogenetic Tree Analysis of WbTTR Proteins

Using IP/MS technique we found that three filaria-specific IgE mAbs target TTR proteins. All TTR proteins which are mainly reported as “hypothetical protein WUBG” share TTR-52 domain with accession number pfam01060. Since these proteins have not been reported as filarial immunogens previously, I sought to take a closer look at this family of proteins and determine if our panel contained IgE mAbs specific to other TTR proteins. All the sequences reported for WbTTR proteins were obtained from NCBI database; the data was cleaned by deleting partial and

repetitive sequences. Multiple amino acid sequence alignment of WbTTR proteins was performed using Clustal Omega program. The family of TTR proteins were found to share a low degree of sequence identity. The pairwise identity percentage between different TTR proteins ranges from 18.1% to 70.06% (Figure 4-1). Afterward, I used a web-based software tool MAB (Methods and Algorithms for Bioinformatics) and MUSCLE algorithm to construct a maximum-likelihood phylogenetic tree of TTR proteins (Figure 4-1).

A



B

	EJW84161.1 (TTR_61)	EJW82905.1 (TTR_05)	VDM07632.1 (TTR_32)	VDM08841.1 (TTR_41)	EJW86116.1 (TTR_16)	VDM13708.1 (TTR_08)	VDM14847.1 (TTR_47)	VDM14190.1 (TTR_90)	EJW78979.1 (TTR_79)	VDM14676.1 (TTR_76)	EJW80404.1 (TTR_04)	VDM13477.1 (TTR_77)	EJW86262.1 (TTR_62)	EJW78295.1 (TTR_95)	EJW85307.1 (TTR_07)	VDM11876.1	EJW82813.1	EJW74314.1	vdm14396.1	VDM11566.1	EJW86117.1	VDM10665.1	EJW78138.1	EJW71285.1	EJW82761.1
EJW84161.1 (TTR_61)																									
EJW82905.1 (TTR_05)	30.8																								
VDM07632.1 (TTR_32)	32.2	27.4																							
VDM08841.1 (TTR_41)	25.4	35.1	33.1																						
EJW86116.1 (TTR_16)	28.1	26.9	23.9	36.9																					
VDM13708.1 (TTR_08)	27.9	43.2	27.7	60.7	33.6																				
VDM14847.1 (TTR_47)	24.1	19.3	24.6	29.4	23.1	30.6																			
VDM14190.1 (TTR_90)	28.2	33.3	30.7	38.2	31.9	30.7	31.0																		
EJW78979.1 (TTR_79)	23.8	28.1	29.5	40.3	33.8	41.5	36.6	39.7																	
VDM14676.1 (TTR_76)	30.0	29.6	31.9	54.5	35.7	44.6	31.2	46.4	49.6																
EJW80404.1 (TTR_04)	31.6	30.3	23.7	43.0	33.8	45.4	28.7	34.5	52.1	48.7															
VDM13477.1 (TTR_77)	24.6	25.8	28.6	43.0	39.1	40.7	34.0	34.6	63.0	50.0	55.9														
EJW86262.1 (TTR_62)	25.0	25.6	26.1	39.1	39.4	42.1	40.0	41.1	60.9	47.5	58.5	70.1													
EJW78295.1 (TTR_95)	30.9	28.8	23.0	29.9	36.8	27.6	29.5	31.0	40.6	30.3	35.5	29.1	30.3												
EJW85307.1 (TTR_07)	25.4	34.3	24.0	30.3	29.9	29.5	29.7	25.7	35.1	29.9	35.1	29.2	30.8	47.4											
VDM11876.1	26.6	29.7	23.6	27.5	28.7	26.1	24.4	27.6	28.2	27.2	21.8	29.1	32.1	27.6	22.3										
EJW82813.1	27.3	26.0	29.1	44.4	39.4	48.8	33.7	37.0	50.9	46.6	45.2	53.2	46.6	30.8	32.1	30.2									
EJW74314.1	28.8	43.2	28.4	66.3	39.0	69.1	30.4	38.7	48.7	49.3	49.3	43.4	44.8	32.1	37.3	24.7	47.4								
VDM14396.1	31.9	24.0	21.8	29.6	33.8	35.5	23.5	28.4	47.5	32.9	43.2	41.5	46.3	46.7	25.7	34.8	35.9	26.4							
VDM11566.1	23.0	18.1	25.6	37.1	40.2	36.9	31.4	34.1	47.4	44.9	40.3	48.0	49.6	34.9	31.0	28.3	41.1	36.1	36.7						
EJW86117.1	26.2	23.5	26.4	45.1	35.8	37.4	28.0	37.9	45.4	41.0	52.0	52.1	51.8	36.2	35.2	22.9	43.0	48.0	40.7	42.6					
VDM10665.1	25.2	20.2	24.8	26.0	26.6	27.3	24.7	29.2	33.9	30.7	37.8	32.1	36.1	43.5	40.7	26.8	28.9	27.9	27.3	29.3	31.0				
EJW78138.1	32.2	27.4	30.6	32.4	30.9	30.3	28.7	26.3	34.4	33.7	33.7	33.0	33.3	45.9	54.9	27.7	34.6	31.6	24.5	29.9	33.3	41.6			
EJW71285.1	30.2	27.5	28.1	35.9	29.0	31.1	25.9	26.5	36.8	35.9	37.1	33.7	35.0	40.0	40.4	29.4	29.0	31.2	25.5	34.0	30.5	38.1	50.0		
EJW82761.1	25.0	27.4	26.7	29.3	26.0	31.9	29.9	43.2	28.9	33.0	25.0	25.8	35.2	34.3	35.3	33.3	25.4	31.0	29.3	26.7	30.7	30.7	29.9	28.6	

Figure 4-1. Phylogenetic tree of *W. bancrofti* TTR proteins. A, All sequences reported for WbTTR proteins were collected from NCBI and a maximum-likelihood phylogenetic tree of TTR proteins were assembled using the MUSCLE algorithm, with partial and repetitive sequences reported in NCBI omitted. Representative TTR proteins we chose for recombinant expression are marked with red boxes. **B,** Pairwise percent identity of *W. bancrofti* TTR proteins.

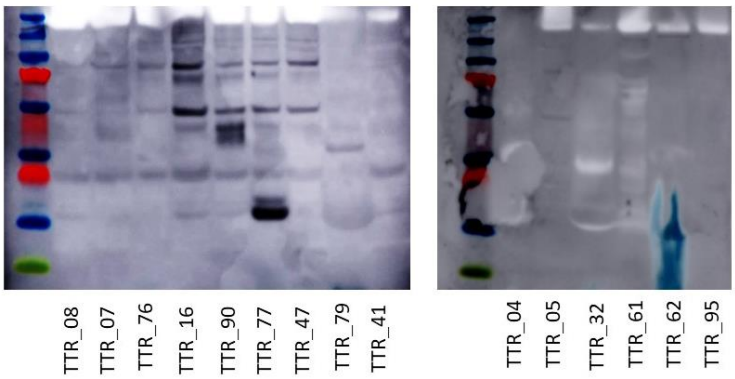
Figure 4-2. Amino acid sequence alignment of WbTTR proteins. Amino acid sequence alignment of WbTTR proteins expressed recombinantly is shown. Conserved amino acids are marked with asterisk.

Cross-reactivity Profile of TTR-specific IgE MAbs

Reactivity of TTR-specific IgE mAbs was tested against TTR proteins on Western blot first (Fig 4-3). Of note was the presence of a dimer band on Western blot of several TTR-specific mAbs, which is an artifact of concentrating recombinant proteins, a similar band around dimer size is present in the IP product of 5H1 and 9C1 (Figures 3-2), while absent form western blot analysis of whole worm lysate.

Our data demonstrates that TTR-specific IgE mAbs show different profiles of TTR-specificity (Fig 4-4). Some were found to be highly specific to one TTR protein, while others showed varying degrees of cross-reactivity across different members of TTR protein family. 1A5 IgE mAb primary target is TTR_77 and it shows cross-reactivity towards TTR_16 and TTR_90. 9C1 IgE mAb is unique in this regard that it seems to only bind to the dimer form of TTR_76 when tested in western blot, it also shows cross-reactivity towards TTR_04 and TTR_41 when tested in ELISA. IgE mAbs 14B2, 11G1 and 10D5 are highly specific to only one TTR proteins; TTR_61, TTR_90 and TTR_32, respectively. Of note are two IgE mAbs, 5H1 and 18H7, that broadly cross-react with 13 different TTR proteins despite marginal amino acid conservation. As some of the TTR-specific antibodies are western blot negative, I had to use another assay, ELISA, to semi-quantitatively assess their binding to TTR proteins. In this assay I coated plates with recombinant TTR proteins and all TTR-specific antibodies were added at a fixed concentration. Finally an HRP conjugated anti-IgE secondary antibody was added and plates were processed by adding TMB substrate. The signal was measured at a single time point. The reported OD 390 nm values are after subtraction of signal from no-antibody wells. 12D4 mAb, specific for WbSXP-1, served as a negative control. Collectively, this data shows that TTR family proteins are the prominent filarial antigens targeted by IgE antibodies (Fig 4-4).

