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Vaccination against filarial nematodes with irradiated larvae provides long-term protection against the third larval stage but not against subsequent life cycle stages

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Abstract

Sustainable control of human filariasis would benefit enormously from the development of an effective vaccine. The ability to vaccinate experimental animals, with reductions in worm burden of over 70%, suggests this aim is possible. However, in experimental vaccinations the challenge is usually administered 2 weeks after the immunisation phase and thus the protection obtained is likely to be biased by persisting inflammation. Using the murine model *Litomosoides sigmodontis*, we increased the time between immunisation with irradiated larvae and challenge with fully infective L3 to 5 months. Significant protection was achieved (54–58%) and the reduced worm burden was observed by 10 days p.i. The developmental stage targeted was the L3, since no nematodes died once they reached the pleural cavity of vaccinated mice, as has been previously shown in short-term protocols. However, larval developmental rate was faster in vaccinated than in primary-infected mice. Immunological assessments were made prior to challenge and then from 6 h to 34 days post-challenge. Samples were taken from the subcutaneous tissue where the larvae were inoculated, the lymph nodes through which they migrate and the pleural cavity in which they establish. Eosinophils were still present although scarce in the subcutaneous tissue of vaccinated mice before challenge. Cytokine and specific antibody production of vaccinated and challenged mice were L3-specific and Th2-biased and greatly exceeded the response of primary-infected mice. The heightened Th2 response may explain the faster development of the filarial worms in vaccinated mice. Thus, long-term vaccination protocols generated a strong memory response that led to significant but incomplete protection that was limited to the infective larval stage suggesting alternative vaccination strategies are needed.

Keywords: Filariasis; Nematode; Litomosoides sigmodontis; Vaccines; Antibody; Cytokine

1. Introduction

Individuals living in endemic areas of filariasis require long-term chemotherapy in order to prevent re-infection and transmission. However, such strategies are difficult to implement on a permanent basis at the population level (Gardon et al., 1997; Molyneux, 2005; Tisch et al., 2005). A crucial complementary approach is the development of an effective vaccine (Hoerauf, 2003). Although immunisation with recombinant antigens has been actively investigated (Fischer et al., 2003; Wu et al., 2004; Perbandt et al., 2005), the most effective protection is still obtained with irradiated larvae. This is consistent with other helminth systems as immunisation with defined antigens of *Schistosoma mansoni* is less effective than with attenuated infective stages (Ganley-Leal et al., 2005).

A variety of animal models have been used to study vaccination against filariasis (Nutman, 2002) and these have relevance to human pathogens because filarial parasites share many biological features including early migration through the lymphatics (Bain and Babayan, 2003). Cross-protection

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between filarial species suggests that even the antigens identified in animal models will be relevant to human infection (Storey and Al-Mukhtar, 1982). Many of the experimental models have been hampered by the inability to develop patent infection in a laboratory mouse, thus limiting the number of tools available for their study (Chusattayanond and Denham, 1986; Abraham et al., 1988; Lucius et al., 1991; Abraham et al., 1992; Bancroft et al., 1993; Lange et al., 1993; Lüder et al., 1993; Taylor et al., 1994; Taylor, 1994). The rodent filaria *Litomosoides sigmodontis* is the only model, which overcomes these obstacles as infection of BALB/c mice with infective L3 progresses through to blood-circulating microfilariae.

Irradiated L3 vaccinations in the *L. sigmodontis* model to date have involved a challenge with fully infective larvae 2 weeks after their last immunisation (Le Goff et al., 1997, 2000b; Martin et al., 2000b; 2001). This strategy routinely leads to around 70% reduction in worm burden. The protective effect is established within 2 days and leads to a reduced percentage of microfilaraemic mice 60 days post-challenge inoculation (Le Goff et al., 2000b). Vaccine-induced protection is abolished when IL-5 is depleted or genetically absent, as well as in B cell deficient mice (Le Goff et al., 1997, 2000a; Martin et al., 2000b; 2001). In addition, degranulated eosinophils are observed at the site of challenge only in mice in which protection has occurred (Martin et al., 2001). Together these data suggest that antibody and eosinophils are the critical players in vaccine-mediated protection. However, because the mice in these studies were challenged within 2 weeks of the last immunisation, there were high antibody and eosinophil levels in the blood and significant numbers of eosinophils in the subcutaneous tissue at the time of challenge (Denham et al., 1984; Wanji et al., 1990, 1994; Le Goff et al., 2000b). The possibility exists that persistent inflammation from the last vaccination dose rather than immunological memory is responsible for the protection observed. We thus designed experiments to test the long-term (6 months) efficacy of irradiated larval vaccines and characterise the immune response they elicit in the *L. sigmodontis*-BALB/c mouse system.

