Exploring the immunology of parasitism--from surface antigens to the hygiene hypothesis

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R. M. MAIZELS*

Centre for Immunity, Infection and Evolution, and Institute of Immunology and Infection Research, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK

(*Received 27 January 2009; revised 9 March 2009; accepted 9 March 2009; first published online 22 May 2009*)

SUMMARY

Helminth immunology is a field which has changed beyond recognition in the past 30 years, transformed not only by new technologies from cDNA cloning to flow cytometry, but also conceptually as our definition of host immune pathways has matured. The molecular revolution defined key nematode surface and secreted antigens, and identified candidate immunomodulators that are likely to underpin parasites' success in eluding immune attack. The immunological advances in defining cytokine networks, lymphocyte subsets and innate cell recognition have also made a huge impact on our understanding of helminth infections. Most recently, the ideas of regulatory immune cells, in particular the regulatory T cell, have again overturned older thinking, but also may explain immune hyporesponsiveness observed in chronic helminth diseases, as well as the link to reduced allergic reactions observed in human and animal infections. The review concludes with a forward look to where we may make future advances towards the final eradication of helminth diseases.

Key words: helminth, nematode, cuticle, immunity, allergy, pathology.

INTRODUCTION

In the past 30 years, all areas of parasitology have been transformed, but perhaps none have emerged from the shadows quite so dramatically as helminth biology. This article presents a personal retrospective of this period, starting from a time before parasite genes or antigens, helper T-cell subsets or cytokines, through to the present day with molecular, biochemical and immunological sciences illuminating many new pathways to control these highly prevalent and damaging diseases.

Helminth parasites include schistosomes (trematodes) and cestodes (such as tapeworms), as well as the nematode roundworms that are the subject of this review. Nematodes include the major gastrointestinal parasites of man (*Ascaris*, hookworm and *Trichuris*) which together infect up to 2 billion people in the world today (Hotez *et al*. 2008); in addition, vector-borne filarial nematodes (e.g. *Brugia* and *Onchocerca*) still infect over 100 million people. To this toll of human health must also be added the immense load of gastrointestinal nematodes in livestock, as well as zoonotic species such as *Toxocara canis* that frequently invade human subjects. Why then has this parasite group not been more urgently prioritized for eradication? To some extent, diseases of developing countries (and generally poorer communities within those countries) were deemed in the past to be less important than those of the Western world. Even in the tropical context, because helminth infections cause relatively little direct mortality, priority was given to diseases with a more lethal profile. Only by calculating the cumulative economic and social costs of disability, growth stunting, fevers and nutritional deficits, can the deleterious effects of helminths be truly appreciated, and appropriate attention paid to the challenge they represent (King *et al*. 2005; Hotez *et al*. 2008).

The fact that a large proportion of infected individuals may, at any one time, appear asymptomatic is also a reflection of a central issue, that helminths can induce a state of immunological tolerance in the host which pre-empts expulsion and, in all probability, minimizes pathological damage. This, together with many other aspects of the complex and varied life histories of nematodes, provides a unique research landscape that mixes molecular and experimental laboratory sciences, with human epidemiology and the ecology of parasite transmission, to describe a dynamic interaction with the host that extends in many cases to overt manipulation of host immunity. Dissecting these relationships from specific molecular partners, to general outcomes at the population level, is an exciting and rewarding enterprise, as may be appreciated from the sections below.

DEFINING PARASITE MOLECULES

While today we have entire genomes with catalogues of parasite proteins, for many years helminth

* Tel: +44 131 650 5511. Fax: +44 131 650 5450. E-mail: rick.maizels@ed.ac.uk

Parasitology (2009), **136**, 1549–1564.
doi:10.1017/S0031182009006106 Printed in the United Kingdom

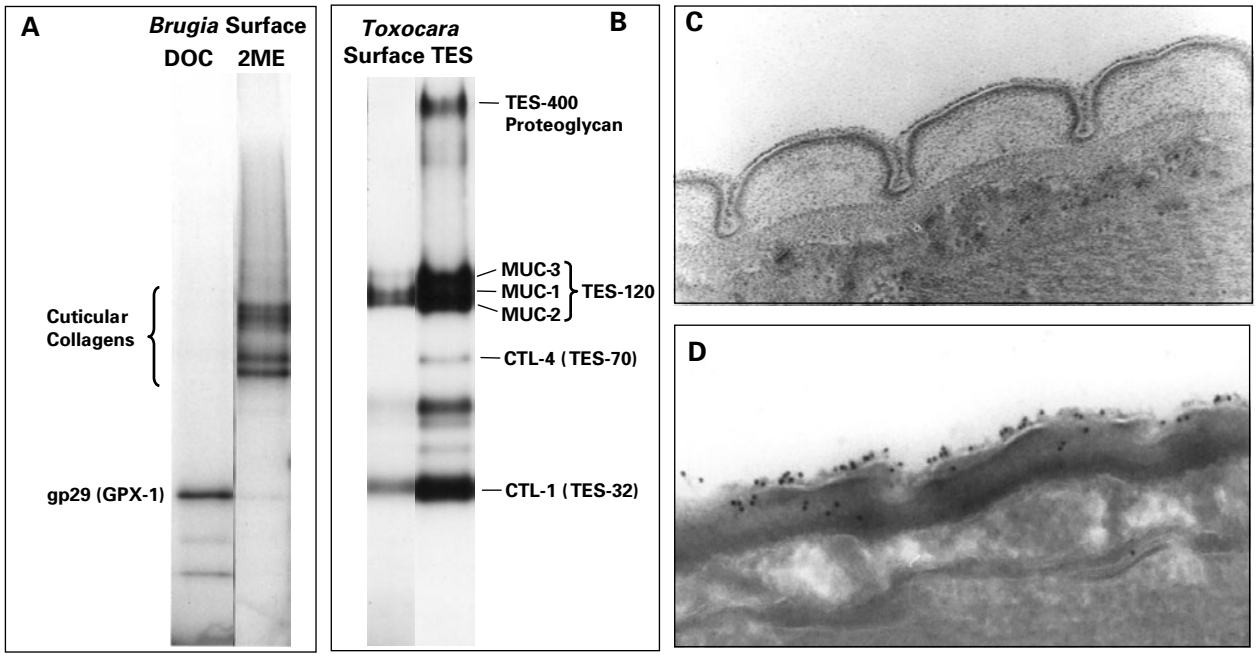

Fig. 1. Surface antigens, secretions and surface coat of parasitic nematodes. (A) *Brugia* adults, surface labelled with iodogen (a non-permeable reagent which iodinates of tyrosine residues) and solubilized in deoxycholate, a non-ionic detergent (DOC) or with SDS and 2-mercaptoethanol (2ME). The major surface glycoprotein, (gp-29, glutathione peroxidase) is released into detergent, while under reducing conditions, the disulphide-linked cuticular collagens are solublilized (Selkirk *et al*. 1989). (B) *T. canis* larvae, surface labelled as (A), or ES proteins labelled with same reagent. Identities to antigens discussed in the text are indicated. Note that the mucins are the major carriers of the dominant trisaccharide glycan. (C) Cryo-electron micrograph showing Ruthenium Red-stained polyanionic surface coat exterior to the cuticle of *T. canis* larvae. (D) Cryo-immunoelectron micrograph localizing binding of monoclonal antibody to the trisaccharide glycan binding to the surface coat (Page *et al*. 1992*b*).