1A5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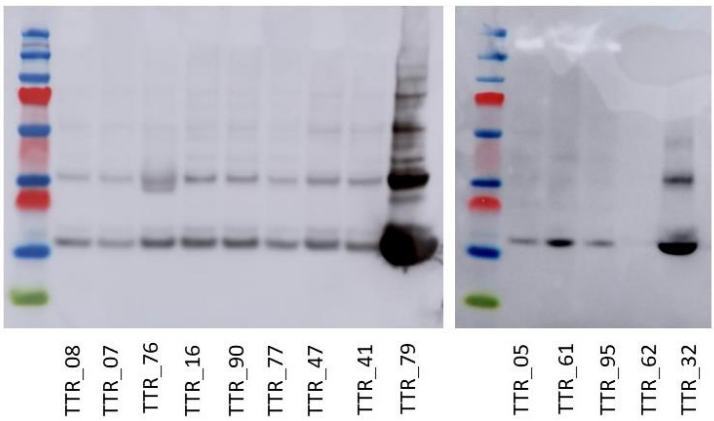
TTR_32
TTR_61
TTR_62
TTR_95

5H1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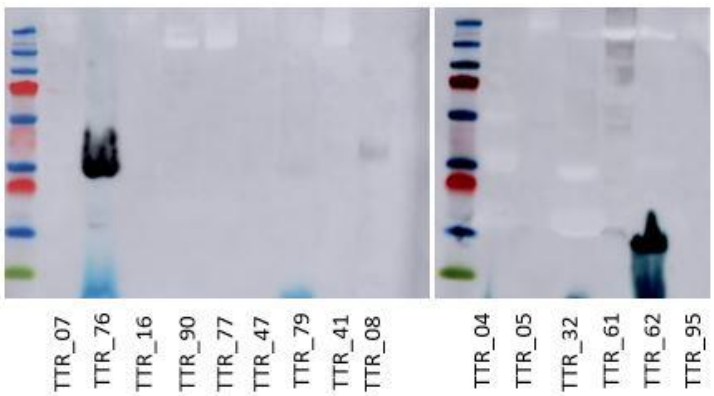
9C1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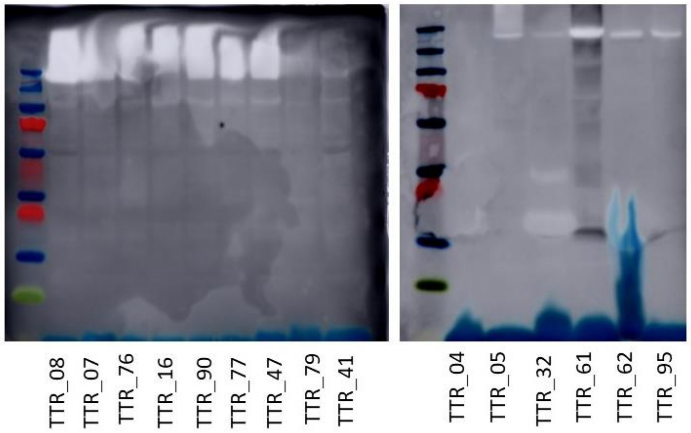
14B2



18H7

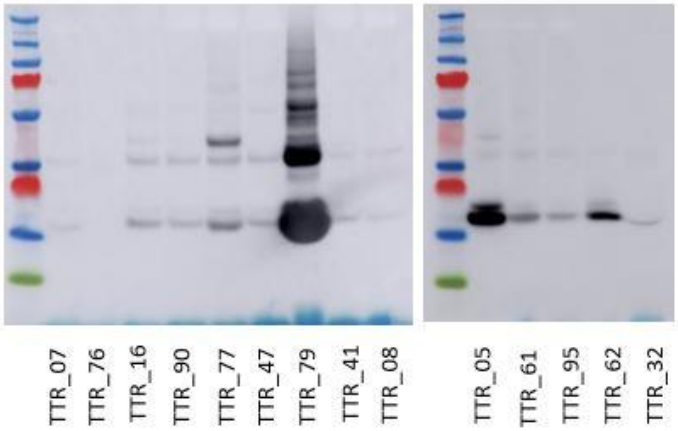


Figure 4-3. Cross-reactivity analysis of TTR-specific IgE mAbs against different TTR proteins in Western blot. Fifteen wbTTR proteins were recombinantly expressed and were tested against five TTR-specific IgE mAbs: 1A5, 5H1, 9C1, 14B2 and 18H7 in a Western blot analysis. Only western-blot-positive antibodies are shown.

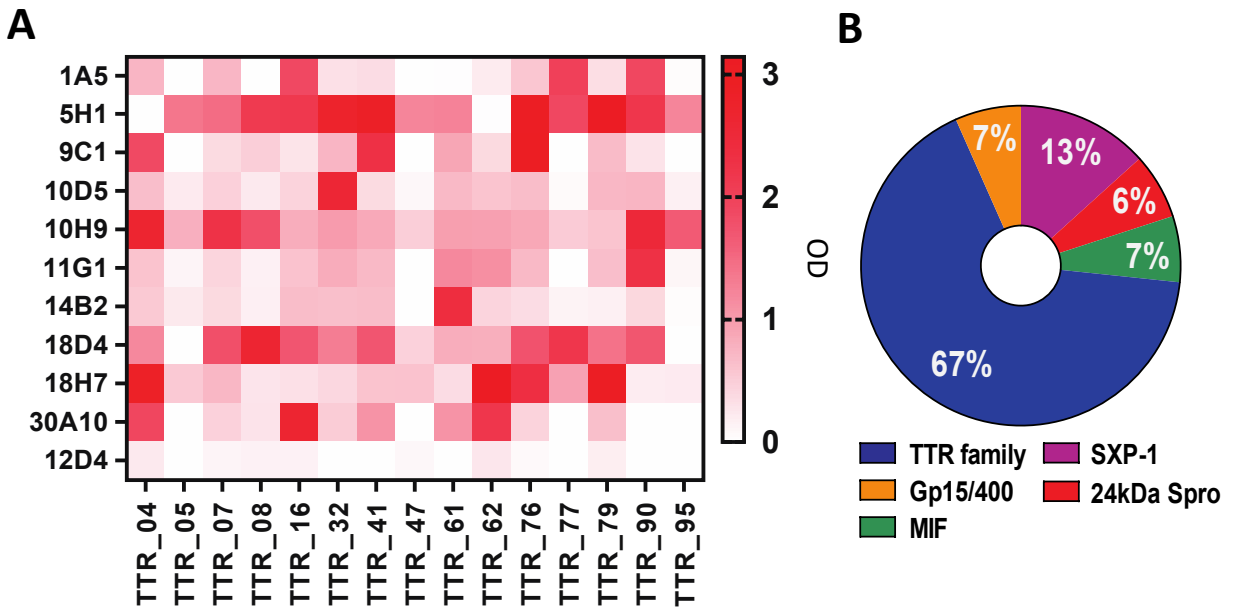


Fig 4-4. TTR family proteins are the prominent filarial antigens targeted by IgE antibodies. A, Semi-quantitative ELISA analysis depicting TTR-specific antibody cross-reactivity profile to multiple TTR proteins. MAb 12D4, a human IgE mAb specific for WbSXP-1, served as negative control. OD, optical density. **B,** Proportion of filaria-specific antibodies binding different filarial antigens.

Although some of the TTR-specific antibodies show binding to multiple TTR proteins, there are differences in the affinity of binding. Representative curves and respective EC_{50} values for antibody binding to each TTR protein is shown in Figure 4-5. 5H1 mAb binds to the TTR_79 with the highest affinity, followed by TTR_76, TTR_41 and TTR_08. 18H7 mAb binds to TTR_62 and TTR_79 with similar affinity followed by TTR_76 and TTR_77. Finally, 9C1 mAb binds to TTR_76 with highest affinity followed by TTR_41.

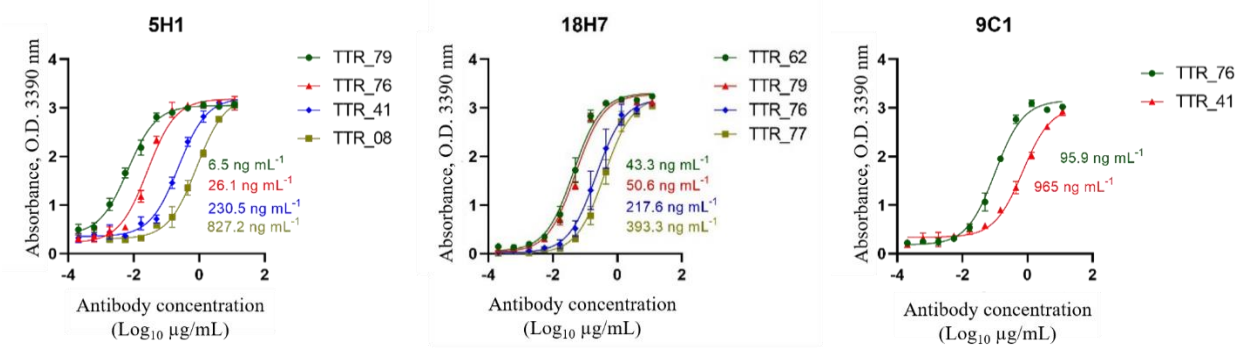


Figure 4-5. Binding of TTR-specific antibodies to different TTR proteins. The ELISA assays using serial dilutions of each antibody against the TTR proteins were performed three times in triplicate. The half maximal effective concentration (EC_{50}) was calculated by curve fitting of the ELISA data. Data are shown as the mean ± SEM.

Recombinant expression of TTR proteins helped me identify more TTR-specific IgE mAbs. So far, I have identified the target protein for 15 IgE mAbs, 10 of which are binding to a member from the TTR family. Together, these results indicate that TTR protein family is the most dominant filarial antigens targeted by the human IgE antibody response (Table 4-1).