2. Materials and methods

2.1. Infection models

2.1.1. Parasite maintenance and vaccination protocol

The *L. sigmodontis* Chandler, 1931 life cycle was maintained as previously described (Diagne et al., 1990; Petit et al., 1992). Briefly, the filarial worms are maintained in the vector *Ornithonyssus bacoti* and the jird *Meriones unguiculatus* on site. Infective larvae (L3) were collected from the vector, counted and placed in RPMI 1640 medium for inoculation. In permissive hosts *L. sigmodontis* larvae moult 7–9 days p.i., then again 28–35 days p.i. and microfilariae can be detected in the peripheral circulation 55 days p.i.

The vaccination protocol consisted of 3 weekly s.c. inoculations of 25 L3 irradiated in the live vector at an absorbed dose of 450 Gray with a caesium source as previously described (Martin et al., 2001). Control mice were inoculated with sham doses containing medium only. Mice were challenged 5 months after the final immunisation with 40 L3 inoculated subcutaneously in the right lumbar region. Mice that received sham immunisation and were subsequently challenged are referred to as primary infected mice.

2.1.2. Mice

Six to seven-week-old female BALB/*c* mice (Harlan Olac, Gannat, France) were used throughout. All mice were kept in micro-isolators and were given sterilised food and water ad libitum. Groups consisted of vaccinated and challenged mice, vaccinated only mice (or vaccinated mice at time point 0 h, H0), primary-infected and naïve mice. Necropsy time points were set at 6 h, 60 h, 10 days and 34 days after challenge. All procedures conformed to the French Ministry of Agriculture regulations for animal experimentation (1987).

2.1.3. Evaluation of parasite survival and development

Recovery of parasites from the pleural cavity was performed by lavage with 10 ml of cold PBS as described previously (Babayan et al., 2003). The protection conferred by vaccination was based on the recovery rates (number of filariae recovered/number of infective larvae inoculated, F/L3) from primary-infected and vaccinated challenged mice: protection (%) = F/L3 (primary infected − vaccinated) × 100/(F/L3 primary infected). All filariae were measured individually with a camera lucida-mounted microscope.

2.2. Tissue sampling, analyses and reagents

2.2.1. Histology and immunohistochemistry

Three mice of each group—naive, primary-infected at H6 p.i., vaccinated only and vaccinated-challenged at H6 p.i.—were fixed in PBS—10% paraformaldehyde. Five micrometer sections of paraffin-embedded skin and subcutaneous tissue were prepared on slides and stained with Hemalun-Eosin-Safran or Giemsa. Eosinophils and neutrophils were counted at objective × 100 as previously described (Le Goff et al., 2000b) whereas mast cells, which were scarcer in the tissue, were counted with a × 40 objective in two series of 10 adjacent fields.

2.2.2. Cell harvesting, identification and in vitro restimulation

Lymph nodes closest to the inoculation site (right inguinal lymph node and iliac + lumbar lymph nodes) were collected 60 h (H60) p.i. and studied separately. Pleural exudate cells (PleC) were isolated from the pleural lavage as described elsewhere (Babayan et al., 2003). After centrifugation at 1500 × *g* for 6 min at 4 °C, the cells were resuspended in 1 ml RPMI 1640 and counted in 0.04% Trypan Blue in PBS (Sigma–Aldrich) using a Malassez haemocytometer containing 1 mm^3 of sample.

The proportions of eosinophils and neutrophils relative to total cells in the pleural exudate were determined microscopically on cytospin cell preparations: a suspension of 100,000

PleC in 150 μl PBS-1% FCS was centrifuged (500 rpm, 3 min, Shandon Cytospin® 3) against slides, and stained with May-Grünwald-Giemsa. Lymph node cells and PleC were further phenotyped by flow cytometry.

Lymph node cells were cultured in triplicate in 96 well plates at 3×10^5 cells/well in RPMI medium 1640 complemented with 10% FCS, 2 mM L-glutamine (Sigma Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies-GiBco BRL) and stimulated for 72 h with soluble extracts from L3 and mixed sex adult filarial (female worms contained microfilariae) at 10 μg/ml or Con A at 5 μg/ml (Sigma) at 37 °C in 5% CO_2 atmosphere.