immunology relied on poorly-defined 'antigen' preparations which were generally solubilized whole worm extracts. In comparison, analyses of B and T cell immune responses had already, by the 1970s, progressed to individual epitopes on key pathogenic or model protein antigens (Maizels *et al*. 1980). It has proven a long path to achieve a similar delineation for helminth antigens. Among the very first steps towards the molecular definition of nematode antigens, was made by Bridget Ogilvie who showed that *Nippostrongylus brasiliensis* altered expression of acetylcholinesterase isoforms in accord with the immune status of the host (Jones and Ogilvie, 1972). This work not only identified individual, functional antigens, but demonstrated that parasites could alter antigen expression in response to environmental conditions within the host. There has since been a direct progression, through tracking surface and secreted antigens, cDNA cloning and now proteomics and genomics, to the modern-day cornucopia of antigens from across the entire range of helminth species.

Surface labelling of nematode antigens

A transformational breakthrough in antigen identification came at the end of the 1970s when Bridget Ogilvie and Michael Parkhouse at NIMR introduced surface labelling as a technique for nematodes. Individual parasite antigens could be visualized for the first time, even if at that time identification at the sequence level was still elusive. It was also startlingly clear that the surface-accessible repertoire was limited to a small number of defined proteins (Philipp *et al*. 1980; Maizels *et al*. 1982), as illustrated in Fig. 1 A and B. Further, each stage in the life cycle presented a different and unique set of antigens, which only rarely cross-reacted with other stages (Philipp *et al*. 1981; Maizels *et al*. 1983*a*). In every case, these surface antigens were highly immunogenic in the host, allowing specific serological responses to be mapped, and where tested were also found to stimulate protective immune responses against the parasite (Grencis *et al*. 1986).

With the advent of cDNA cloning, nematode surface antigens were conclusively identified, such as the gp29 glutathione peroxidase from *Brugia malayi* (Maizels *et al*. 1989; Cookson *et al*. 1992), also independently discovered by Eileen Devaney in *B. pahangi* (Devaney, 1988), and the TES-32 C-type lectin of *T. canis* (Maizels *et al*. 1984; Loukas *et al*. 1999), which has homologues in hookworms and other nematodes (Loukas and Maizels, 2000).

In 1981, in collaboration with Felix Partono of the University of Indonesia, we had begun to examine the antigens of human filarial parasites, and

the immune responses to them. We were able to show that all the lymphatic filariae, including *Brugia timori* and *Wuchereria bancrofti*, expressed highly similar sets of surface antigens which were also strongly cross-reactive in terms of human antibody recognition (Maizels *et al*. 1983*a*, *b*, 1986; Morgan *et al*. 1986). These findings sparked curiosity into why there should be antigenic conservation within this set of parasites, rather than the diversity that might be expected under the pressure of the specific immune response. One possible answer is that filarial parasites induced some form of immunogical tolerance, favouring conservation of 'tolerised' specificities (Maizels and Lawrence, 1991).

Labelling experiments also investigated the structure of the nematode surface, which is an unconventional structure with a collagen-rich extracellular matrix bounded by a lipidic epicuticle. Interestingly, in *Trichinella spiralis* the epicuticle is not a conventional lipid bilayer, but is arranged as cylindrical lipid bundles based on a hexagonal pattern (Gounaris *et al*. 1996). In *T. canis*, the fact that most surface antigens were also secreted (Maizels *et al*. 1984) led to the discovery of a labile surface coat, external to the epicuticle (Fig. 1C and D), involved in parasite immune evasion (Page *et al*. 1992*b*).

Secreted ES antigens

In 1975, Don de Savigny published a simple technique for culturing *T. canis* larvae, in serum-free medium, which allowed the collection of significant quantities of 'excretory-secretory' (ES) antigens (de Savigny, 1975). In collaboration with Don, we identified the ES proteins (Maizels *et al*. 1984), as did the group of Bob Grieve (Badley *et al*. 1987*b*), and once cDNA libraries became available each of these antigens were cloned (Maizels *et al*. 2000). *In vitro* biosynthetic labelling of secreted proteins with radioactive amino acids (Parkhouse *et al*. 1985) allowed identification of ES products from species and stages which are available in only small numbers, such as from the infective larvae of *W. bancrofti* (Maizels *et al*. 1986). While the technologies of the 1980s allowed Gek-Eng Kwan-Lim to visualize the broad array of adult *B. malayi* ES antigens (Kwan-Lim *et al*. 1989), nearly 20 years elapsed before these were matched to genome sequence data by proteomics (Hewitson *et al*. 2008). The detection of ES products in the serum of infected humans (Des Moutis *et al*. 1983) and animals (Maizels *et al*. 1985), also proved important in developing diagnostic circulating antigen tests (Weil *et al*. 1997).

T. canis provided an unrivalled model system in which to study the relationship between surface and secreted proteins, because larvae could be recovered from eggs in the laboratory, and cultured at 37 °C for many months in serum-free medium. With Malcolm Kennedy and Huw Smith, we generated monoclonal antibodies which demonstrated that most ES components were associated with the parasite surface (Maizels *et al*. 1987*b*). Tony Page then used these antibodies in immuno-electron microscopy to show that while the C-type lectin TES-32 is integrally distributed in the larval cuticle, the mucin-like glycoproteins (TES-120, see below) are synthesized in internal secretory glands (Page *et al*. 1992*a*) and on release form a loosely-attached surface coat which envelops the parasite (Page *et al*. 1992*b*). Immune attack, for example by eosinophils, results in shedding of the surface coat and parasite escape (Fattah *et al*. 1986; Badley *et al*. 1987*a*). Similar surface coats can be observed in a number of other nematode species (Blaxter *et al*. 1992).