Table 4-1. List of filarial proteins targeted by human IgE mAbs

IgE mAb	Subject code	ImmunoCAP reactivity (kUA/L)	Binding to filarial worm extract		EC_{50} (ng/mL)	Mass spectrometry	Antigen name	Sequence ID
			ELISA	Western blot				
12D4	P3	5.3	+	+++	24.6	WbSXP-1	Antigen WB14	AF063940.1
4E1	P3	0.5	-	+++	144.6	WbSXP-1	Antigen WB14	AF063940.1
9C1	P3	1	+	+++	95.9	TTR-76	?	VDM14676.1
12C2	P3	0.3	+	+++	95.9	TTR-76	?	VDM14676 .1
5H1	P1	1.4	++	+++	6.5	TTR-79	?	EJW78979 .1
18H7	P1	0	+	+++	43.3	TTR-62	?	EJW86262.1,
1A5	P1	14.2	++	+++	ND	TTR-77	?	VDM13477.1
10D5	P5	31	++	-	ND	TTR-32	?	VDM07632.1
11G1	P2	4.8	+	-	ND	TTR-90	?	VDM14190.1
10H9	P1	28.6	++	-	ND	TTR-04	?	EJW80404.1
14B2	P1	23.9	+	+++	ND	TTR-61	?	EJW84161.1
30A10	P4	2.5	+	-	ND	TTR-16	?	EJW86116.1
18D4	P1	3.5	+	-	ND	TTR-08	?	VDM13708.1
2E6	P1	465.7	+++	+++	7.7	Ladder protein, gp15/400	Nematode polyprotein allergen-1 (NPA-1)	AAG31482.1
4E9	P3	57.5	+++	-	26.7	Macrophage Migration	MIF-1	EJW88743.1

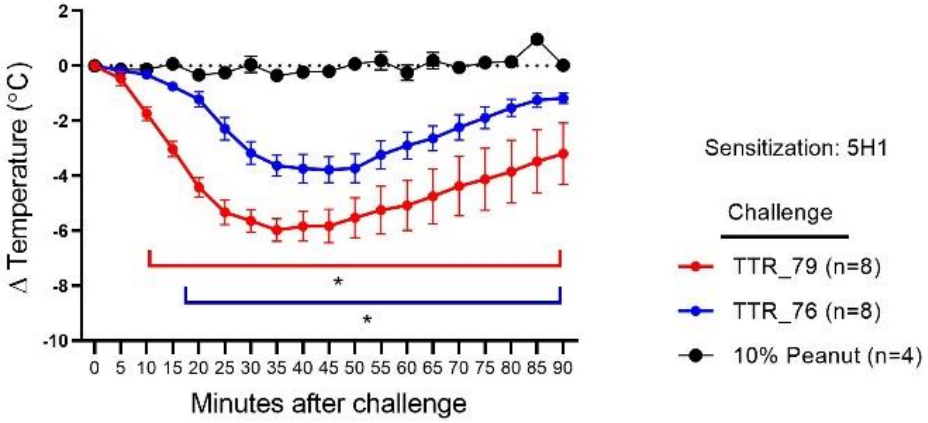
						Inhibitory Factor (MIF)		
11H12	P1	12	++	+++	1.7	24kDa secreted protein	Alt-1, P22	VIO90327.1
ND: Not determined. Due to the low expression of their target proteins EC50 calculation was not possible.*Some of the TTR-specific IgE mAbs (such as 5H1, 9C1 and 18H7) show various levels of cross-reactivity with other members of TTR family, the most prominent antigen is listed.								

Functional Analysis of TTR-Specific IgE MAbs in FcεrIα–Transgenic Mouse Model

5H1 and 18H7 IgE mAbs exhibit anaphylactic efficacy *in vivo* against purified recombinant TTR proteins. I collaborated with Dr. Stokes Peebles' group to examine the functionality of filaria-specific IgE mAbs *in vivo* using a human FcεRIα–transgenic mouse model of passive systemic anaphylaxis. In this model mice expressing the human FcεRIα are first sensitized with an i.p. injection of either 5H1 mAb or 18H7 mAb (100 μg total IgE per injection). These antibodies were selected because of their ability to bind TTR protein dimers, and therefore could in theory cross-link FcεRI to trigger mast cell degranulation. On day three following sensitization, mice sensitized with 5H1 or 18H7 antibodies were challenged via an intraperitoneal injection with recombinant TTR proteins or peanut allergen extract to act as a control. The timing of the challenge injection was determined by previous observations and relate to the timing of peak FcεRIα induction following sensitization with human IgE. In this model drop in core body temperature is measured as a sign of anaphylaxis. Here, mice that were challenged with injection of recombinant TTRs exhibited a significant fall in temperature, compared to the control group challenged with peanut

allergen extract. When we used a cocktail of 5H1 and 18H7 for sensitization of mice instead of one antibody, no synergistic effect was seen between 5H1 and 18H7 IgE mAbs.

A



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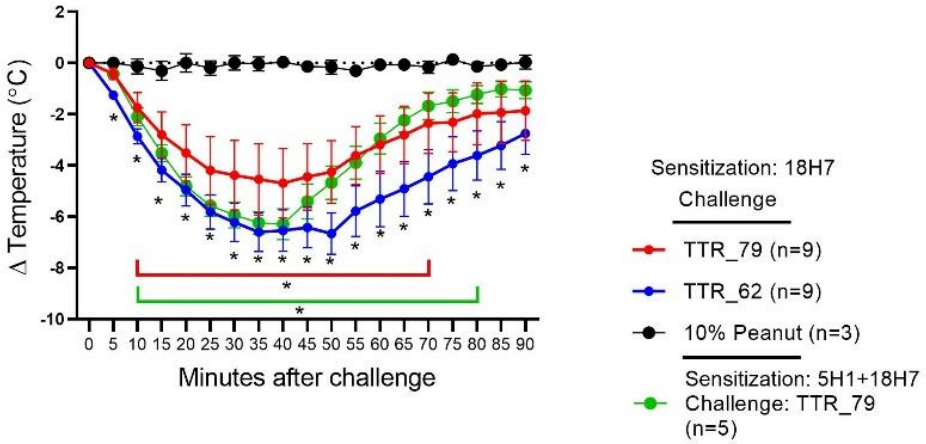


Figure 4-6. Filarial antigens induced anaphylaxis in IgE mAb sensitized human FcεRIα–transgenic mice. Mice were sensitized with a 100 μg i.p. injection of human IgE mAb 5H1 or 18H7 or both. Three days following sensitization, mice were injected i.p. with 50 μg of correspondent antigen TTR_76, TTR_79, TTR_62 or a control allergen (10% peanut extract). The change in body temperature was monitored using an implanted temperature probe for 90 minutes. A paired 2-tailed t test assuming unequal variance was used to compare the change in body temperature of experimental group with the control allergen group at each

time point. Time points with calculated P values less than 0.05 are highlighted by an asterisk. Data are mean ± SEM of each experimental group. There are n mice in each experimental group.

Discussion

Despite the significant increase in the IgE level in the course of parasitic infection, there is very little information about the natural inducers of this antibody isotype. Here, I sought to determine the predominant filarial antigen targeted by the majority of filarial-specific IgE mAbs. In previous chapter I characterized filaria-specifc IgE mAbs and I reported five distinct filarial antigens targeted by human IgE mAbs, WbSXP-1, gp15/400, MIF, 24 kDa secreted protein and TTR family of proteins. As three out of eight filaria-specific IgE mAbs immunoprecipitated a unique member of TTR protein family and since this family of protein has not been reported as an immunogen before, I wanted to take a closer look at other members of this family of proteins. There are 21 protein sequences reported for *W. bancrofti* TTR proteins in NCBI. I initially performed an amino acid sequence alignment of WbTTR proteins to identify any conserved area that might be responsible for binding of these TTR-specific mAbs. As there was not much homology among WbTTR proteins, sequence identity ranging from 18.1% to 70.06%, a phylogenetic tree was assembled using the MUSCLE algorithm. Based on the shape of the phylogenetic tree constructed, 15 different WbTTR protein sequences were expressed recombinantly in SHuffle T7 bacterial cells. The TTR proteins showed variable expression level with TTR_62 and TTR_79 showing the best expression level and TTR_47, TTR_61 and TTR_05 showing the lowest level of expression. I tested all IgE mAbs obtained from patients with filarial infection against recombinant TTR proteins regardless of their initial reactivity profile against filarial worm extracts in hope of finding those TTR-specific IgE mAbs that were missed in the primary screenings either due to the low

concentration of a specific TTR protein in whole worm lysate or due to their low affinity for the antigen. I was able to identify 10 TTR-specific IgE mAbs, some of which showed cross-reactivity against different TTR proteins. IgE mAbs 5H1 and 18H7 are probably the most interesting ones due to their broad cross-reactivity against 13 different TTR proteins despite their marginal amino acid conservation. The half maximal effective concentration (EC_{50}) values for 5H1 binding to different TTR proteins range from 6.5 ng/mL to 827.2 ng/mL. In the case of 18H7 the EC_{50} of binding to different TTR proteins range from 43 ng/mL to 400 ng/mL.