2.2.3. Quantitative assessment of cytokine and transcription factor gene expression

Lymph nodes from three primary infected or vaccinated-challenged mice were excised aseptically at H6 p.i., pooled in each group and stored in Trizol® (Invitrogen) at −80 °C until required. The samples were thawed, homogenised and the RNA extracted according to the manufacturer's protocol. Following treatment with DNAse1 (Ambion Ltd, Huntingdon, UK) cDNA was synthesised using MMLV reverse transcriptase (Stratagene, Amsterdam, The Netherlands). Quantification of the cytokines IFN-γ, IL-4, IL-5, IL-13 and of the transcription factors T-bet and GATA-3 cDNA was performed by quantitative PCR using the LightCycler (Roche Diagnostics Ltd) with the following forward and reverse primers, and annealing and reading temperatures, respectively: IFN-γ: 5′-AAGACTGTGATTGCGGGGTTG-3′, 5′-GAGCGAGTTATTTGTCATTCGGG-3′, 60, 86 °C; IL-4: 5′-CCACGGATGCGACAAAAATC-3′, 5′-TGGTGTTCTTCGTTGCTGTGAG-3′, 60, 83 °C; IL-5 5′-CAATGAGACGATGAGGCTTCCTG-3′, 5′-ACCCCCACGGACAGTTTGATTC-3′, 60, 79 °C; IL-13: 5′-GCTGAGCAACATCACACAAGACC-3′, 5′-AGGGAATCCAGGGCTACACAGAAC-3′, 60, 87 °C; T-bet: 5′-GCCAGGGAACCGCTTATATG-3′, 5′-GACGATCATCTGGGTCACATTGT-3′, 50, 84 °C; GATA3: 5′-CTACGGTGCAGAGGTATCC-3′, 5′-GATGGACGTCTTGGAGAAGG-3′, 55, 82 °C; β-actin: 5′-TGGAATCCTGTGGCATCCATGAA-3′, 5′-TAAAACGCAGCTCAGTAACAGTC-3′, 54, 86 °C. PCR amplifications were performed in 10 μl reactions, containing 1 μl cDNA, 0.3 μM primers and 5 μl of Qiagen hotstart *Sybr*green (Qiagen) using the following conditions: 15 min hotstart at 95 °C, 15 s denaturation at 95 °C, 20 s annealing of primers and 15 s elongation at 72 °C, for 40–60 cycles (see conditions given above). mRNA transcription was normalised against β-actin. Eight serial dilutions of cDNA pooled from each sample were used to create a standard curve (arbitrary units) against which mRNA transcription was normalised and expressed as a ratio to that of β-actin mRNA.

2.2.4. Litomosoides. sigmodontis abundant larval transcript (Ls-alt) cloning and protein production

For reverse transcription (RT)-PCR, first-strand cDNA was produced from total *L. sigmodontis* RNA and *L. sigmodontis* Abundant Larval Transcript Ls-ALT amplified by PCR (Accession number DQ451171). The predicted mature Ls-ALT protein was expressed in the Pet29 T vector (Novagen) as a fusion protein with histidine and S tag peptides. Expression was induced with 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) at 37 °C for 3 h. Bacteria were pelleted and sonicated and the supernatant was taken for metal-chelating affinity chromatography on His-Bind resin (Novagen). The Ls-ALT-containing fractions were quantified using a Bradford assay according to the manufacturers recommendations, and stored at −20 °C until required.

2.2.5. Cytometric bead assay, ELISA, FACS, antibodies and reagents

The concentrations of mouse chemokine MCP-1 and cytokines IL-10, IL-6, IL-12p70, TNF-α and IFN-γ were measured in the serum of mice, as per the manufacturers recommendations (BD Cytometric Bead Array, BD Biosciences), except for an additional washing step included before the addition of phycoerythrin (PE) Detection Reagent in order to prevent any interference of haemoglobin in the later acquisition steps.

Additionally, ELISAs were performed on serum, the first microlitre of pleural lavage fluid and on culture supernatants in triplicate with the following purified and biotinylated monoclonal antibody pairs and corresponding recombinant cytokines. R4-6A2 and XMG1.2 for IFN-γ, BVD4-1D11 and BVD6-24G2 for IL-4, TRFK5 and TRFK4 for IL-5, MP5-20F3 and MP5-32C11 for IL-10 were purchased from Becton–Dickinson–Pharmingen. Antibody 38213 (catalogue no. MAB413 and BAF413) for IL-13 was purchased from R&D Systems (Abingdon, UK). ELISAs were performed as recommended by the manufacturer. For the detection of IgG1 and IgG2a, 96-well plates were coated overnight at 4 °C with adult worm extract at a final concentration of 5 μg/ml obtained as described previously (Maréchal et al., 1997) or at 10 μg/ml with Ls-ALT. The plates were then incubated with mouse sera (1:800) and detected with biotinylated anti-IgG1 (A85-1, BD-Pharmingen) or anti-IgG2a (R19-15, BD Pharmingen). The reactions were all revealed with Amdex streptavidin–horseradish peroxidase conjugate (Amersham) followed by the addition of tetramethylbenzidine and H_2O_2 reagents (KPL Laboratories, Gaithersburg, MA) and the OD measured at 450 nm.