Once identified, the challenge has been to determine the functional properties of ES components. ES material is known to have significant immunological properties; for example, Martin Holland showed that *N. brasiliensis* ES (NES) is an intrinsically potent Th2-inducing adjuvant (Holland *et al*. 2000) while Billy Harnett's laboratory have demonstrated that the major component of ES from the rodent filaria *Acanthocheilonema viteae* (ES-62) directly interferes with host immune cell signalling, offering therapeutic routes to the treatment of immunological disorders such as arthritis (Harnett *et al*. 2005) and allergy (Melendez *et al*. 2007). Many ES products are proteases, fulfilling key roles in invasion and nutrition (Williamson *et al*. 2006), but their profile as immunomodulators is less certain. For example, although NES contains proteases (Healer *et al*. 1991), they are not responsible for its Th2-driving properties (Holland *et al*. 2000). Similarly, while ES-62 is a leucine aminopeptidase, its immunoregulatory activity is attributable to phosphorylcholine modifications and not to enzymatic activity (Goodridge *et al*. 2007).

cDNA cloning and the spliced leader

The second transformational change took place from the mid-1980s with the introduction of molecular cloning of cDNA transcripts (Selkirk *et al*. 1987). Screening *B. pahangi* expression libraries with monospecific antisera, Murray Selkirk's laboratory isolated clones for key surface-associated antigens of microfilariae (MF22, SHP-1) (Selkirk *et al*. 1991) and adults (gp29 and gp15/400) (Cookson *et al*. 1992; Tweedie *et al*. 1993). A new platform for parasite biology and immunology was created with a rapid succession of collagens, muscle and heat shock proteins, enzymes, inhibitors and lipid-binding proteins being identified by investigators around the world. Pleasingly, the Selkirk laboratory also sequenced the acetylcholinesterases of *N. brasiliensis* first discovered by Bridget Ogilvie (Blackburn and Selkirk, 1992; Grigg *et al*. 1997). By differential screening of an *Onchocerca volvulus* cDNA library with sera from

onchocerciasis and lymphatic filariasis patients, Jan Bradley isolated a set of *O. volvulus*-specific clones, providing both immunodiagnostic probes and potential immunomodulators (Bradley *et al*. 1991). Most of the individual parasite proteins studied in the field today were first characterized during this era of discovery.

C. elegans molecular biology was developing rapidly at this time, for example with the discovery of a perfectly-conserved 22-nt spliced leader (SL) sequence which is *trans*-spliced onto the 5′ end of >50% of mRNA transcripts (Blumenthal and Thomas, 1988). David Gems exploited this feature to amplify mRNA from *T. canis* by PCR using SL and oligo-dT (complementary to the poly-A tail at the 3′ end of all mRNAs) primers. He found 2 abundant mRNAs, one of which was a phosphatidylethanolamine (PE)-binding protein (Gems *et al*. 1995), and the other a mucin (Gems and Maizels, 1996), 1 of 3 associated with the surface-coat (Loukas *et al*. 2000*b*). The SL 'trick' was then used to great effect on *B. malayi* by Bill Gregory to discover the *abundant larval transcript* (ALT) proteins (Gregory *et al*. 1997), subsequently shown to be a prominent larval-specific antigen shared by all filarial parasites, and a promising vaccine candidate (Gregory *et al*. 2000). Returning the compliment, Mark Blaxter applied parasite surface labelling techniques to *C. elegans* (Blaxter, 1993).

Era of transcriptomes, genomes and proteomes

The late 1990s saw the development of systematic sequencing of cDNA libraries, termed Expressed Sequence Tags (ESTs). For *B. malayi*, this analysis confirmed that the SL-bearing *alt* transcripts were indeed very highly expressed by L3 larvae (Blaxter *et al*. 1996). More directed cDNA sequencing projects were then aimed to capture major products of *N. brasiliensis* (Harcus *et al*. 2004) and *T. canis* (Tetteh *et al*. 1999), as well as – foraying into cestodes – *Echinococcus granulosus* (Fernández *et al*. 2002). In the case of *N. brasiliensis*, for example, we were able to show that signal sequence-bearing proteins, presumed to include most of the secreted (ES) products, had a greater proportion of novel sequences with no similarity to *C. elegans* or other database entries; this was interpreted as showing a greater diversification among proteins interacting with the host (Harcus *et al*. 2004). With large-scale transcriptomics now accomplished across many members of the nematode phylum (Parkinson *et al*. 2004), a rich new resource for helminth research had been made available.

The size of nematode genomes, typically $5–30 \times 10^7$ bp, has proved an obstacle to full genome sequences until recent advances in high-throughput sequencing. In addition, there are varying degrees of polymorphism within each parasite species (Maizels and Kurniawan-Atmadja, 2002; Redman *et al*. 2008), which can frustrate genome assembly. Nevertheless, a real success in the field has been the publication of the draft sequence of *B. malayi* in 2007 (Ghedin *et al*. 2007). This dataset has allowed us to apply proteomics to nematode ES products; for example, recently James Hewitson and Yvonne Harcus identified some 80 proteins in adult *B. malayi* ES (BES), including a number detailed below (Hewitson *et al*. 2008). For our laboratory, the focus has been on identifying which of these many predicted products and/or known secreted proteins may be immunomodulatory (Maizels *et al*. 2001*a*, *b*). In Fig. 2, and in a later part of this review, I summarize our findings on candidate modulators which have been obtained from a variety of approaches, ranging from *in vitro* testing of recombinant proteins, to heterologous expression by transfection into *Leishmania* for assessment of immunological functions *in vivo* (Maizels *et al*. 2008).

HUMAN FILARIASIS – TOLERANCE AND IMMUNITY

The central immunological feature of filarial infection is antigen-specific hyporesponsiveness, the inverse relationship between the presence of parasites and T-cell reactivity, such that peripheral T cells from microfilaraemic carriers fail to mount a proliferative response to parasite antigen *in vitro*. Maria Yazdanbakhsh's group in Leiden, collaborating together with colleagues at the University of Indonesia, had shown that unresponsiveness ablated IFN-γ and IL-5 responses (Sartono *et al*. 1997), while IL-4 and IL-10 were largely intact. Hence, immunomodulation was relatively selective and targetted 'effector' cytokines rather than the regulatory mediators. Maria's group also showed that responsiveness can be regained following drug treatment, demonstrating that hyporesponsiveness is maintained by live parasites (Sartono *et al*. 1995). Hyporesponsiveness can, as first mooted by Eric Ottesen (Ottesen, 1984) be considered a form of immunological tolerance; these ideas were developed first with Rachel Lawrence, in terms of how tolerance may break down and immunopathology develop (Maizels and Lawrence, 1991) and more recently with Maria Yazdanbakhsh within a wider framework of a regulatory T-cell network (Maizels and Yazdanbakhsh, 2003). The key unanswered question is what factors influence the propensity to develop a tolerance-inducing regulatory T-cell response, or an immunity/pathology-conferring effector response.

