The L3 of Brugia induces a Th2-polarized response following activation of an IL-4-producing CD4−CD8− αβ T cell population

Julie Osborne and Eileen Devaney
Department of Veterinary Parasitology, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK

Keywords: cellular activation, cytokines, helminth parasites, T_{h1}/T_{h2}

Abstract
Lymphatic filariasis in man is characterized by a profound bias in the immune response. Parasite-specific T_{h1} responses are dramatically down-regulated while T_{h2} responses dominate. We have used the infective larval stage of the nematode parasite Brugia pahangi, a potent T_{h2} inducer in naive mice, to examine cytokine production during the initiation phase of the response. For comparative purposes, the early cytokine transcription pattern elicited by microfilariae (mf), another life cycle stage of the parasite known to induce a primary T_{h1} response, was analysed in parallel. At 24 h post-infection (p.i.) a burst of IL-4 transcription was detected in the draining popliteal lymph node of L3-infected animals. IL-4 was the only cytokine transcript detectable at this early time point and was not present in mf-infected mice. From day 4 p.i. onwards, the L3 elicited a T_{h2} response as defined at the level of cytokine mRNA and protein production by CD4 cells. In contrast, mf stimulate high levels of IFN-γ mRNA at day 4 p.i. in the absence of IL-4 or IL-10 induction. Cell selection analysis indicated that IL-4 produced at 24 h derived from a population of CD4−CD8− αβ T cells. These results suggest that triggering of an unusual double-negative T cell population to secrete IL-4 at the very outset of infection with the L3 of B. pahangi may be the critical factor favouring the development of antigen-specific T_{h2} cells in response to this stage of the parasite.

Introduction
Lymphatic filariasis is a chronic infection of man, caused by the mosquito-borne nematode parasites of the genera Wuchereria and Brugia. The infection is initiated by the bite of a mosquito carrying third stage larvae (L3) in the head and mouthparts. The L3 migrate to the nearest lymphatic vessel where they mature into afferent lymphatic-dwelling adult worms that mate and release first stage larvae or microfilariae (mf) into the bloodstream. When ingested by a feeding mosquito, the mf develop to the L3, which may be transmitted to the human host at the next blood meal.

The infection in humans is characterized by a profound bias in the T_{h1} cell response of infected individuals, such that parasite-specific T_{h1} responses are dramatically impaired, while T_{h2} responses dominate (1–3). This phenomenon is most pronounced in individuals with circulating mf and little or no clinical signs of disease (4). The mechanisms underlying the immune dysregulation in the human population remain poorly understood. Our work has focused on the analysis of immune responses elicited by the L3, as this is the infective form for humans and, in addition, is the likely target of an age-dependent protective immunity (5). In this study we have exploited the capacity of the L3 of the filarial nematode Brugia pahangi to stimulate a strong T_{h2} response in naive mice (6), to examine T_{h} cell polarization during the early period after infection.

Naive CD4 T cells can differentiate into cross-regulatory T_{h1} or T_{h2} effector cell types upon encounter with specific antigen (7,8). Many studies, both in vivo and in vitro, have indicated that cytokines present at the time of T cell priming mediate this differentiation. In particular, IL-12 and IL-4, by acting directly on stimulated naive CD4 T cells in vitro, support the development of T_{h1} and T_{h2} cells respectively.
Basophils (30–34). These results are supported by the observation that in vivo treatment with anti-IL-4 antibody markedly diminished the appearance of T\(_h\)2 cells (13–15). Furthermore, infection of IL-4 knockout mice with the helminth parasites Nippostrongylus brasiliensis (16), Brugia malayi (17) or Schistosoma mansoni (18) resulted in impaired T\(_h\)2 responses. Conversely, the constitutive expression of IL-4 in transgenic mice of Leishmania-resistant background renders them susceptible to Leishmania major infection following activation of a T\(_h\)2 response (19).

Since these studies clearly demonstrate that IL-4 is essential for the development of T\(_h\)2 cells in vivo, the source and the stimulus for the requisite primary production of IL-4 is the subject of intense interest. Recent results from several systems have identified subpopulations of T cells that are capable of prompt IL-4 production in response to a variety of stimuli. These include unusual T cell populations that express the NK1.1 marker, and which comprise CD4\(^+\) (20), CD4\(^+\)CD8\(^-\) \(\alpha\beta\) T cells (21) and NK1.1\(^+\) γδ T cells (22). The majority of NK1.1 T cells are positively selected on class I or class I-related molecules, like the non-polymorphic molecule CD1, and have restricted TCR chain usage (23). In other systems, the early production of IL-4 has been attributed to ‘conventional’ T cell populations including both CD4\(^+\) T cells (24–28) and γδ T lymphocytes (29), although the expression of NK1.1 on these populations was not examined. Furthermore, there are non-T cells sources of preformed IL-4 that could act at the initiation of immune responses, including mast cells and basophils (30–34).