It has long been a debate over IgE mechanism of action in helminth infection. It is shown that intestinal IgE antibodies can block *Trichinella spiralis* attachment to intestinal epithelium of infected rat. So, they propose that mast cell degranulation appears to be a dispensable part of the intestinal IgE function [242]. There is evidence that IgE and eosinophils act together in the immune response against parasites. For example it is shown that IgE induces ADCC directed against *Schistosoma mansoni* larvae in vitro. The proposed mechanism of action is claimed to be activation of eosinophils via IgE binding to FcεrI and eosinophil degranulation not mast cell activation [243]. Another study has shown the *in vivo* protection of rats from *S. mansoni* infection as a result of IgE-mediated platelets activation. [244]. Furthermore, it is shown that filarial parasites at all life stages can be killed by ADCC *in vitro*. It is proposed that FcεrI bound IgE antibody attaches to the parasite surface, recruiting immune cells such as macrophages, eosinophils and neutrophils to the site of parasite infection, so they can kill parasites through secretion of toxic mediators. However, the exact *in vivo* mechanism of action is not known [245]. Here we show for the first time that TTR-specific IgE mAbs function potently and can induce anaphylaxis like symptoms by engaging FcεRI receptors on the surface of mast cells. Our *in vivo* studies show that FcεRI transgenic mice that were sensitized with 5H1 mAb displayed reduction in body temperature following challenge with

either TTR_76 or TTR_79. Similarly, 18H7 mAb induced body temperature drop when mice were challenged with TTR_79 or TTR_62. Our findings provide further insight about the mechanism of action of human IgE antibodies developed in response to helminth infection.

Together, these results show that all IgE targeted filarial antigens were excretory secretory proteins (ESP), with a family of previously uncharacterized proteins, the transthyretin-related antigens (TTRs), acting as the dominant inducer of the filaria-specific IgE antibody response. The ability of TTR-specific mAbs to engage FcεRI receptors on the surface of mast cells further emphasizes the importance of this family of proteins.

Methods

Phylogeny of WbTTR proteins. Amino acid sequences of all TTR-52 domain-containing proteins reported for *W. bancrofti* were derived from National Center for Biotechnology Information database (NCBI) using TTR52 domain accession number pfam01060 and filtering for *W. bancrofti* sequences. Phylogenetic analysis and phylogeny estimation were performed using the web-based software tool MAB (Methods and Algorithms for Bioinformatics), and the maximum-likelihood phylogenetic tree was constructed; for this purpose the MUSCLE algorithm was used. To reach a more rational phylogenic tree, partial and repetitive sequences reported in NCBI were not included.

Production of TTR-specific IgE mAbs. Purified IgE mAbs, designated 1A5, 5H1, 9C1, 10D5, 10H9, 11G1, 14B2, 18D4, 18H7, 30A10 and 12D4, were produced by hybridoma cell expression and were used for immunoblotting assay, EC_{50} studies, cross-reactivity studies, and functional studies.

Cloning, expression and purification of recombinant TTR proteins. Recombinant His-tagged TTR proteins produced in bacterial expression system were used for Western blot analysis, EC_{50} studies, cross-reactivity studies, and functional studies.

Recombinant filarial protein expression was described previously in Chapter III, but in brief, DNA sequences encoding Transthyretin related proteins (GenBank: EJW82905.1 [TTR_05], EJW84161.1 [TTR_61], VDM07632.1 [TTR_32], EJW80404.1 [TTR_04], EJW78295.1 [TTR_95], EJW86262.1 [TTR_62], EJW85307.1 [TTR_07], VDM14847.1 [TTR_47], EJW86116.1 [TTR_16], EJW78979.1 [TTR_79], VDM13477.1 [TTR_77], VDM14190.1 [TTR_90], VDM14676.1 [TTR_76], VDM13708.1 [TTR_08], and VDM08841.1 [TTR_41]) were amplified from a synthetic gene construct (Genscript) and were inserted into the expression vector pET-28a (with an N-terminal hexahistidine tag). The constructed plasmids were used to transform SHuffle® T7 Competent E. coli (NEB, C3029J). Transformed bacteria were incubated at 37ºC while shaking at 220 rpm until reaching OD (at 600 nm) of 0.5-0.8. After inducing protein expression (1 mL 1 M IPTG) hours, cultures were grown for 20 hrs at 16°C while shaking at 220 rpm. Bacterial cells were removed by centrifugation and after cell disruption in Avesti EmulsiFlex C3 benchtop homogenizer proteins were purified by metal affinity chromatography using TALON resin (Takara, 635504).

Cross-reactivity analysis of TTR-specific antibodies. ELISAs were performed to semi-quantitatively assess binding of TTR-specific mAbs to different TTR proteins. Recombinant TTR proteins were coated to ELISA plates overnight at 4°C at a minimum concentration of 10 µg/mL. After blocking, 25 µL of primary antibody was incubated for 1 hour at a concentration of 2 ng/µL in blocking buffer. Bound antibodies were detected using HRP-conjugated mouse anti–human IgE Fc secondary antibody (Southern Biotech, 9160-05, 1:1,000 dilution) and 3,3′,5,5′-

tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific). Color development was measured at 390 nm after 10 min using a Molecular Devices plate reader. Reported OD values are after subtraction of signal from wells containing block. The 12D4 mAb served as negative control.

Half maximal effective concentration (EC_{50}) analysis. The experiments to determine EC_{50} value was performed as described previously in Chapter III. Briefly, ELISA plates were coated with the purified TTR protein at estimated concentration of 10 μg/mL, after blocking, For each experiment repeat, three-fold dilutions of IgE mAb was prepared in triplicate starting from 12 μg/mL. MAb dilutions were added to the wells, followed by 1 hour incubation of 1:1000 dilution of an HRP-conjugated mouse anti–human IgE Fc secondary antibody. After incubating plates with substrate solution (TMB) for 10 min, color development was measured at 390 nm using a Molecular Devices plate reader. To calculate EC_{50} values on Prism software (GraphPad) mAb concentration was log-transformed and a sigmoidal dose–response nonlinear regression model was fit.

In vivo efficacy of TTR-specific IgE mAbs 5H1 and 18H7. Experimental groups human FcεRI–transgenic mice [B6.Cg-Fcεr1α^{tm1Knt} Tg(FCER1A) 1Bhk/J] (stock 010506) were used in this study. Specific characteristics of transgenic mice were explained previously in Chapter III. Mice were passively sensitized with i.p. injection of 100 μg total of either 5H1 (n=20), 18H7 (n=21) or a cocktail of 50/50 5H1 and 18H7 (n=5) IgE mAbs and challenged three days later by i.p. injection of 50 μg purified recombinant TTR protein of interest or 10% peanut extract as negative control. Mice sensitized with 5H1 IgE mAb were challanged with TTR-76 (n=8), TTR-79 (n=8), and 10% peanut extract (n=4). Mice sensitized with 18H7 IgE mAb were challanged with TTR-62 (n=9), TTR-79 (n=9), and 10% peanut extract (n=3). Finally, mice sensitized with a cocktail of 50/50 5H1 and 18H7 (n=5) IgE mAbs were challanged with TTR-79, due to the strong binding of both

5H1 and 18H7 IgE mAbs for this TTR protein. Mice core body tempreature was monitored for 90 min using implanted temperature probes and any significant tempreature drop was recorded.

Statistics. For ELISA studies, data from three repeats of assays performed in triplicate were used to calculate mean relative fluorescence units. The descriptive statistics mean ± SEM was determined. To calculate EC_{50}, I log-transformed antibody concentrations and did fit sigmoidal dose-response non-linear model. Statistical analysis of ELISA was performed using Prism v9.0 (GraphPad) software. In mouse studies, a paired 2-tailed t test assuming unequal variance was used to compare the change in body temperature of experimental group with the control allergen group at each time point (Microsoft Excel Office Professional Plus 2016). Time points with calculated P values less than 0.05 were labeled and considered significant.

CHAPTER V

DEVELOPMENT OF FILARIASIS DIAGNOSTICS USING RECOMBINANT FILARIAL ANTIGENS

Introduction

Lymphatic filariasis affects more than 50 million people and is currently endemic in 72 countries. Additionally, over 800 million people live in endemic areas and are at risk of developing LF. Lymphatic filariasis is a mosquito-borne infection which makes it much harder to control the spread of this disease. As WHO currently has an ongoing program to eliminate lymphatic filariasis as a public health issue by 2020 (GPELF), further emphasizes the importance of developing diagnostic assay for LF to monitor the progress of GPELF activities. Traditional laboratory diagnosis for LF was relying on microfilaria detection via microscopic examination of stained thick blood films [116], but the inconvenience of nighttime blood sample collection, limited sensitivity, and low throughput nature of this assay makes it a an inferior assay to use. There are some serological tests available that are based on crude worm lysate, however, they are not standardized and results are not reproducible. In addition, they are not specific for filarial infections because they can cross-react with phosphorylcholine determinants present in many human pathogens [246]. So, antibody- and antigen-based assays would be the method of choice in assessing endemicity and exposure in children and travelers. In addition, these assays will be crucial for monitoring efficacy of MDA and for post-treatment surveillance program.

Circulating Filarial Antigens (CFA) diagnostic test is an antibody-based assay that is designed to detect circulating antigens of adult *W. bancrofti* worms. In these tests an IgG mAb, Og4C3, which is generated in response to *Onchocerca gibsoni* antigens is used to capture circulating antigen of *W. bancrofti* [121], [122]. Therefore, these tests are mainly used for detection of active filarial infections in endemic populations. On the other hand, antigen-based assays utilize recombinant

filarial antigens in an ELISA to detect circulating antibodies to filarial infections (mainly IgG4 subclass due to the low concentration of IgE in the serum). The recombinant filarial antigens used in developing these antigen-based assays are antigens WbSXP-1, which shows 84% homology to *Brugia malayi* BmSXP-1 [225], [247] Bm14 [124], [125], BmR1[126] and Wb123[127]. Some of these immunoassays are now available in a rapid test format, so they can be easily used in the field for the diagnosis and surveillance of the filarial elimination program [226], [248]. While these tests are incapable of differentiating between active infection and past infection, they would be great tools to assess endemicity, and to test exposure in children and travelers.