In addition to hyporesponsiveness, Eric Ottesen's laboratory also highlighted the extraordinarily high levels of parasite-specific IgG4 antibodies present in microfilaraemic patients (Hussain *et al*. 1987). IgG4 is a poorly-researched isotype which represents <5% of normal serum immunoglobulins, and does not

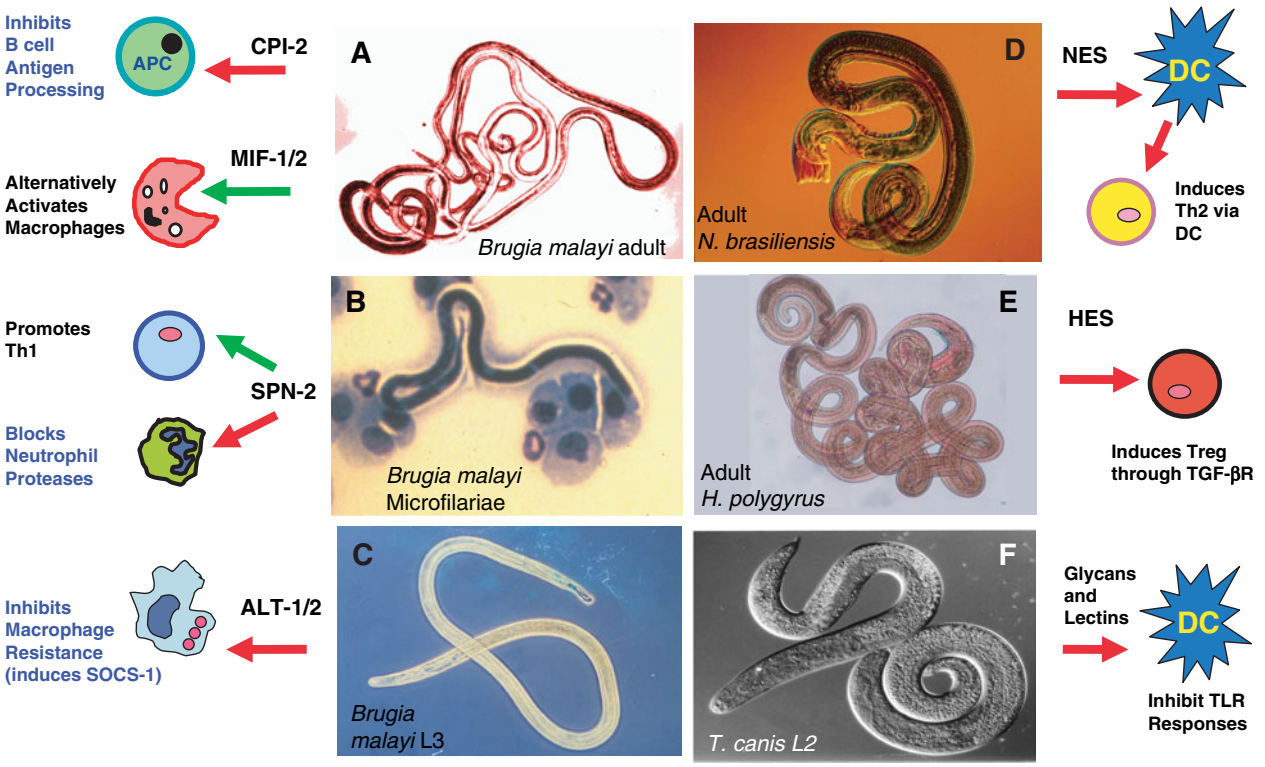

Fig. 2. Immunomodulators secreted by nematode parasites and their host target cells. (A) *B. malayi* adult worms: CPI-2, MIF-1/2. (B) *B. malayi* microfilariae: SPN-2. (C) *B. malayi* infective larvae (L3): ALT-1/2. (D) *N. brasiliensis* adult worms: NES. (E) *H. polygyrus* adult worms: HES. (F) *T. canis* adult worms: glycans and lectins. Images are from our own laboratory, with thanks to Bill Gregory (A), Judith Allen and Andrew MacDonald (B), Bill Gregory and Sinclair Stammers (C), Julie Healer and Sinclair Stammers (D), Constance Finney (E) and Tony Page (F). See text for details of all mediators mentioned.

bind Fc receptors that activate effector mechanisms. In filariasis, however, the antibody response may be dominated by IgG4, and indeed with Karen Day (then at Imperial College) we showed that there is a significant positive correlation between microfilarial counts and IgG4 titres (Kwan-Lim *et al.* 1990). Qualitatively, multiple antigenic species are recognized by this isotype (Kurniawan *et al.* 1998), consistent with a 'blocking antibody' hypothesis that in filariasis effector mechanisms may be disabled by the high concentration of IgG4 antibodies. Recently, Achim Hoerauf's laboratory has established that IgG4 is specifically up-regulated by IL-10, which may be produced by regulatory T cells (Satoguina *et al.* 2005). Hence, IgG4 levels may reflect the degree of immune regulation in a helminth infection, as much as they reflect worm load.

With the development of circulating antigen assays in filariasis, Karen Day applied these to testing epidemiological concepts. Using changes in circulating antigen levels over time to quantify adult worm numbers, and thereby the acquisition of new infections, she showed that adults gained a form of concomitant immunity to incoming larval infections with *W. bancrofti* (Day *et al.* 1991*b*). Together with Bill Gregory, we showed that acquired immunity coincided with non-IgG4 antibody to the surface of infective larvae (Day *et al.* 1991*a*), to a specificity distinct from the major protein antigen components. Hence the antibody profile in a 'tolerant' individual is very different from that of an 'immune' subject, although there is no evidence as yet from human studies that immunity to filariasis is antibody mediated.

NEMATODE ANTIGENS AND MODULATORS

These profound effects on the host immune system spurred interest in the molecular products of helminths which may modulate immunity. The following section, and Fig. 2, summarise the major players identified so far.

Cystatins and serpins

Protease inhibitors secreted by nematodes do not simply regulate activity of parasite enzymes, as 2 major ES protease inhibitor families appear to act as immunomodulators by targetting host enzymes. One set, the cystatins (cysteine protease inhibitors) include CPI-2, a 15 kDa surface and secreted protein of *B. malayi* larvae and adult worms (Gregory *et al.* 1997; Gregory and Maizels, 2008), as well as *O. volvulus* and *A. viteae* (Hartmann and Lucius, 2003). Although homologues from plants to vertebrates inhibit papain-like cysteine proteinases,

only mammals have forms which block asparaginyl endopeptidase (AEP), a key enzyme in antigen processing, through a second inhibitory site. Remarkably, CPI-2 has evolved the same AEP-inhibitory motif, and hence can interfere with antigen processing in human cells (Manoury *et al*. 2001). Consistent with the hypothesis that parasite cystatins have evolved AEP inhibition as an immune interference strategy, Janice Murray showed that *C. elegans* homologues lacked both the sequence motif and AEP inibition (Murray *et al*. 2005). One unsolved puzzle is how CPI-2, secreted from extracellular *Brugia* parasites, accesses intracellular antigen-processing compartments of mammalian cells; an untested possibility is that a unique 20-aa N-terminal extension of the filarial CPI-2 protein, absent from other cystatins, is involved in uptake or entry into host APCs.