Here we show that the L3 of Brugia induces a rapid (24 h) burst of IL-4 mRNA transcription in a CD4\(^+\)CD8\(^-\) [double-negative (DN)] \(\alpha\beta\) T cell population in the draining lymph node (LN) of BALB/c mice. This response is specific to the L3 and could not be detected following infection with mf. The subsequent response to L3, from day 4 onwards, is exclusively T\(_h\)2 as defined at the level of cytokine mRNA and protein production by CD4\(^+\) cells. This is the first evidence that DN \(\alpha\beta\) T cells promptly express IL-4 mRNA in response to infection with a filarial nematode. The results presented suggest one mechanism by which filarial parasites bias the primary T cell response towards the T\(_h\)2 pathway.

Methods

Parasites and antigen

*B. pahangi* L3 were harvested from infected mosquitoes (*Aedes aegypti*, refm) using standard methods (35), washed in sterile HBSS and counted. Mf were obtained from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) by extensive washing in HBSS and separated from host cells by centrifugation over Histopaque-1077 (Sigma, St Louis, MO). Mf were washed twice in HBSS, resuspended and counted. Soluble extract of *B. pahangi* adult worms was prepared by homogenization in RPMI on ice. The suspension was centrifuged at 10,000g for 30 min at 4°C. The supernatant was sterilized by filtration through a 0.45 µm Spin-X filter unit (Costar, Cambridge, MA), assayed for protein concentration using the BioRad method (BioRad, Richmond, CA) and stored at –70°C until use.

Animals and experimental infections

Six-week-old male BALB/c mice were purchased from Harlan-Olac (Bicester, UK) and were maintained in filter-topped cages. Groups of five to 10 mice were injected with either 30 L3 or 6 x 10\(^4\) mf into each hind footpad. These numbers of parasites were selected based on previous studies with alternative routes of infection (6,36). Control mice were given a sham injection using the same volume of HBSS.

RNA extraction

At specified time points after infection the draining popliteal LN (popLN) were removed, frozen immediately in liquid nitrogen and ground to a fine powder. In most experiments 10–20 popLN were pooled. Total RNA was extracted using Trizol (Gibco, Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions, DNase treated (DNase I amplification grade kit; Gibco) to remove contaminating DNA and quantified by the intensity of ethidium bromide staining on agarose gels.

Cell selections and FACS analysis

In the selection experiments, the popLN were removed at 24 h p.i., teased to a single-cell suspension and incubated with mAb against mouse CD4 (RMA-5; PharMingen, San Diego, CA), CD8 (53-5.8, PharMingen), Thy-1.2 (53-2.1; PharMingen) or anti-TCR γδ (GL3; PharMingen). Cells were magnetically selected using either the appropriate secondary antibody coupled to magnetic particles (Advanced Magnetics, Cambridge, MA) followed by three rounds of exposure to a magnetic field or using the appropriate midi-MACS beads (Miltenyi Biotec, Dusseldorf, Germany), according to the manufacturer’s instructions. During sorting cells were kept at 4°C to ensure mRNA stability. At 24 h p.i. with L3 the T cell population was selected from the single cell suspension using T cell enrichment columns (R & D Systems, Minneapolis, MN). PopLN from L3-infected animals were enlarged at 24 h p.i. and cell recoveries were in the order of 3- to 5-fold greater from these animals compared to HBSS-injected mice. In additional experiments, pop LN were removed from L3-infected or control animals at day 4 p.i. and CD4\(^+\) cells separated using magnetic beads as described before. RNA extraction was performed on the separated populations as before. The purity of the designated selections was assessed using FITC-conjugated rat anti-mouse CD4 (RMA-5 IgG1; PharMingen), FITC–conjugated rat anti-mouse CD8 (53-5.8 IgG2a, PharMingen), FITC–conjugated rat anti-mouse Thy-1.2 (53-2.1 IgG2a; PharMingen) or hamster anti-mouse TCR γδ (GL3 IgG; PharMingen) followed by FITC–conjugated mouse anti-hamster IgG (G192-1; PharMingen) and exceeded 91–100%. Isotype-matched standards FITC–rat IgG1 (R3-34, PharMingen), FITC–rat IgG2a (R2a01; Caltag, South San Francisco, CA), FITC–rat IgG2b (R35-38; PharMingen) or FITC–hamster anti-mouse TCR γδ alone were used to control for non-specific staining. Fluorescence intensity of stained cells was measured on an Epics Elite FACS (Coulter, Miami, FL).