Analyses of cell types were performed by flow cytometry using the following rat anti-mouse antibodies. Fluoresceine isothiocyanate (FITC)-conjugated anti-CD19 (ID3), PE-conjugated anti-CD5 (53–7.3), FITC-conjugated anti-CD4 (RM 4–5), PE-conjugated anti-CD8 (58–6.7), Cy-Chrome®-conjugated anti-CD3 (17A2), APC-conjugated anti-CD25 (PC61) were purchased from BD-Pharmingen (Le Pont de Claix, France). PE-conjugated anti-F4/80 (C1:A3-1) was purchased from Tebu–Caltag (Le Perray-en-Yvelines, France). Data were collected using a FACScalibur® flow cytometer and analysed using the Cell Quest software (Becton–Dickinson).

2.3. Statistical analyses

The non-parametric Kruskal–Wallis *H*-test was used to compare the medians of groups of mice over several time

points and Dunn's multiple comparison test, which includes a multiple test correction, was used when those medians differed. Additionally, when only two groups were compared, the Mann–Whitney *U*-test was used in all cases except for comparing filarial worm lengths for which Student's *t*-test for unpaired groups was used. Interaction plots were used to compare the effects of different culture conditions on cytokine production between each experimental group (Crawley, 2002).

A difference was considered significant when $P<0.05$. R (Ihaka and Gentleman, 1996) and GraphPad Prism® software packages were used for data analyses and graphics.

3. Results

3.1. Vaccination conferred long-term protection against incoming larvae, but accelerated their growth

Mice challenged 5 months after immunisation with 3 weekly doses of irradiated larvae, exhibited significant protection as compared to primary-infected mice (Fig. 1A and B). However, the amount of protection was lower than that seen in short-term vaccination protocols (Le Goff et al., 2000b; Martin et al., 2001). Reduced nematode recovery rates in vaccinated challenged mice as compared to primary-infected mice had already occurred by D10 p.i., with a $54\pm0.6\%$ protection in vaccinated mice ($P=0.04$). The recovery rates had not diminished any further at D34 p.i. and vaccinated mice exhibited $58\pm0.6\%$ protection ($P=0.02$) at that time point (Fig. 1B).

L. sigmodontis filariae reach the pleural cavity of mice between D4 and D5 p.i., at which point they are all still at the L3 stage, then moult to L4 at D7 p.i. and again to adults at D28 p.i. In order to assess whether any filariae had died in the pleural cavity, we analysed the granulomas found in primary-infected and vaccinated mice for their contents at D10 p.i. and D34 p.i. Only one dead worm was found in a granuloma at D10 p.i. and because it was an adult parasite, it must have been derived from an immunisation rather than challenge dose. All other granulomas contained only empty filarial cuticles that are shed during the moulting process (Attout et al., submitted). Because no filariae from the challenge doses had died in the pleural cavity, we can assume that larvae were killed prior to reaching the pleural cavity, in the s.c. tissue or lymphatic vessels.

In addition to measuring total parasite recovery we measured nematode length to assess whether vaccination had induced stunting of developing parasites as observed in other nematode infections (Goyal and Wakelin, 1993; Loukas et al., 2004). In fact, we found the opposite. Nematodes in vaccinated mice grew faster than in primary-infected mice. At D10 p.i., female lengths were 1587 ± 78 μm in vaccinated mice and 1401 ± 30 μm in primary-infected mice (mean ± SEM, $P=0.045$) while male lengths were 1495 ± 40.5 μm in vaccinated mice and 1321 ± 36.3 μm in primary-infected mice ($P=0.008$) (Fig. 1C). At that time point, similar proportions of larvae had reached the fourth larval stage (L4): $93.3\pm4.6\%$ (average between mice) in the vaccinated mice and $92\pm7\%$ in primary-infected mice. At D34 p.i., the nematodes from the two groups had reached similar lengths: 42 ± 1.3 mm in vaccinated mice and 39.4 ± 1 mm in primary-infected mice for female nematodes and 17.82 ± 1.22 mm in vaccinated mice