In the late 1990s, Xingxing Zang started a project to identify T-cell-stimulating antigens from *B. malayi*. He identified a serine protease inhibitor (serpin) SPN-2, which was also the most abundant transcript from the microfilarial (Mf) stage. In collaboration with a leading serpin laboratory, we found that SPN-2 selectively inhibited 2 neutrophil proteases (Zang *et al*. 1999), but not a range of other serine proteases tested. Subsequently, another group noted that the SPN-2 sequence varied from the consensus for inhibitory proteins, and reported lack of inhibitory activity for SPN-2. Irrespective of this controversy, SPN-2 selectively drives Th1 responses (Zang *et al*. 2000), an interesting finding in view of the fact that *B. malayi* Mf are one of the few helminth forms which induce Th1 *in vivo* (Lawrence *et al*. 1994). SPN-2 is a major target of antibody in infection, and presence of the IgG4 isotype to this antigen can be considered diagnostic of active infection (Zang *et al*. 2000), as antibody levels decay rapidly following chemotherapeutic clearance of parasites. Notably, no *C. elegans* homologues encode a signal sequence, indicating that *B. malayi* has evolved to secrete the serpin for a new function within the host (Zang and Maizels, 2001).

Glycans, mucins and lectins

Carbohydrate specificities play a major role in the immunology of infections. In the case of *T. canis* ES, we found that it comprised approximately 40% carbohydrate by weight (Meghji and Maizels, 1986); subsequent characterization of the glycan structures by mass spectrometry, the first for a parasitic nematode, identified a dominant *O*-linked trisaccharide similar to mammalian blood group sugars, but modified with 1–2 additional *O*-methyl groups (Khoo *et al*. 1991). These trisaccharides have more recently been synthesized (Amer *et al*. 2003) and confirmed as major antigens in infection (Schabussova *et al*. 2007). Monoclonal antibodies reactive to the trisaccharides also bind to the mucin glycoproteins produced by the major larval secretory glands (Page *et al*. 1992*a*), and bind the labile surface coat of the parasite (Page *et al*. 1992*b*). Whether the mucins and glycans interact further with the immune system once they are secreted seems likely but has yet to be documented.

Alex Loukas subsequently discovered, by protein sequencing, that *T. canis* TES-32 was a C-type lectin (CTL), with calcium-dependent carbohydrate specificity (Loukas *et al*. 1999), presaging a much wider presence in helminth secretions of this family of carbohydrate binding proteins (Loukas and Maizels, 2000; Loukas *et al*. 2000*a*). Because immune system receptors such as DC-SIGN are members of the same gene family, the possibility arose that secreted parasite CTLs interfere or compete with host lectins for ligands, thereby blocking host immunity. If so, it would constitute a fascinating role reversal, as similar CTLs are employed in *C. elegans* to mediate immunity against bacterial infection. In a further functional twist, the surface-bound CTL of the marine nematode *Laxus* captures symbiotic bacteria required for sulphur metabolism (Bulgheresi *et al*. 2006). It is notable that while CTLs are prominent in the secretions of *T. canis* and hookworms, they are poorly represented among the filarial parasites; however, adult *B. malayi* secrete an abundant S-type lectin (galectin), which while bearing no structural similarity to CTLs, might serve a similar physiological role *in vivo* (Hewitson *et al*. 2008).

Cytokine homologues

The immunosuppressive cytokine TGF-β belongs to an evolutionarily ancient extracellular signalling family that also includes bone morphogenetic proteins (BMPs). From observations of filarial parasite hyporesponsiveness, and with identification in *C. elegans* of the TGF-β homologue DAF-7, we hypothesized that *B. malayi* may express a functionally active mimic of TGF-β. Natalia Gomez-Escobar used degenerate PCR to isolate a *B. malayi* homologue (TGH-1), with a 7-cysteine active domain as found in the BMPs, which is primarily expressed in developing larvae (Gomez-Escobar *et al*. 1998). A second homologue more like DAF-7 and human TGF-β in containing a 9-cysteine active domain emerged from ESTs; moreover, transcription of this gene (TGH-2) was maximal in the developmentally-arrested Mf and mature adult stages, which were found to secrete the protein (Gomez-Escobar *et al*. 2000). More recently, we have found ES from *Heligmosomoides polygyrus* contains an active TGF-β mimic able to induce immunosuppressive regulatory T cells (Grainger *et al*., manuscript in preparation). Currently we are testing whether this biological activity is indeed attributable

to a nematode homologue of the mammalian gene so central to immunoregulation.

A second ancient evolutionary family, which encompasses both mammalian cytokines and parasite homologues, is that of the Macrophage Migration Inhibitory Factor, or MIF (Vermeire *et al*. 2008). The first member of this family from *B. malayi* (MIF-1) was reported by Alan Scott's laboratory (Pastrana *et al*. 1998), and Xingxing Zang isolated a second homologue, MIF-2, which was subsequently crystallized in Edinburgh (Zang *et al*. 2002). Because of the apparent contradication for a tissue-dwelling parasite to produce a potentially pro-inflammatory mediator, we examined the activity of MIF in more detail. *In vivo*, MIF was able to induce the production of Ym-1, a key product of alternatively activated macrophages (Falcone *et al*. 2001). Most recently, Lidia Prieto-Lafuente established that in the presence of IL-4, MIF loses ability to induce IL-12 and TNF, and instead promotes the development of these suppressive macrophages (Prieto-Lafuente *et al*. 2009).

Novel proteins – ALTs, VALs and vaccines

Many parasite products involved in establishment in the host will not be recognized or functionally categorized by sequence alone. We were intrigued by novel gene transcripts that are highly expressed in infective larval stages, as judged by their abundance in EST datasets. For *B. malayi*, 2 such families stood out at levels >1%, the abundant *trans*-spliced transcripts *alt-1* and *alt-2* mentioned above, and a Venom Allergen-Like (VAL) homologue similar to *Ancylostoma* secreted protein (ASP).

ALT-1 and -2 proteins are approximately 80% identical to each other. Homologues exist in all filarial nematodes, for example in *O. volvulus* in which Ted Bianco's laboratory showed ALT proteins stockpiled in the glandular oesophagus of the vector-borne infective larva (Wu *et al*. 2004). ALT proteins are the foremost filarial vaccine candidates, with data from several laboratories showing protection in animal models (Gregory *et al*. 2000). We wished, however, to test the immunomodulatory properties of ALT proteins, as the dominant parasite products at the first encounter with the immune system. With Natalia Gomez-Escobar, we adopted the strategy of transfection of *Leishmania* parasites, to allow *in vitro* effects on macrophages and *in vivo* outcomes in mice to be evaluated (Maizels *et al*. 2008). When either ALT-1 or ALT-2 were expressed as a transgene in *L. mexicana*, growth in macrophages was enhanced; cellular mRNA analyses showed higher levels of SOCS-1 expression due to ALT transfection. Because SOCS-1 inhibits IFN-γ signalling, the effects of ALT may therefore dampen Th1 effector mechanisms. Consistent with this hypothesis, the virulence of ALT-expressing lines was significantly amplified, with up to 3-fold more parasites *in vivo* (Gomez-Escobar *et al*. 2005).