RT-PCR detection of IL-4, IL-10, IL-2 and IFN-γ mRNA

The reverse transcription reaction was carried out in a total volume of 20 µl. 2 µl (1 µg) of random hexamer primers
IL-4-producing DN T cells in Brugia infection

In these experiments mice injected with 30 L3 or HBSS were killed at day 10 p.i. and the popLN draining the infected footpads were removed aseptically. Single-cell suspensions were prepared by forcing the nodes through fine nylon mesh into RPMI (1640 Dutch Modification containing 10% heat-inactivated FCS, 5 mM glutamine, 5 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin, all from Gibco). The number of viable cells was assessed by Trypan blue exclusion. CD4⁺ cells were separated from the total popLN population by positive selection using the mini-MACS system according to the manufacturer’s instructions (Miltenyi Biotec). The effectiveness of separation was determined by FACS analysis at >95%. The purified CD4⁺ cells were then plated out in duplicate 1 ml cultures at 4×10⁵/ml in the presence of 6×10⁵ irradiated syngeneic spleen cells/ml with antigen (10 µg/ml). Similarly unseparated popLN cells from L3-infected mice and uninfected controls were incubated at 1×10⁷/ml in the presence of antigen (10 µg/ml). Culture supernatants were harvested after 48 h and the levels of IL-2, IFN-γ, IL-4, IL-5 and IL-10 were measured by specific two-site ELISA using antibody pairs purchased from PharMingen. Results are expressed as U/ml by reference to commercially produced standards of rIL-2 (Sigma), rIL-4, rIL-5, rIFN-γ (PharMingen) or rIL-10 (Genzyme, Cambridge, MA). The limit of detection for each assay was defined as the mean + 3 SD of 16 wells containing medium only.

Results

L3 and mf induce a contrasting pattern of cytokine gene expression early after infection

BALB/c mice were infected in the hind footpads with L3 or mf and killed 24 h, 4 days or 7 days later. Control animals received an equivalent volume of HBSS. Analysis of cytokine mRNA levels in the draining popLN by semi-quantitative RTPCR revealed a highly stage-specific and polarized response from the outset of infection (Fig. 1). The most striking observation was the early burst in IL-4 transcription within 24 h p.i. with L3. It was the only cytokine transcript detectable at this time point and was not induced following infection with mf. In the representative experiment shown in Fig. 1, there was a 10-fold increase in IL-4 transcription in L3-infected mice over uninfected controls. The increase in abundance of IL-4 transcripts varied from experiment to experiment, but was always in the range of 10- to 20-fold. The full time course with both L3- and mf-infected mice was repeated twice with similar results. In addition, the significant increase in IL-4 expression at 24 h p.i. in L3-infected animals compared to uninfected controls was consistently observed in all the cell selection experiments (see, e.g. Figs 2 and 3). By days 4 and 7 p.i. with L3, IL-4 and IL-10 mRNA levels were elevated, in the absence of any IFN-γ induction. This contrasts with the situation following infection with mf where elevated levels of IFN-γ, but not IL-4 or IL-10, were detected at day 4. By day 7 p.i. with mf the cytokine profile showed a low level of IL-4 and IL-10 transcription (2- to 3-fold more than uninfected controls), but was dominated by high levels of IFN-γ mRNA production (30-fold more than uninfected controls). However,
IL-4-producing DN T cells in Brugia infection

Fig. 1. Cytokine mRNA expression in the popLN at early time points after infection with *B. pahangi* L3 or mf. BALB/c mice were injected with 30 L3 (top), 6×10^4 mf (bottom) or HBSS and popLN harvested at the indicated time points for RNA extraction. RT-PCR, electrophoresis, Southern blotting, hybridization and autoradiography were performed as described in Methods. mRNA levels for each designated cytokine were expressed relative to the level in the popLN of uninfected animals, that were assigned a value of 1. cDNA concentrations were standardized in individual samples by normalizing to the constitutive gene β-actin, that did not show changes >2-fold between compared samples. The results represent one of two comparable time course experiments.

Both mf and L3 induced identical kinetics of IL-2 mRNA induction, peaking at day 4 and rapidly declining to baseline levels by day 7 p.i.