I wanted to develop a new highly sensitive diagnostic test to identify patients with active filarial infection or prior exposure to filariasis using serum samples. I screened for antigens that were targeted by naturally occurring IgE antibodies. I have developed a Western blot test for the detection of filarial-specific IgE in filariasis cases caused by *W. bancrofti*, *B. malayi*, *O. volvulus* and *L. loa* infections utilizing immunodominant filarial antigens expressed recombinantly. These findings show that a mixture of rationally selected antigens can be used to develop an antigen-based test for the diagnosis of filariasis to replace older serological and microscopy tests. Although this assay is presented in the form of immunoblotting assay here, the filarial antigens discovered here can be used to develop a much more sensitive diagnostic tool by using Phadia ImmunoCAP platform.

I acknowledge Dr. Thomas Nutman, NIH, Bethesda, for providing patients serum and *B. malayi* whole-worm lysate used for my study, Dr. Heather Stockdale Walden; University of Florida College of Veterinary Medicine for providing adult male and female *D. immitis* worms, NIAID/NIH Filariasis Research Reagent Repository Center (FR3; Athens, GA; www.filariasiscenter.org) for providing *B. malayi* worms, Dr. Benjamin Spiller; pharmacology

department at Vanderbilt University Medical Center for his help with expression of recombinant filarial antigens, and past members of the Smith laboratory for developing and optimizing the hybridoma technique that allowed me to isolate large panels of human IgE mAbs.

Development of A Western blotting Test using Recombinant Filarial Antigens to Detect Filarial-specific IgE in the Blood of Patients with Filarial Infection

Due to the low concentration of IgE antibodies in the blood of patients, we decided to develop a Western blotting based assay because it offers greater sensitivity compared to ELISA assay. All antigens that we had any proof that are positive in western blot analysis were included in this assay. Another obstacle was to have all antigens at approximate similar concentration of truly folded antigen, which is not the same as having them all at the same protein concentration. So the standardization was done by applying the mixture of IgE mAbs that were originally used to identify these targets (1A5, 2E6, 4E1, 5H1, 9C1, 11H12, 14B2 and 18H7) at concentration commensurate to their binding strength. As shown in Figure 5-1, all recombinant proteins are present in such way that bands are observable and none of them is over exposed. In order to make comparison easier all low expressing TTRs are placed in one Western Blot and the rest of proteins are included in another blot.

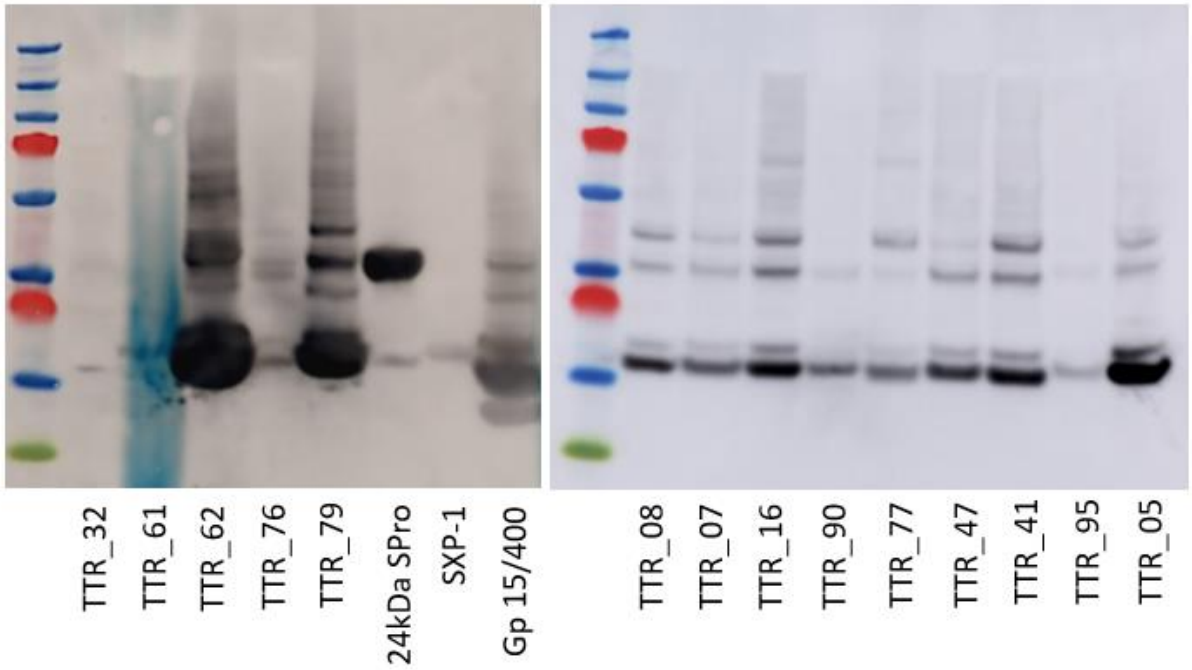


Figure 5-1. Western blot analysis for standardization of recombinant proteins. All recombinant proteins were analyzed by immunoblotting against a cocktail of 1A5, 2E6, 4E1, 5H1, 9C1, 11H12, 14B2 and 18H7 IgE mAbs to ensure that optimum concentration from each antigen is present.

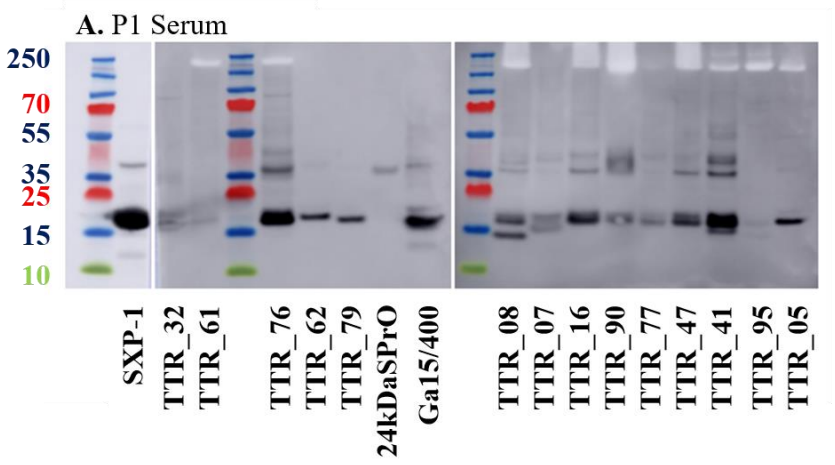
Detection of Filaria-specific IgE Antibodies in Sera from Patients with Filariasis

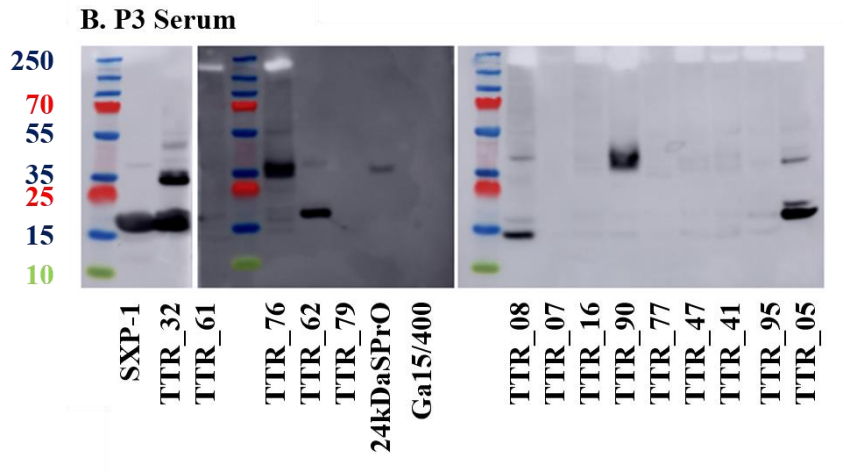
We next sought to test the presence of filaria-specific IgE antibodies in the sera from patients with filaraissis. Recombinant filarial antigens were Western blotted using sera from patients with filariasis and results are given in Fig 5-2. Presence of a clear band around the known molecular weight for each antigen or the molecular weight of its dimer form in the Western blot was considered positive. We had access to sera samples from five different patients with the clinical record of filariasis, three of which we had PBMC samples from and produced IgE mAbs from. IgE mAbs that were obtained from P1 and their correspondent antigens are as follows: 1A5 (TTR_77), 10H9 (TTR_04), 11H12 (24kDa secreted protein), 14B2 (TTR_61), 18H7 (TTR_62 & TTR_79),