Nematode VAL/ASP proteins are similarly elusive in function. Homologues range from pathogenesis-related proteins in plants through to testis-specific proteins in mammals, but have no assigned biological function. It is possible that they represent a conserved structural framework compatible with a variety of functional activities. The first nematode homologue (*Ancylostoma* secreted protein) was discovered by Peter Hotez' laboratory (Hawdon *et al*. 1996), and a closely-related product is now in human vaccine trials. Since then, VAL/ASP proteins have been shown to be dominant ES products of many organisms, such as *H. contortus* (Yatsuda *et al*. 2003). In our laboratory, Janice Murray isolated the *B. malayi* VAL protein and showed it to be highly immunogenic although its vaccine efficacy, under the conditions tested, was less potent than that of ALTs (Murray *et al*. 2001).

Surprisingly, when we applied the transcript-led approach to *T. canis* (Tetteh *et al*. 1999), we found not only the major secreted lectins and mucin apro-protein, but also a set of 4 abundant novel transcripts (ANTs) with similarity to each other only in their 3′ untranslated regions. No protein corresponding to these transcripts could be identified, but 27 nt in the 3′ UTR bore similarity to the *lin-4* suppressive micro-RNA of *C. elegans*. The possibility of post-transcriptional suppression of protein translation was supported by transfection of *C. elegans* with GFP reporter constructs containing the ANT 3′ UTR, which resulted in sequence-specific suppression of GFP expression (Callister *et al*. 2008). Hence, empirical proteomic analysis is crucial before interpreting parasite expression profiles based on cDNA alone.

Phosphorylcholine (PC)

The presence of PC in somatic antigens from *N. brasiliensis* had been discovered in the 1970s (Péry *et al*. 1979). Remarkably, many monoclonal antibodies raised to filarial antigens were PC-specific, and reacted with a circulating parasite antigen (Forsyth *et al*. 1985; Maizels *et al*. 1987*a*). In human patients, PC is found on a high molecular-weight proteoglycan-like complex, while in the rodent filaria *A. viteae*, PC occurs on one secreted protein, leucine aminopeptidase (LAP) or ES-62. More recently, James Hewitson identified that in *B. malayi*, PC is bound not to LAP but to a distinct secreted protein, *N*-acetylgalactosamine transferase (Hewitson *et al*. 2008). Interestingly, in another rodent filarial species (*Litomosoides sigmodontis*), the PC specificity is replaced by dimethylethanolamine (DMEA), containing one less methyl group (Hintz *et al*. 1998). This microheterogeneity in the presentation of PC-like epitopes may relate to differences in

innate recognition systems of the respective host species.

THE METAMORPHOSIS OF NEMATODE IMMUNOLOGY

From the modern perspective, helminth immunology 30 years ago was a blank canvas; infection was associated with eosinophilia, mastocytosis and IgE, but the interconnectedness of these phenomena was not obvious. Mechanisms of immunity were interpreted from *in vitro* assays or by *in vivo* transfer of unseparated lymphocyte populations or infection sera likely to have contained cytokines. The transformation of this field came through both technical and conceptual revolutions. The identification of cytokines and their receptors, monoclonal reagents and gene-targetted (knockout) mice, and the development of fluorescent cell staining and fast cell sorting have all provided precise tools to dissect immunity to parasites. Moreover, with the delineation of Th1 and Th2 in 1986 by Coffman and Mosmann (Mosmann *et al*. 1986), helminth immunology also became conceptually alive.

Nematodes clearly provoked strong Th2 responses, including IL-4 and IL-5, stimulating IgE and eosinophilia. Immunity to gut nematodes was shown to be Th2-dependent by Kathryn Else and Richard Grencis in Manchester for *Trichuris muris* (Else *et al*. 1994) and by Fred Finkelman, Joe Urban and colleagues for other gastrointestinal species with a range of *in vivo* cytokine manipulations (Finkelman *et al*. 1997). Interestingly, different Th2-dependent mechanisms were then found to be involved in immunity to each species studied, with *N. brasiliensis*, for example, being most sensitive to IL-13-induced goblet cell mucin production, and *Strongyloides ratti* expulsion requiring mast cell activation (Maizels and Holland, 1998). Whether Th2 immunity is sufficent against tissue-dwelling nematodes, however, was far less clear (Allen and Maizels, 1997); Rachel Lawrence had shown that *B. malayi* microfilariae in fact drive Th1 responses (Lawrence *et al*. 1994), and IL-4-deficient animals are not more susceptible to infection (Lawrence *et al*. 1995). Mice deficient in IL-4R (and also unresponsive to IL-13) are likewise resistant to *B. malayi* larval infection (McSorley *et al*. 2008). Hence, control of tissue helminth infections requires an important non-Th2 component. With very recent recognition of other T-cell subsets in infection, this issue is now being re-examined.

The Th1/Th2 paradigm also seemed, at first, highly applicable to human filariasis, with a unarguable antigen-specific Th2 bias in infected patients (Sartono *et al*. 1996). However, the paradox remained that Th2 responses often co-existed with active helminth infections, calling into question their protective effect. Moreover, cytokine responses did not accord exactly with expectations, with intact IL-4 responses in the absence of IL-5 (Sartono *et al*. 1997). Two lines of argument could be offered to account for these discrepancies; first, that patients showed a 'modified Th2' similar to that observed in desensitized allergic patients (Platts-Mills *et al*. 2001), in which effector cytokines such as IL-5 are shut down; or that a 'third force' of suppressive, regulatory T cells controlled both Th1 and Th2 responses (Sakaguchi, 2000), and were involved in the maintenance of host hyporesponsiveness to parasite infection (Hoerauf and Brattig, 2002; Maizels and Yazdanbakhsh, 2003). As explained below, both models may apply in parallel.

Dendritic cells and macrophages

In the first encounter between pathogens and the innate immune system, dendritic cells (DCs) shape the ensuing response, through recognition of pathogen molecules and selective induction of T-cell differentiation. A central paradigm is that bacterial LPS, ligating TLR4, upregulates surface costimulator molecule expression and induces IL-12 release, driving a Th1 outcome. Adam Balic asked whether DCs exposed to NES would represent the opposite side of the coin, eliciting a Th2 bias, and indeed this was the case (Balic *et al*. 2004). Interestingly, the capacity to drive Th2 responsiveness was not accompanied by the upregulation of surface markers observed in LPS stimulation, but by blocking the ability of LPS to induce IL-12 p70, as has also been observed for other helminth products (Cervi *et al*. 2004). Because NES actively promotes Th2 differentiation, even in the presence of Th1-inducing bacterial products, we argued against a 'default' hypothesis in which Th2 resulted from an absence of DC stimulation (MacDonald and Maizels, 2008). More recently, with the focus shifting to DC populations *in vivo*, Katie Smith in our laboratory has shown that nematode infection switches intestinal DCs into a predominantly tolerogenic subset.