The L3-induced IL-4 burst at 24 h is produced by a CD4^-CD8^- T cell population

In an attempt to define the cellular source of the early IL-4, popLN cells from mice 24 h p.i. with L3 were separated into defined cell populations using specific mAb coupled to magnetic beads. In separate experiments mAb to CD4, CD8 and Thy-1.2 were used to perform the purification. The purity of the designated selections exceeded 91% (CD4), 97% (CD8) or 96% (Thy-1.2) as assessed by FACS analysis. RNA was extracted from the positive and negative fractions and from the unseparated popLN population, reverse transcribed, and the resultant cDNA was analysed for IL-4 transcripts and

Fig. 2. Cellular source of the IL-4 burst at 24 h p.i. with *B. pahangi* L3. BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 24 h. In separate experiments popLN from infected animals were selected into positive (+) and negative (-) populations using mAb to CD4, CD8 or Thy-1.2 coupled to magnetic beads. RNA was isolated from unseparated (US) and separated populations, and analysed for the expression of IL-4 and β-actin mRNA by RT-PCR as described in Methods. Unseparated popLN cells were analysed from uninfected animals (Con). Comparable results were obtained in independent experiments.

Fig. 3. IL-4 expression at 24 h p.i. in the γδT cell-depleted population. BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 24 h. PopLN from infected animals were depleted of γδ cells using a mAb to γδ TCR coupled to magnetic beads. RNA was isolated from unseparated (US) and the γδ T cell-depleted population (γδ-), and analysed for the expression of IL-4 and β-actin mRNA by RT-PCR as described in Methods. Since IL-2 mRNA was shown to be up-regulated in the γδ- population (see Results), analysis of RNA extracted from γδ- cells was omitted from this experiment. Unseparated popLN cells were analysed from uninfected animals (Con). Comparable results were obtained in independent experiments.
IL-4-producing DN T cells in Brugia infection

Fig. 4. Cytokine gene expression by CD4+ and CD4− cells at day 4 p.i. with B. pahangi L3. BALB/c mice were injected with 30 L3 or HBSS and popLN harvested after 4 days. Cells were separated into CD4+ and CD4− populations. Cytokine mRNA analysis was performed on unseparated (US) or separated populations and band intensities were quantified and expressed as described in legend to Fig. 1.

At day 4 p.i. with L3 popLN CD4+ cells express IL-2, IL-4 and IL-10 mRNA

In order to determine whether the burst of IL-4 transcription at 24 h p.i. with L3 results in a Th2 response in the draining LN, a CD4 selection was performed on popLN cells at day 4 p.i. The results of this experiment (Fig. 4) show that CD4+ cells clearly contribute to the IL-4 signal and also to the elevated levels of IL-2 and IL-10 mRNA by day 4 p.i. However, the CD4− population still significantly contributes to the IL-4 and IL-2 signal at day 4 p.i. Consistent with the results of the time course, no IFN-γ signal is detectable in either the CD4+ or CD4− populations at day 4 p.i.

By day 10 p.i. with L3 popLN CD4+ cells secrete T\(\gamma\)2 cytokines exclusively

The results described above indicate that L3 induce a dominant T\(\gamma\)2 response, at the mRNA level, from the outset of infection. However, it was important to confirm whether T\(\gamma\)2 cytokines were actually secreted in response to infection with the L3 by this route. To achieve this, popLN were removed at day 10 p.i., by which time an antigen-specific Th cell response can be detected in vitro upon re-stimulation of lymphocytes. CD4+ cells were purified using mini-MACS columns and the profile of cytokine secretion monitored in response to stimulation with parasite antigen. Figure 5 shows that popLN cells from L3-infected mice produce exclusively T\(\gamma\)2 cytokines and that CD4+ cells were the source of IL-4, IL-5 and IL-10. No antigen-specific IL-2 or IFN-γ was detected (data not shown). Therefore, measurement of cytokine protein
secretion at day 10 p.i. correlates well with the analysis of cytokine mRNA production.