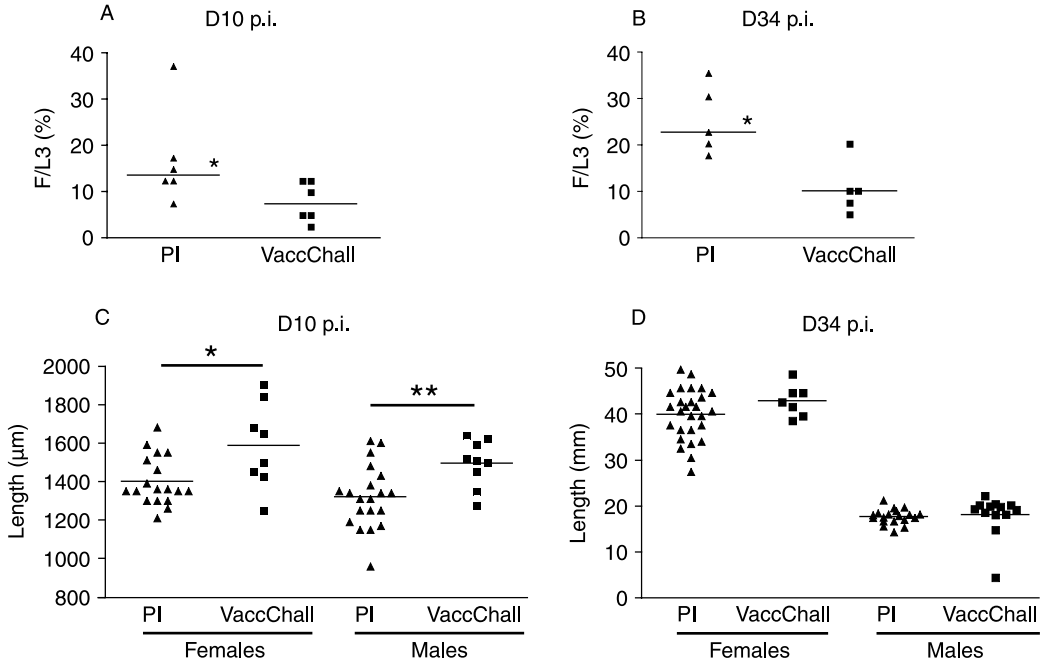

Fig. 1. Survival and growth of *L. sigmodontis* in primary-infected (PI, triangles) and vaccinated challenged (VaccChall, squares) mice. (A) Recovery rates (F/L3) in vaccinated or primary infected mice at day 10 post-inoculation (D10 p.i.) and D34 p.i. Data shown are individual mouse recovery rates expressed in percentages. Horizontal lines represent medians; *, $P<0.05$ (Mann–Whitney's *U*-test). (B) Nematode lengths in vaccinated and primary-infected mice at D10 p.i. and D34 p.i. L4, fourth stage larvae (third stage and moulting larvae not included); Lf, lengths of female nematodes; Lm, lengths of male nematodes; *, $P<0.05$; **, $P<0.01$ (Student's *t*-test).

Table 1
Subcutaneous infiltrated cells at the site of L3 inoculation

		Eosinophils	Neutrophils	Mast cells[a]	Macrophages
H0	Naïve	1 [1–13]	14 [1–18]	2 [2–3]	12.5 [10–15]
	Vacc	13 [6–19]	24 [0–27]	4 [2–8]	10 [0–14]
H6	PI	4.5 [0–7]	137.5 [33–213][b]	1 [0–4]	8.5 [4–17]
	Vacc	14 [0–37]	99 [62–165][b]	4.5 [1–13]	10.5 [1–34]

Polynuclear eosinophils, polynuclear neutrophils, mast cells and macrophages infiltrated in the subcutaneous tissues of vaccinated (Vacc) or primary-infected (PI) mice before challenge (H0) or 6 h p.i. (H6). Numbers express the mean counts of each cell type per 10 ×100 microscopic fields of three mice in each group, except mast cells.

[a] Average number of mast cells in 10 ×40 microscopic fields. Extreme values are between square brackets.

[b] Increased neutrophil infiltration between H0 and H6 ($P<0.06$, Mann–Whitney U-test).

and 17.38 ± 0.37 mm in primary-infected mice for male nematodes (Fig. 1D). However, 100% of the filarial nematodes in the vaccinated mice were adults ($n=20$), whereas 93 ± 5% ($n=49$) had reached that stage in the primary-infected mice at that time point.

3.2. Subcutaneous tissue eosinophilia persisted at a low level 5 months after immunisation

Since our data suggested that larval killing was occurring prior to arrival in the pleural cavity, we chose to assess cell recruitment in the s.c. tissue. The number of infiltrating cells was counted in thin sections of the s.c. tissue (Table 1). Eosinophils were found in the s.c. tissue of vaccinated mice prior to the challenge, at approximately a twofold higher level than in control mice that had received only media as mock inoculations. However, these numbers were about seven times lower than those previously observed in short-term vaccination protocols (Le Goff et al., 2000b; Martin et al., 2001). Six hours after the challenge, the number of eosinophils had not increased in either mouse group (Table 1). Densities of mast cells were irregular and low. But 6 h after challenge, there was a non-significant trend towards increased mast cell numbers in vaccinated mice. Neutrophil numbers were low in both groups before challenge and steeply increased afterwards (10-fold), regardless of immunisation status. The similarity in cell numbers and types recruited to the site of inoculation between vaccinated and primary-infected mice does not rule out a role for any particular cell population in protection as the activation state of these cells may be more critical than the actual cell number.