An unusual innate immune population was discovered by Judith Allen in the peritoneal cavity of *Brugia*-infected mice. These were macrophages which exerted a broad and profound anti-proliferative effect on target cells (Allen *et al*. 1996), and which required IL-4 but not IL-10 to develop (MacDonald *et al*. 1998), While the mechanism of suppression is still unknown, it is cell contact-dependent (Loke *et al*. 2000). This new Th2-associated macrophage was similar to the 'alternatively activated' macrophage identified by Saimon Gordon at Oxford (Gordon, 2003), and was further linked to the synthesis of a chitinase-like molecule Ym1, among other characteristic proteins. From the initial finding in *Brugia*-infected mice, the study of alternatively activated macrophages has grown into a major field bringing together immunity, wound healing, obesity and innate recognition (Loke *et al*. 2007; Siracusa *et al*.

2008). A functional role for these cells in protective immunity against *H. polygyrus*, while in its larval phase in the gut wall, has been demonstrated (Anthony *et al*. 2006). A key question is what stimulates the development of this phenotype, and Lidia Prieto-Lafuente has very recently shown that the combination of IL-4 and MIF (whether from host or parasite) can induce suppressive, Ym-1-expressing macrophages *in vitro* (Prieto-Lafuente *et al*. 2009).

Regulatory T cells and tolerance

The Regulatory T cell (Treg) has represented the latest conceptual tidal wave in immunology, and the concept of a suppressive population inhibiting both Th1 and Th2 was one which resonated immediately in the human filariasis context (Doetze *et al*. 2000). In 2001 Matthew Taylor started testing whether, in an animal model, Tregs blocked Th2 immunity to *L. sigmodontis*. Matt showed that ablation of Treg activity, using anti-CD25 and anti-GITR antibody treatment *in vivo*, significantly reduced worm load (Taylor *et al*. 2005). This was the first immunological intervention able achieve a measure of 'cure' in a susceptible infected host. While anti-CD25 alone abated Treg numbers, additional anti-GITR or anti-CTLA-4 (Taylor *et al*. 2007) were required to kill parasites. Because infected mice displayed antigen-specific hyporesponsiveness, which was reversed by the combined antibody treatment, we suggested that CD25+ Tregs tolerize the effector T-cell population, which requires re-activation through GITR ligation or CTLA-4 blockade (Taylor *et al*. 2007).

Mark Wilson and Constance Finney then studied Tregs in the model system of *H. polygyrus*, well characterized by Jerzy Behnke and David Pritchard at Nottingham. Mark found a significant rise in Treg numbers during infection, which Constance demonstrated was accompanied by raised expression of CD103 (considered to be a Treg activation marker) and increased suppressive effect per cell (Finney *et al*. 2007). Mark Wilson used *H. polygyrus* to explore the interaction between airway allergy and helminth infection, because this parasite does not transit the lungs. Infection resulted in a profound suppression of inflammation (measured, for example, by eosinophil infiltration) even in animals rendered fully allergic prior to infection. Most importantly, CD4+ CD25+ Tregs from infected, allergen-naive animals can, on transfer to an allergen-sensitized but uninfected recipient, suppress airway allergy (Wilson *et al*. 2005). Thus, helminth-induced Tregs were shown functionally to inhibit bystander reactivities, contributing to our perception of the 'hygiene hypothesis' discussed below.

The question remains as to whether helminths stimulate expansion of pre-existing 'natural' Tregs, specific for self antigens, or induce the differentiation of new 'adaptive' Tregs from naive peripheral cells. We suspect that both may occur (Fig. 3). Matt Taylor showed that *L. sigmodontis* activates the natural Treg subset early in infection (Taylor *et al*. 2009), while *B. malayi* can drive bystander (ovalbumin-specific) T cells into a regulatory phenotype (McSorley *et al*. 2008). Most recently, John Grainger has found that *H. polygyrus* ES contains a TGF-β-like activity which, acting through the TGF-β receptor of mouse T cells, can convert non-regulatory cells into 'adaptive' Tregs, confirming that some nematodes at least have evolved to exploit the host regulatory network (Grainger *et al*., manuscript in preparation).

IMPACT OF INFECTION ON ALLERGIES AND AUTOIMMUNITY – REVISING THE HYGIENE HYPOTHESIS

The inverse epidemiological association between helminth infections and allergies been has often remarked upon, but was not well substantiated until Anita van den Biggelaar in Maria Yazdanbakhsh's group showed that schistosome-infected Gabonese schoolchildren had lower Th2-dependent allergic reactivity than their uninfected classmates (van den Biggelaar *et al*. 2000). This very significant work intimated a causal link between infection and reduced allergy which demanded more than a simple Th1 *vs* Th2 model, because schistosomes themselves elicit a potent Th2 response.

Maria Yazdanbakhsh's work was crucial for the reformulation of what had earlier been proposed as the 'Hygiene Hypothesis'. Although originally conceived in terms of diminished bacterial stimulation of Th1 resulting in stronger pro-allergic Th2 responses, the hypothesis required revision to account for mounting Th1-mediated autoimmune diseases in countries with the more aseptic Western lifestyle, as well as the ability of Th2-inducing helminths to counter allergy. By incorporating a regulatory T cell network, able to dampen both Th1 autoimmunity and Th2 allergy, and dependent on extraneous organisms, a new version of the Hygiene Hypothesis emerged (Yazdanbakhsh *et al*. 2001, 2002), which can be integrated with a model of immunoregulation in infection as shown in Fig. 3.

To test this hypothesis, Mark Wilson set up his studies described above and confirmed that nematode-driven Treg expansion could mediate suppression of airway allergy (Wilson *et al*. 2005). A number of other groups have shown reductions in both allergic and autoimmune pathologies in mice infected with helminth parasites (reviewed in Wilson and Maizels, 2004), and abated human autoimmune pathologies in helminth infection have also been reported (Correale and Farez, 2007; Elliott *et al*. 2007). However, the modifying effects of infection are not limited only to parasites, or even only to pathogenic organisms. For example, among