Discussion
The Th2 bias in lymphatic filarial infection is well documented, but the mechanisms underlying this phenomenon are difficult to define in the human population. In this study we employed semi-quantitative RT-PCR to analyse cytokine induction during the first few days of infection of BALB/c mice with B. pahangi L3. Measurement of cytokine mRNA levels in defined cell populations revealed that the L3 stimulates a burst of IL-4 gene transcription from a population of DN (CD4\(^-\)CD8\(^-\)) \(\alpha\beta\) T cells, within 24 h of infection. The cell population producing IL-4 was defined as a DN \(\alpha\beta\) T cell by magnetic cell selections using mAb to CD4, CD8, Thy-1.2 and \(\gamma\delta\) TCR, and by negative selection using T cell enrichment columns. Unfortunately, it was not possible to directly determine IL-4 expression in positively selected \(\alpha\beta\) T cells since magnetic separation using antibodies to the TCR resulted in T cell activation, as evidenced by the up-regulation of IL-2 mRNA, a cytokine that is not produced at 24 h p.i. in the unselected population. The early burst of IL-4 is followed by a polarization of the response in the draining popLN in the Th2 direction, with both IL-4 and IL-10 mRNA levels elevated at day 4 and day 7 p.i., in the absence of any IFN-\(\gamma\) induction. As would be predicted, by day 4 p.i. CD4\(^+\) cells contribute to the increase in IL-4 and IL-10 transcription. Consistent with the gene expression data, CD4\(^+\) popLN cells from L3-infected animals at day 10 p.i. secrete IL-4 and IL-10, but no IL-2 or IFN-\(\gamma\), when re-stimulated in vitro with parasite antigen.

While the focus of the study was on the early response to L3, mice were also infected with mf since injection of live mf has been shown to induce a primary Th1 response at day 12/14 p.i. (6,36). In contrast to the results obtained with the L3, the pattern of cytokine gene expression following infection with mf was dominated at day 4 p.i. by high levels of IFN-\(\gamma\) mRNA, in the absence of any IL-4 or IL-10 transcription. Both L3 and mf elicit a similar pattern of IL-2 mRNA transcription, peaking at day 4 p.i. Since the burst of IL-4 at 24 h p.i. was undetectable in mf-infected mice, we propose that L3-induced early IL-4 production by the DN T cell population is important for stage-specific Th2 development.

This early kinetic analysis of cytokine gene expression has allowed the mechanism underlying the Th2 polarized response elicited by L3 to be clarified. The infection in humans is a constant feature, NK1.1 cells express the distinctive skewed V\(\beta\) repertoire (49), the absolute requirement for NK1.1 marker, it was not possible to confirm whether the DN T cell in this present study was NK1.1. However, preliminary experiments in C57BL6 mice (which express NK1.1) and in BALB/c mice demonstrate that IL-4 is induced in the spleen within 6 h of i.v. injection of L3. Therefore, it should be possible in future studies to determine whether these cells express NK1.1 and/or display the restricted V\(\beta\) usage, and, further, to define the relationship between NK1.1 expression and IL-4 production.

Discussion
IL-4-producing DN T cells in Brugia infection

DN \(\alpha\beta\) T cells are also induced in response to Listeria monocytogenes (47) and the majority of IL-4 producing NK1.1\(^+\) T cells that are activated in response to Salmonella infection are CD4\(^+\) CD8\(^-\) T cells (48). However, although these cells express the distinctive skewed V\(\beta\) repertoire (49), the majority do not bear the NK1.1 marker. This finding may be due to the fact that the surface phenotype of NK1.1 cells is not stable. Although the canonical TCR repertoire remains a constant feature, NK1.1\(^+\) CD4\(^+\) T cells appear to lose expression of NK1.1 and CD4 following activation through their TCR (49). Furthermore, engagement of the NK1.1 molecule on NK1.1\(^+\) T cells is believed to elicit an opposing signal (IFN-\(\gamma\)) to activation of these cells via their TCR (IL-4). However, the absolute requirement for NK1.1\(^+\) T cells in initiating Th2 responses is controversial (50–52) and recent studies suggest that multiple cell types may exist that are capable of providing the stimulus for Th2 responses in vivo (53).

These studies have shown that a DN T cell population is implicated in the early production of IL-4 triggered by infection with the L3. Our future studies will seek to define more precisely the phenotype of the early IL-4-secreting cells using a variety of knockout strains of mice and to identify the stage-specific L3 antigen(s) capable of triggering IL-4 production.
IL-4-producing DN T cells in Brugia infection

Through its prompt production of IL-4 this cell type could represent a form of innate immunity that may play a central role in the Th2 bias in filarial infection.

Acknowledgements

This study was supported by a grant from the MRC. E. D. is a Wellcome Trust University Lecturer. We would like to thank Richard Gencris and Paul Gasride for critically reading the manuscript, Colin Chapman for help with the infections, and Linda Andrews and Brian Willett for help with the FACS analysis.

Abbreviations

Abbreviations

References


1590 IL-4-producing DN T cells in Brugia infection


48 Enomoto, A., Nishimura, H. and Yasunobu, Y. 1997. Predominant appearance of NK1.1+ T cells producing IL-4 may be involved in the increased susceptibility of mice with the beige mutation during Salmonella infection. J. Immunol. 158:2268.