3.3. Vaccination induced higher amounts of IgG1 than of IgG2a

To assess antibody responses quantitatively in these mice, we performed ELISA to two antigens, soluble adult extract and recombinant ALT (abundant larval transcript). ALT is expressed at high levels by infective larvae of filarial nematodes and is a strong candidate for vaccine development (Allen et al., 2000; Gregory et al., 2000; Wu et al., 2004). Vaccinated mice all had detectable antibody to soluble adult worm extract before challenge, and those amounts did not increase 6 or 60 h after the challenge (data not shown). However, by D10 and D34 p.i., amounts of both IgG1 and IgG2a increased considerably more in vaccinated challenged mice than in primary-infected mice (Fig. 2A). Additionally, vaccinated mice showed a strong bias towards a Th2 response with much higher amounts of IgG1 than IgG2a at D10 and D34 p.i.

No specific anti-Ls-ALT IgG1 and IgG2a antibodies were detected in vaccinated only mice (H0), but they were detected in all irradiated L3-vaccinated challenged mice, with peaking concentrations at D10 p.i., whereas in primary infected mice anti-ALT antibodies were low to undetectable throughout (Fig. 2B). Although anti-ALT antibodies were virtually undetectable 5 months after the last immunisation, the response to challenge in vaccinated mice suggests that irradiated larvae do produce ALT and elicit ALT-specific immunological memory.

3.4. Initial mixed gene expression profile in lymph nodes is followed by Th2 dominated L3-specific cytokine production shortly after challenge of vaccinated mice

To generate a more detailed picture of the T helper (Th) response in vaccinated and in primary-infected mice upon challenge, we analysed the ratio of transcription factors T-bet (Th1) to GATA-3 (Th2) mRNA expression (Chakir et al., 2003), as well as that of IFN-γ mRNA (Th1) and IL-4, IL-13, IL-5 mRNA (as Th2 cytokines) in inguinal lymph node cells at H6 p.i. All measured cytokines (of both Th1 and Th2) were approximately fourfold more abundantly expressed in vaccinated challenged mice than in primary-infected mice (data not shown). The T-bet to GATA3 ratio indicated a stronger bias towards a Th1 phenotype in lymph nodes of vaccinated than of primary-infected mice 6 h after challenge.

To assess cytokine secretion, we restimulated the cells from lymph nodes that drain the site of L3 inoculation with L3 and adult worm extracts at H60 p.i. The highest specific response detected was the production of IL-5 in response to L3 extract in vaccinated challenged mice, whereas no or very little IL-5 was produced under any other condition (Fig. 3B). A high concentration of IL-4 by cells stimulated with Con A and low production of IFN-γ support the overall Th2 bias that developed in the vaccinated challenged mice (Fig. 3A and D) despite evidence for early Th1 gene transcription. Additionally, adult extract but not L3 extract induced relatively high IL-10 production in all groups except primary infected mice

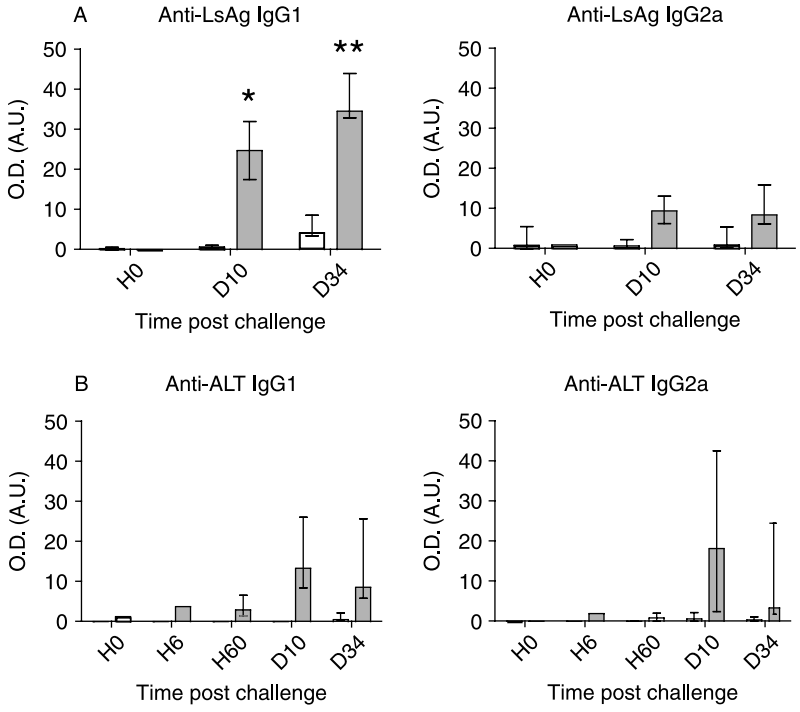

Fig. 2. Soluble *L. sigmodontis* extract and *L. sigmodontis* Abundant Larval Transcript (Ls-ALT)-specific immunoglobulin production in vaccinated and primary-infected mice. Concentrations of IgG1 and IgG2a specific for soluble extract of adult *L. sigmodontis* (A) and Ls-ALT (B) are shown. Antibody levels of primary-infected (open bars) and vaccinated (grey bars) mice are shown before challenge (H0), at D10 p.i. and at D34 p.i. Serum was diluted 1/800. Error bars represent the interquartile range around the median; *, $P<0.05$; **, $P<0.01$ (Kruskal–Wallis *H*-test with Dunn's multiple comparison post-test).