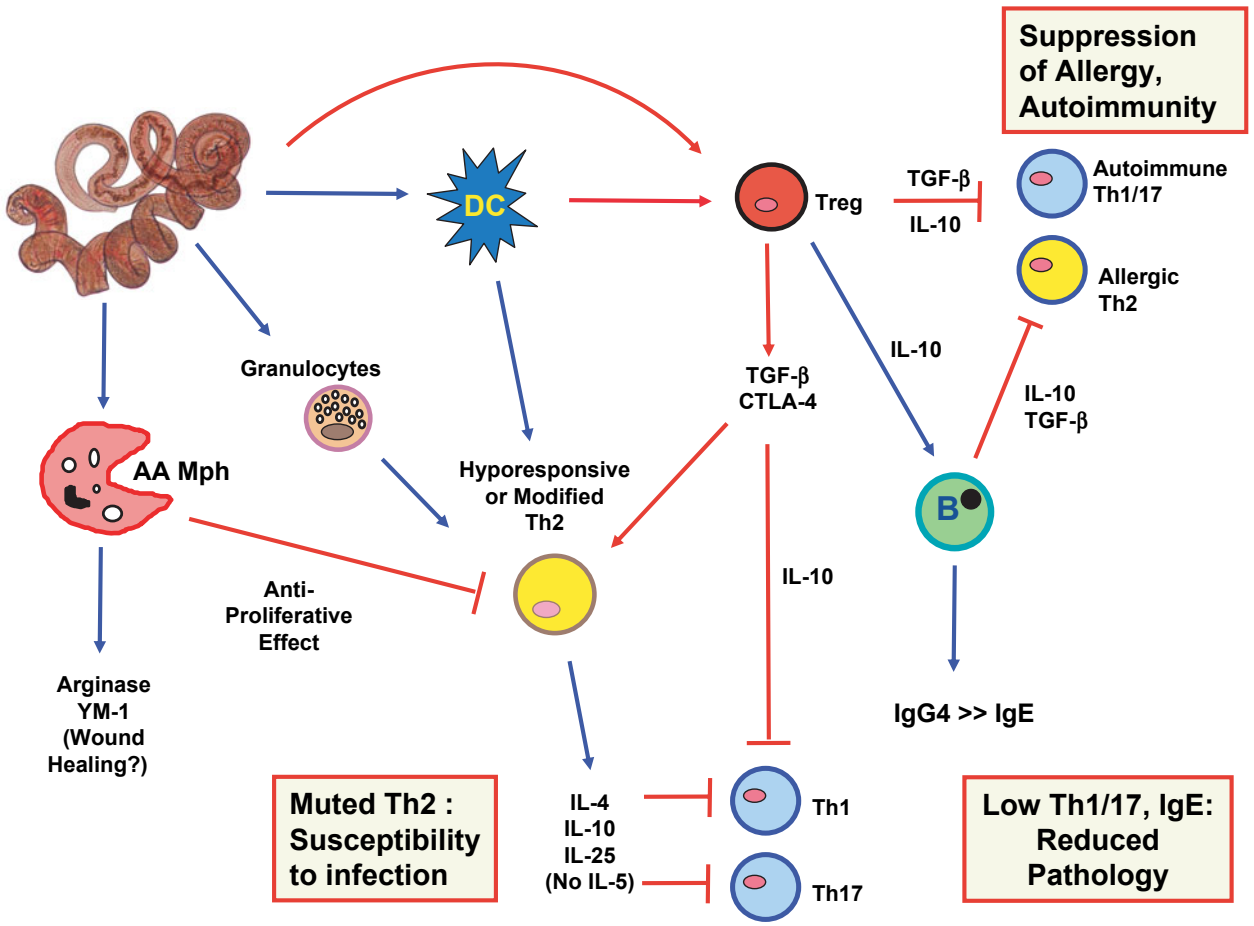

Fig. 3. Immunoregulation in nematode infections and the 'Hygiene Hypothesis'. Infection generates both activating signals (blue arrows), regulatory signals (red arrows) and inhibitory interactions (red bars). Hence, a Th2 response is generated through innate immune system cells, but in chronic infection adopts a modified or muted phenotype in which proliferative responses are poor (due to either or both alternatively activated macrophages and the effects of regulatory T cells). The Th2 response is sufficient, however, to inhibit pro-pathogenic Th1 and Th17 responses, again in combination with Treg activity. IL-10 switches B cells into IgG4 production, reducing IgE, and minimising overall pathology of infection. The high levels of Tregs (induced via DC, or directly by parasite molecules), together with B cells, create a regulatory environment which inhibits pro-allergic and autoimmune T cells from mounting a response. The observed associations between infections and protection from allergies and autoimmunity may be attributed to a regulatory network such as that depicted here.

commensal bacteria, particular species are associated with immunoregulatory protection (Mazmanian *et al*. 2008). Perhaps there is even a parallel between bacterial commensals and the better-adapted helminth parasites, if both have mastered the art of conditioning the host immune system (Maizels, 2005).

CONCLUSION

While in 30 years our knowledge of helminth parasites has changed beyond recognition, the prevalence of the diseases they cause remains a critical problem. Fortunately, the challenge of disease control has now been embraced by agencies such as the Global Progamme for Elimination of Lymphatic Filariasis and the Bill and Melinda Gates Foundation, but there remains great potential to transform the profile of both parasitic and immunological diseases. In particular, the following areas can be highlighted.

First, the new genomes as well as the availability of *C. elegans* genetics, should greatly facilitiate elucidation of biochemical pathways and new drug candidates. A notable achievement of 2008 was the description of new anthelmintics targetting the nematode acetylcholine receptor (Kaminsky *et al*. 2008). More distant, but attractive, drug targets would be immunomodulatory molecules, if neutralization potentiated the immune response to infection. We may also look forward to exploiting parasite immunomodulators as drugs in their own right to ameliorate inflammatory diseases such as allergy and autoimmunity.

Second, the onward march of cellular immunology is generating a constant stream of new models, reagents and tools which are being applied to helminth infections; just as the regulatory T-cell concept could explain an important aspect of the immunobiology of infection, so new cell types, cytokines or

signalling pathways still to be defined will be instrumental in constructing the full picture of the host-nematode interaction. In addition, novel helminth products may lead us to new immune system receptors and pathways which have evolved specifically to combat helminth infections, enriching our knowledge of the immune system itself.

Third, another dividend of the genomic revolution will be the definition of nematode antigens at the level of key epitopes involved in tolerance and immunity. Coupled with the distinction between (for example) parasite-specific and self-specific regulatory T cells, and the identification of nematode-associated molecular patterns that may trigger the innate immune system, vaccines can be fine-tuned to maximize appropriate immune responsiveness and avoid deleterious outcomes (whether excessive pathology or excessive regulation).

It is perhaps overly optimistic to expect that all of these goals will be met in the next 30 years; but with renewed efforts now being placed in treatment of human infections on the global scale, and the speed of scientific advance still accelerating, it is tempting to speculate that with an unrecognizably deeper understanding of helminths, and far stronger array of practical tools for disease control, we will by then be close to eradication of helminths as a major global health problem.

I owe a huge debt of gratitude to all my many colleagues, mentors and students involved in the research described here, first at Mill Hill (1979–1983) then Imperial College London (1983–1995) and more recently at Edinburgh; I have not had room to detail all their many contributions. Space has limited the extent to which I have been able to refer to a great number of contemporary publications in this area. I would also like to thank the MRC and the Wellcome Trust in particular for supporting this research over many years.

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