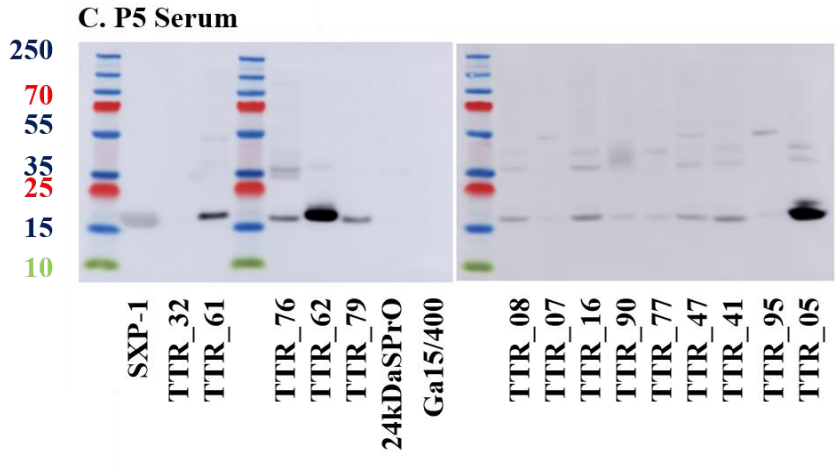
2E6 (Ladder protein, gp15/400), 5H1 (TTR_79) and 18D4 (TTR_08). IgE mAbs that were obtained from P3 and their correspondent antigens are 9C1 (TTR_76), 12C2 (TTR_76), 12D4 (WbSXP-1), 4E1 (WbSXP-1) and 4E9 (MIF). IgE mAb that was obtained from P5 and its correspondent antigen is 10D5 (TTR-32). First, I tested the binding of sera from patients that we had produced IgE mAbs from against recombinant antigens. Therefore, if our diagnostic test works correctly, at least targets of mAbs derived from each patient must be positive in their serum study as well. Two of the serum samples used were from patients with TPE diagnosis that we did not make IgE mAbs from, so they will serve as an unknown group to test our diagnostic method on a random population of patients with filariasis. 100% (5/5) of serum samples were positive for WbSXP-1, which further confirms that prior research was correct in introducing SXP-1 protein as a good mean for detection of filarial infection. 80% (4/5) of serum samples were positive for 24kDa secreted protein, which has never been reported before as a prominent filarial antigen. 20% (1/5) of serum samples were positive for Gp15/400. Looking at TTR proteins as a family together 100% (5/5) of serum samples were positive for at least one of the members of this protein family. Looking at each member of this protein family alone 80% (4/5) serum samples were positive for TTR_05, %40 (2/5) serum samples were positive for TTR_07, 100% (5/5) of serum samples were positive for TTR_08, 80% (4/5) of serum samples were positive for TTR_16, 60% (3/5) of serum samples were positive for TTR_32, 80% (4/5) of serum samples were positive for TTR_41, 80% (4/5) of serum samples were positive for TTR_47, 40% (2/5) of serum samples were positive for TTR_61, 80% (4/5) of serum samples were positive for TTR_62, 100% (5/5) of serum samples were positive for TTR_76, 80% (4/5) of serum samples were positive for TTR_77, 60% (3/5) of serum samples were positive for TTR_79, 100% (5/5) of serum samples were positive for TTR_90, 20% (1/5) of serum samples were positive for TTR_95. Prominent reactivity against TTR family of proteins,

WbSXP-1, 24kDa secreted protein, and Gp15/400, further confirms that antigens discovered here in an unbiased manner can be combined to develop robust diagnostics (Figure 5-2).

In order to provide initial evidence that IgE response induced during parasitic infection is distinct from IgE response elicited by allergic reactions, I used sera from patients with allergy to mold and shellfish as negative controls. While these sera reacted strongly with the allergen extracts mold and shellfish, respectively, none of them showed any reactivity with recombinant filarial antigens. Including sera from allergic patients in this test further proves the specificity of our diagnostic test and refutes the possibility of any glycan based cross-reactivity that might be a concern. In order to make the comparison between serum reactivity in patients with filariasis and allergic control individuals easier I generated a binary heatmap showing patterns of sera reactivity against filarial antigens (Figure 5-3).




B. P3 Serum
250
70
55
35
25
15
10
SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05


C. P5 Serum
250
70
55
35
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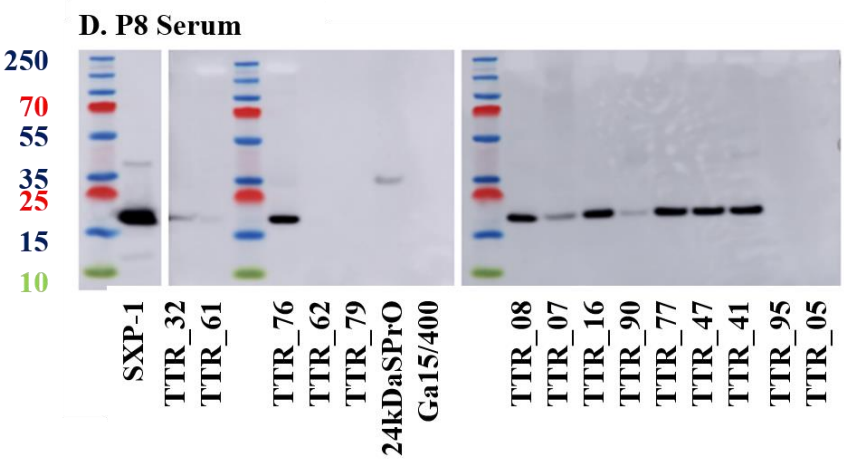
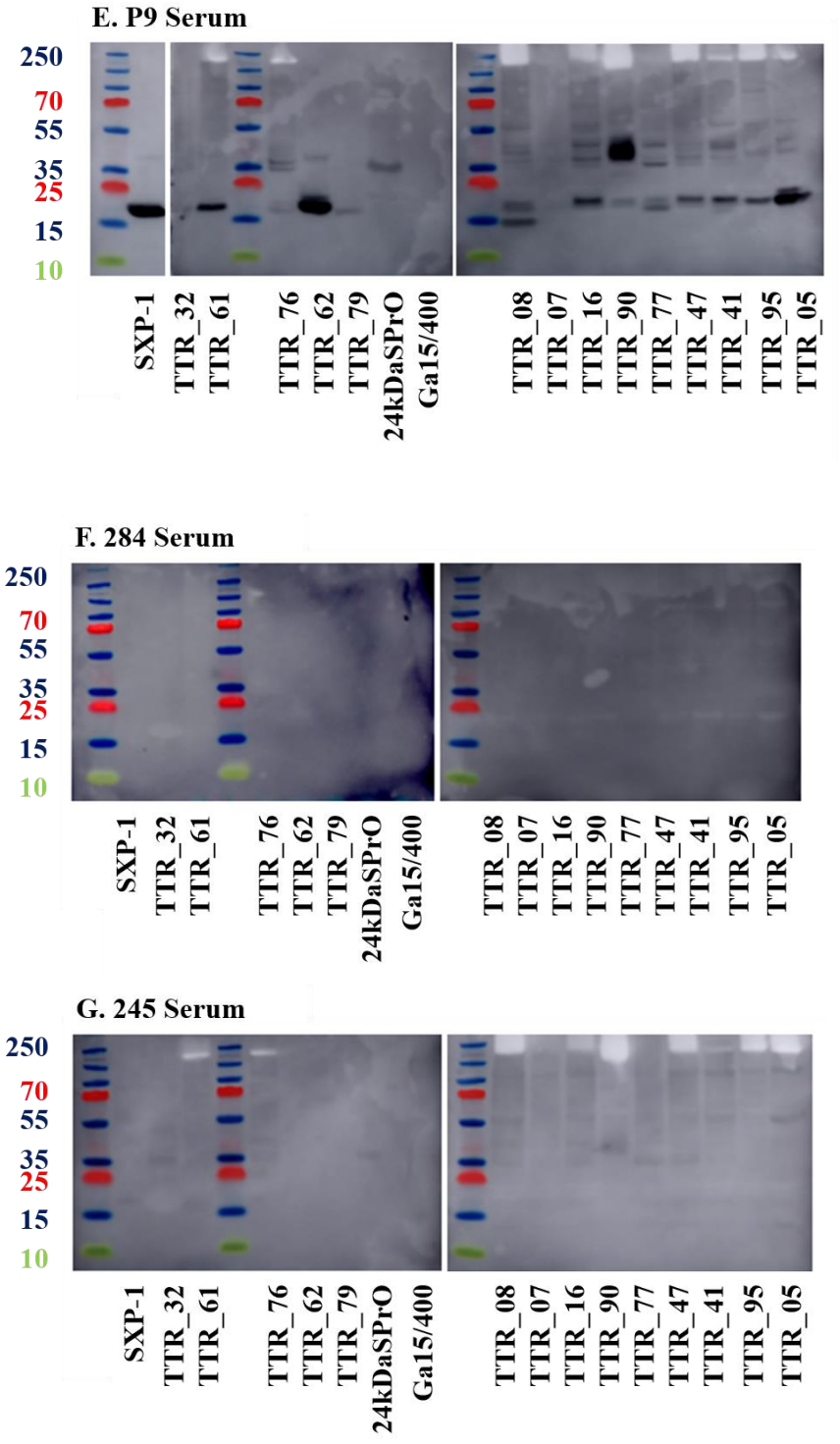
15
10
SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05


D. P8 Serum
250
70
55
35
25
15
10
SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05


E. P9 Serum
250
70
55
35
25
15
10
SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05
F. 284 Serum
250
70
55
35
25
15
10
SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05
G. 245 Serum
250
70
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15
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SXP-1
TTR_32
TTR_61
TTR_76
TTR_62
TTR_79
24kDaSPrO
Ga15/400
TTR_08
TTR_07
TTR_16
TTR_90
TTR_77
TTR_47
TTR_41
TTR_95
TTR_05

Figure 5-2. Western blot analysis of sera from patients with filaraissis against different filarial antigens. Filarial proteins were expressed in T7 SHuffle *E.coli* and used for western blot analysis using sera from patients with filariasis. Sera from patients with mold allergy and shellfish allergy served as negative controls. **A,** P1, **B.** P3, **C.** P5, **D.** P8, **E.** P9. **F.** mold-allergic patient 284, **G.** shellfish- allergic patient 245.