(Fig. 3C). However, cytokine production in the lymph nodes revealed an unexpected pattern: lower in vitro production of cytokines in response to Con A was detectable in the cells from mice that had received L3s (whether vaccinated only or challenged) than in those of naïve controls. This might be due to immunomodulation by the filariae and by their somatic extracts.

The analysis of serum cytokines at H6 p.i. showed an increase of IL-6 concentrations in both primary infected and in vaccinated challenged mice compared to naïve and vaccinated only mice (Fig. 3E), whereas concentrations of MCP-1, TNF-α, IL-12p70, IL-10 and IFN-γ did not increase at that time point (data not shown). No changes in MCP-1, TNF-α, IL-12p70, IL-4, IL-6, IL-10 nor IFN-γ serum concentrations were seen at H60 p.i. in both vaccinated and primary-infected mice. Sixty hours p.i., detectable amounts of IL-5 were measured in the serum of all challenged mice whether vaccinated or not (Fig. 3F).

We also examined the pleural lavage fluid for cytokines. No significant differences were seen at H0 and H60 prior to L3 reaching the cavity (not shown). However, Th 2 cytokines IL-4, IL-5 together with IL-10 were found in higher concentrations in the pleural lavage fluid of vaccinated challenged mice than of primary-infected mice at D10 p.i. (Fig. 4). All cytokines increased from D10 p.i. to D34 p.i. and IFN-γ and IL-4 remained higher in the vaccinated mice, whereas concentrations of IL-5 and IL-10 had reached similarly high concentrations in both groups of mice (Fig. 4). Pleural concentrations of IL-13, measured at H0, H60, D10 and D30 p.i. showed no difference between naive, vaccinated and primary-infected mice (data not shown).

3.5. Large quantities of cells were recruited to the pleural cavity of vaccinated mice following challenge but decreased to that of primary-infected mice by day 34 p.i.

Before challenge the total number of pleural exudate cells (PleC) was higher in the vaccinated mice than in naive mice. Upon challenge, the vaccinated mice then recruited vast numbers of cells to their pleural cavity, over 25×10^6 in total at D10 p.i., approximately threefold the number of PleC in the primary infected mice. At D34 p.i. that number had fallen to $\sim11\times10^6$ in the vaccinated challenged mice, whereas it had not increased any further in the primary-infected mice, which remained at $\sim8\times10^6$. The various cell populations among the PleC were altered by the vaccination in a similar way: B cells (both B1 and B2 subsets), T cells (particularly the potential regulatory CD4+CD25+subset), eosinophils and macrophages were all found in higher numbers in vaccinated challenged mice than in primary infected mice at D10 p.i., but not at D34 p.i. (Fig. 5). Conversely, neutrophils, not present in significant numbers at D10 p.i. or before, tended to be less numerous in the vaccinated mice than in the primary-infected mice at D34 p.i. ($P=0.09$, Fig. 5). Nonetheless, as no difference in recovery rate was observed between D10 and D34 p.i., the PleC were not responsible for the protection measured.