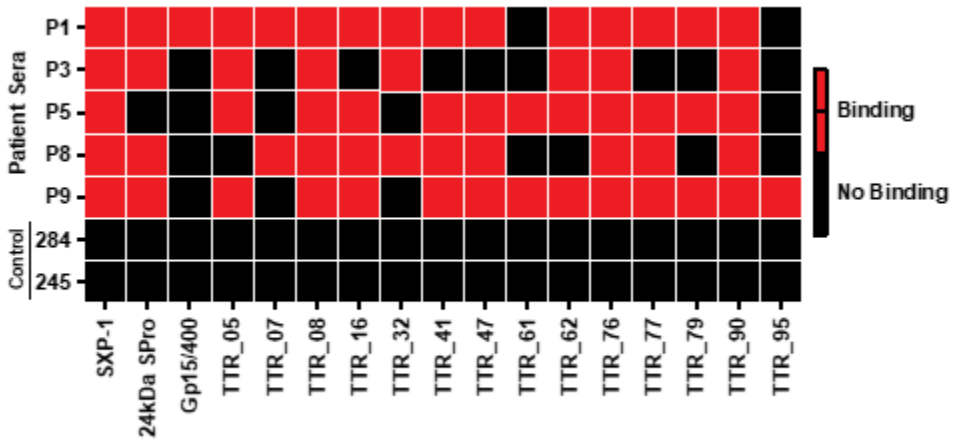

Figure 5-3. Binary heatmap showing distinctive pattern of sera reactivity against filarial antigens. This heatmap shows presence (red) or absence (black) of serum antibody against filarial antigens tested in Western blot. Binding assay were performed using western blot analysis of sera from patients with filariasis against recombinant filarial antigens. Two serum samples from mold and shellfish allergic patients served as negative control.

Here, I report a comparative study on the reactivity patterns of sera from patients with filarial infection against immunodominant filarial antigens using western blot analysis. In order to rule out non-specific bindings, I tested serum from patients with different allergic diseases against filarial antigens and no proteins were recognized.

Discussion

IgE antibodies are known to be a correlate of protection against disease caused by various helminthic parasites as evidenced by epidemiologic data showing that anti-parasite IgE responses can be protective against hookworm infections [249], [250], *Trichuris* [251], *Ascaris* [252], [253] and *schistosomes* [254]–[260]. However a major gap in knowledge in the field of parasitology is the underlying helminth proteins targeted by human IgE antibody response.

Excretory-secretory products (ESP) of the filarial parasites have been used in developing diagnostics. However, the individual components of the ESP remained largely uncharacterized which might be due to the technical/practical constraints and/or low abundance of these proteins. [218], [219]. WbSXP-1 is the only filarial antigen that was used in antigen-based diagnostic tests. Since the discovery of this filarial antigen, a significant number of papers were published trying to implement SXP-1 protein in a variety of diagnostic assays, aiming for higher sensitivity and specificity. For example, this research has evaluated the use of synthetic peptides of WbSXP-1 for the diagnosis of human lymphatic filariasis. [261]. Another study developed a capture assay using high-affinity monoclonal antibodies for WbSXP-1 [262]. Our serological assay further shows that WbSXP-1 is a suitable filarial antigen to be used in diagnostics because 100% of infected individuals showed reactivity to it, while no allergic individual showing positive signal.

In this chapter, I have compared the serum antibody response to filarial infection. I obtained sera from five patients with filarial infection, three of which we have generated IgE mAbs from previously. All the filarial antigens identified, except for MIF, were tested in a Western blot for reactivity against sera from patients with filariasis. The recombinant proteins showed distinct reactivity with the sera from patients with filariasis, whereas no reactivity was observed with the sera from allergic individuals. This study further shows that filarial antigens identified here can be

used in developing filariasis diagnostics with improved sensitivity and specificity. Prospective testing of this concept is underway to develop a true point of care diagnostic by using ImmunoCAP platform combined with the filarial antigens discovered here.

Methods

Research subjects. We analyzed serum samples from 5 subjects with prior history of filarial worm infection and 2 subjects with clinical history of mold and shellfish allergy, as negative control. Subjects P1, P8 and P9 are diagnosed with tropical pulmonary eosinophilia (TPE), while subjects P3 and P5 are diagnosed with loiasis. Of note, PBMC samples from patients P1, P3 and P5 were previously used for generation of IgE mAbs.

Production of recombinant filarial proteins. The design and expression of recombinant filarial proteins for binding studies are described in chapter II. WbSXP-1 protein was expressed in BL21 (DE3) bacterial expression system, while, all other recombinant proteins, TTR family, Gp15/400, 24kDa SPro, were expressed in SHuffle T7 competent *Ecoli* (NEB, C3029J) to ensure the correct formation of disulfide bonds. Gp15/400 or ladder protein exists as a tandem repeat of sequences, so I generated the shortest repeat of sequence for this protein which is 15 kDa.

Production of filarial-specific IgE mAbs. Purified IgE mAbs 1A5, 5H1, 9C1, 12C2, 14B2, 10H9, 10D5, 18D4, 18H7, 11G1, 30A10, 12D4, 4E1, 2E6 and 11H12 from hybridoma cell expression was used for standardization of immunoblotting assay.

Western blot analysis of filarial patient sera. Recombinant filarial antigens were mixed with loading buffer, separated by SDS-PAGE on 4-12% gradient gel (Thermo Fisher Scientific, NP0322BOX) along with a prestained protein ladder (Thermo Fisher Scientific 26616) under

nonreducing/nondenaturing conditions. The antigen components were electrophoretically transferred to PVDF membranes (Thermo Fisher Scientific, LC2005) at 40 V for 150 min. After blocking with 5% nonfat dry milk in PBS overnight at 4°C, membranes were incubated with 1:10 dilution of patient serum or a cocktail of IgE mAbs (1A5, 5H1, 9C1, 12C2, 14B2, 10H9, 10D5, 18D4, 18H7, 11G1, 30A10, 12D4, 4E1, 2E6 and 11H12) in blocking buffer for 1 h at room temperature. The bound antibodies were probed with murine anti–human IgE-peroxidase conjugate secondary antibody (Southern biotech, 9160-05) at 1:1000 dilution in blocking solution and subjected to 1 h incubation at room temperature. Blots were washed 3 times with PBS between each step. Blots were visualized using chemiluminescent substrate (Supersignal Pico; Thermo Scientific, 34577) on an Amersham™ Imager 600.

CHAPTER VI
SUMMARY AND FUTURE STUDIES

Summary

Lymphatic filariasis (LF) is a high morbidity disease known as the second leading cause of long-term disability worldwide. Lymphatic filariasis is a mosquito-borne infection caused by three closely related filarial worms *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori* and is presently endemic in 72 countries. More than 50 million people are suffering from LF, a third of whom show disfigurement. Additionally, over 800 million people live in endemic areas and are at risk of developing LF [78]. Although filariasis control measures, such as Mass Drug Administration (MDA), are already in place, rapid re-infection and the dramatic rise in drug resistance are raising serious concerns. Thus, like with all other helminth infections, there is an urgent need for an effective vaccine. In addition, development of a specific and sensitive rapid diagnostic assay for filarial infection is highly desired as it can be used to measure the prevalence of filarial disease in endemic and nonendemic areas.

While elevated levels of IgE antibodies is the hallmark of the humoral response to helminth infections, including lymphatic filariasis [172], knowledge about the underlying antigenic triggers of this response and their protective function remains limited. Epidemiological data shows that anti-parasite IgE responses can be protective against hookworm infections [249], [250], *Trichuris* [251], *Ascaris* [252], [253] and *schistosomes* [254]–[260]. Early serologic studies introduce IgE antibodies as being ‘nonspecific’[173] and others have focused on the allergen-like proteins such as tropomyosin, to find a link between helminthic disease and allergy [174]. The limitations of these studies stem from the difficulty, if not impossibility, of studying IgE using human immune serum given the complexity and exceedingly low concentration of antigen-specific IgE. The best

way to study the role it plays in the human anti-helminth immune response is to study it as a naturally occurring IgE monoclonal antibody (mAb).

Our group has developed methods to allow for the very first time the identification, amplification, and ultimately the generation of stable cell lines, human hybridomas, from the very rare population of helminth-specific B cells in peripheral blood of infected human subjects. Here, I performed an unbiased study of the IgE antibodies associated with filariasis and identified several filarial antigens targeted by the IgE response with strong diagnostic and vaccine potential. I present here the isolation and characterization of the first panel of human IgE mAbs to filarial worms. I isolated a total of 56 IgE mAbs, 26 of which showed reactivity against filarial worms. The filaria-specific IgE mAbs can be used to standardize filariasis diagnostics and filarial worms extracts. I was able to identify the target antigen for 16 of those filaria-specifc IgE mAbs. In order to find a link between filarial infection and allergic diseases, all IgE mAbs were tested against common allergens but no cross-reactivity was observed.

2 IgE mAbs, 4E1 and 12D4, targeted WbSXP-1, a 15 kDa secretory protein, which is a member of the SXP/RAL-2 protein family. There are proteins from various nematodes that are members of SXP/RAL-2 protein family including almost all filarial worms (BmSXP-1, DiSXP-1, OvSXP-1) and *Ascaris suum* (AS16) [263]. WbSXP-1 has been used in commercial diagnostic tests and in vaccine studies for lymphatic filariasis [226], [227]. Previously, it was reported that patients with filarial infection are only developing IgG4 response to WbSXP-1 [225]. So, here for the first time I am reporting WbSXP-1 as a target of human IgE response.

2E6 IgE mAb targets a protein called ladder antigen-like or gp15/400, which is a surface-associated glycoprotein. [232]. This surface-associated glycoprotein forms a ladder like pattern on Western blot because it is derived from cutting and processing of a precursor of approximately

400 kDa size. 2E6 IgE mAb only reacted towards gpl5/400 of *B. malayi and W. bancrofti* and not against *D. immitis*, which is in line with the results from study showing that the tandem unit sequence for *D. immitis* gp l5/400 is not similar to other filarial worms [233]. The homologue of gp l5/400, in allergens is ABA-1, which is the most abundant protein in body fluid of *Ascaris suum* and is an excretory secretory protein [215], [216]. No putative biological function is evident for this protein.

4E9 IgE mAb targets MIF, which is a major immunological mediator of mammalian inflammatory conditions [236]. MIF proteins from Brugia malayi (BmMIF-1 and -2) are biochemically and immunologically well characterized, and their three dimensional crystal structures are resolved and published. Despite considerable phylogenetic gap between human and filarial worms, the crystal structure and biological activity is highly conserved between a host's cytokine and its parasite.

Ten IgE mAbs, 1A5, 5H1, 9C1, 10D5, 10H9, 11G1, 14B2, 18D4, 18H7 and 30A10 targeted a single family of proteins, which are secretory proteins with approximate molecular weight of 15kDa and with unknown function. TTR proteins are a nematode-specific family. Except from the fact that TTRs are upregulated in the ESP of parasitic nematodes including *B. malayi* [229], this family of protein has never been studied as an immunogenic protein. One study reports that TTR-52 functions as apoptosis mediator in *C.elegans* [230]. Another study on plant parasite nematode *Meloidogyne javanica* reports TTR MjTTL5 protein interfering with host immune system[231]. My data shows that two thirds of filaria-specific IgE mAbs are targeting this family of proteins with two IgE mAbs, 5H1 and 18H7, exhibiting broad cross reactivity across different TTR proteins. Therefore, I would speculate that TTR family of proteins should play an important role in parasite life cycle.

Helminthic parasites are masters of regulation and that is why they have such a long life span in their host, there is a theory that these regulatory molecules are either excretory–secretory products (ESP) or are on the cuticle of worms in direct contact with host immune system [264]. Interestingly, all the filarial antigens identified hare all excretory–secretory products (ESP). So one would speculate that human IgE response is correctly targeting immunoregulatory molecules produced by parasitic worm during the course of infection.

The allergy field has shown that IgE antibodies activate mast cells and basophils via binding to Fc receptors resulting in the release of mediators of inflammation such as histamine, heparin, proteases, leukotrienes and prostaglandins, etc. However, the helminth field has put more emphasis on IgE's role in eliciting antibody-dependent cell-mediated cytotoxicity (ADCC) by monocytes, macrophages, eosinophils [69], [70] and antibody-dependent cell-mediated phagocytosis (ADCP) by macrophages and monocytes [71], [265], [266]. Here, we show that TTR-specific mAbs can induce anaphylaxis like symptoms in FcεRI transgenic mice by engaging FcεRI receptors on the surface of mast cells. Our findings provide proof of concept for filarial vaccine development.

In conclusion, my data suggest that IgE axis has evolved to protect mammals against multi-cellular parasites. Therefore a better understanding of IgE response during helminth infection not only will help us eliminate this disease as a public health problem, but also will help us combat other IgE mediated phenomena such as allergy, a modern world epidemic.

Future Directions: Towards Anti-filaria Vaccines

The key focus of current research is to 1) better understand the humoral immune response to filarial infection, 2) build a strategy for rational vaccine design against filarial worms, 3) develop a more sensitive serodiagnostic assay for filariasis. Based on my results and conclusions, I have proposed

future directions aimed towards rational design of filarial vaccines and diagnostics to eliminate LF as a public health problem.

Immune Response to Filarial Worms in an Endemic Normal Cohort

In Chapter II, I reported isolation of the first panel of human IgE mAbs from patients with filariasis and tropical pulmonary eosinophilia. We used this cohort because of their high level of IgE in the serum, with the hope that it gives us a large population of IgE memory B cells to develop panels of IgE mAbs from. Our data demonstrates that the frequency of IgE expressing B cells range from 6 to 14 cells per 10 million PBMCs. I isolated 56 naturally-occurring human IgE mAbs from patients with filariasis and TPE regardless of their specificity. These IgE antibodies were further tested for their binding to filarial antigens obtained from *B. malayi* and *D. immitis* worms using Western blot analysis, ELISA and *B. malayi* ImmunoCAP and 46% of the IgE mAbs (26 out of 56) were positive in at least one of these screens. Some of these filaria-specific IgE mAbs were further pursued for target identification. I have discussed the limitations of our current strategy, and proposed using *W. bancrofti* as a source of antigen if available. Now that all the methods and techniques are developed and optimized, a future direction for this work would be to study a cohort of endemic normal (EN) individuals in order to characterize the nature of antibody responses that actually protected those individuals from having circulating microfilaraemia. It is already shown that they mount protective T-cell responses to filarial antigens [267]. So, even a serological comparison between a cohort of EN and this cohort would be insightful.

Role of Filarial Excretory Secretory Proteins in Human Immune Response to Helminth

Strategies employed by eukaryotic pathogens (macroparasites) such as helminths to evade immune system are different from those employed by microparasites as they cannot reproduce as fast as such as viruses, bacteria and protozoa or undergo rapid antigenic variation [268]. In contrast to

microparasites that induce acute and short-lived infections, followed by long lasting protective immunity, macroparasites induce gradual chronic infections along with a regulated host immune response over long time periods [269].

In Chapter III and IV, I focused on characterizing the filaria-specific IgE antibodies and identified the major antigens targeted in the course of filarial infection. I have identified 14 distinct filarial antigens targeted by 16 filaria-specific IgE mAbs. Of 16 filaria-specific antibodies, 10 IgE mAbs were targeting a single family of proteins called transthyretin related proteins (TTR). A common theme between these proteins was that they were all excretory secretory proteins. A future direction for these studies would be to identify the function of these proteins and the role they play in parasite life cycle. If all these proteins are present in the secretome of parasites and they are targeted by human immune responses, they must serve an essential function in worm life cycle.

Development of More Sensitive LF Diagnostics

As MDA is progressing towards elimination of LF as a public health issue, there is a renewed interest in developing highly specific and sensitive diagnostic assay for LF to monitor the progress of GPELF activities. Currently, the only filarial antigen used in commercial antibody-based assays is WbSXP-1. My results introduces new filarial antigens, MIF, gp15/400, 24 kDA secretory protein and TTR family of proteins that can be combined with ImmunoCAP technology to develop promising diagnostic tools for this disease.

Development of Filarial Vaccines

Epidemiological studies show that there is a reduced rate of parasitic infection in patients with high level of antigen-specific IgE. The opposing group argue that there is similar amount of IgE in patients with chronic pathology and Endemic Normal (EN) individuals, therefore IgE may not

be the effector arm in anti-helminth immunity. It should be noted that the profound immunosuppression induced by filarial worms can mask the role IgE is playing in defense against parasites.

Exposure to filarial worms induced IgE mAbs that target various TTR proteins, some of which showed broad cross-reactivity towards different TTR proteins. My analysis of two of such mAbs, 5H1 and 18H7, revealed that these mAbs function by binding to the FcεRl present on the surface of mast cells and inducing mediator release similar to the IgE mechanism observed in allergy. I hypothesize TTR-specific IgE antibodies are likely to function in preventing establishment of infection by inducing a type one hypersensitivity response at the site of new parasite entry. A future direction for these studies would be to test this hypothesis in a *Dirofilaria* dog model or in a *Brugia* mouse or gerbil model. Unfortunately, parasite cannot establish patent infection in commonly used inbred laboratory strains of mice (C57BL/6J and BALB/cByJ) as they will eliminated prematurely [270]. Some studies have postulated development of SCID mice as a filarial macrofilaricide screening model to screen pre-clinical candidate macrofilaricides such as ABZ or DEC. [271], [272]. However, some studies show that Mongolian gerbils which are used for production of adult filarial worms can be better animal models for macrofilaricidal drug screening [273] and for testing potential filarial vaccines [274]. Although filarial worms do not complete the full life cycle in them, filarial worms develop from L3 stage to adult male and female worm stage residing in peritoneal cavity and have been used as animal models for *Brugia malayi, Brugia pahangi* and *Strongyloides venezuelensis* infections [275]–[277]. So, I propose administration of recombinant IgE mAbs and their correspondent antigens together and separately followed by subcutaneous (SC) administration of *Brugia malayi* or *Brugia pahangi* L3 stage. Differences in the number of adult

worms developed after 120 days reflects the potential preventive role of IgE antibodies and/or filarial antigens.

Strategic Planning to Control Parasitic Worms

Here, I am reporting a roadmap to tackle filarial infection as a public health issue through a step by step process of studying IgE response in patients infected with this disease, identifying important filarial antigens and then integrating those antigens into developing preventive vaccines and more robust diagnostic tools used in immunosurveillance of endemic areas undergoing treatment. This approach can be replicated to address other parasitic worm infections. In addition, considering the similarities that exist between parasitic worms, which was reflected in the antigens identified here as well, it is possible to find an elimination strategy that can work for the majority of parasitic worms. The impact of this control strategy on public health would be saving billions of dollars in MDA and helping 1.5 billion people who are suffering from some kind of helminth infection. It worth mentioning that parasitic infection is a zoonotic, which means that it can be transmitted to human via animals. So, even though many parasitic infections are eradicated in the developed countries, animals still get infected with parasitic worms regularly and they can transmit those diseases. For example consuming undercooked meat from pigs infected with *Trichinella* can infect human. In addition, consuming water or food contaminated with stool of infected cows and pigs can infect human and cause symptoms such as diarrhea, muscle aches, and fever. So, better understanding of parasitic worm infections can also saves millions of dollars in anti-parasite treatments given to livestock and pets.

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