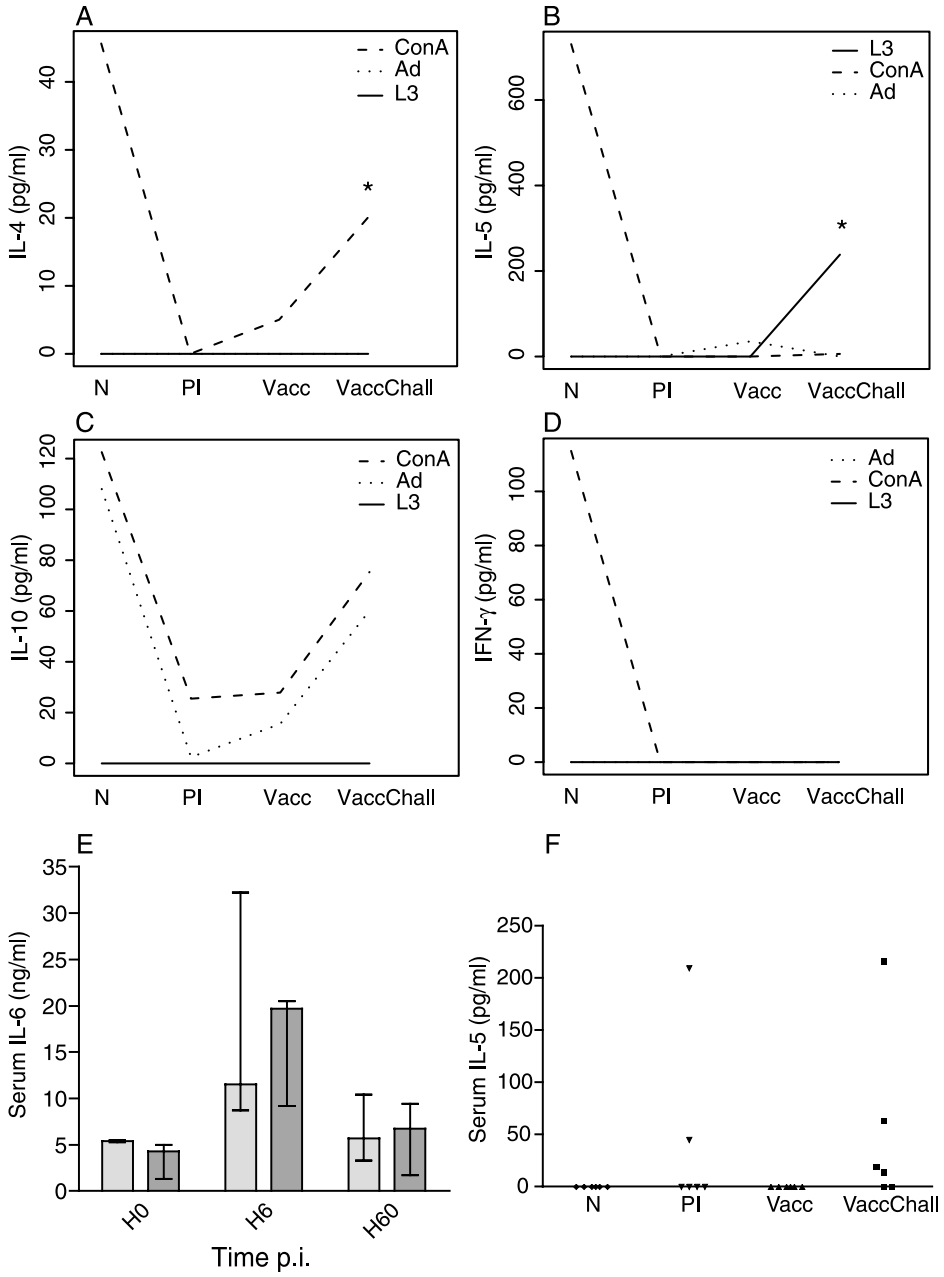

Fig. 3. Early serum and in vitro lymph node cell cytokine concentrations in primary-infected and vaccinated mice. (A–D) Interaction plots demonstrating the effect of vaccination and challenge on in vitro production of IL-4 (A), IL-5 (B), IL-10 (C), IFN-γ (D) by lymph node cells 60 h p.i., under each given stimulus. N, naïve; PI, primary-infected; Vacc, vaccinated only and VaccChall, vaccinated and challenged mice. Cells were stimulated with L3 extract (L3), adult extract (Ad) or Con A. Each data point represents the median concentration of cytokine per mouse group normalised by subtracting medium-only values. Non-parallel lines mean that the production of cytokines by vaccinated or challenged mice is affected differently by the culture conditions, suggesting a specific response (e.g. the production of IL-5 by vaccinated mice increased specifically upon challenge in response to L3 antigen but not to adult antigen). For visual ease, no error bars are provided but * indicates statistical difference of the medians between culture conditions. (E) Concentrations of IL-6 detected in the serum of non-vaccinated (light grey) and vaccinated (dark grey) mice before challenge (H0), 6 and 60 h p.i. (H6, H60). Bars are medians, errors bars cover the full range of data. (F) Concentrations of IL-5 60 h p.i. in the serum of naïve (N), primary-infected (PI), vaccinated only (Vacc) or vaccinated and challenged (VaccChall) mice 60 h p.i. *, $P < 0.05$ (Kruskal–Wallis H-test and Dunn's post-test).

4. Discussion

Previous studies using irradiated larvae to vaccinate against *L. sigmodontis* have allowed us to characterise the mechanisms and anatomical site of protection. Indeed, the vaccine-induced killing targets the L3 in the subcutaneous tissue shortly after inoculation and requires the presence of IL-5, B cells and degranulated eosinophils (Le Goff et al., 1997; Martin et al., 2000b; 2001). However, in those studies mice were challenged only 2 weeks after the immunisation.

In the present study, we show that vaccination against a filarial parasite protected mice over a longer period, namely 5 months. The level of protection achieved in our study (54–58%) was slightly below that obtained in the short-term

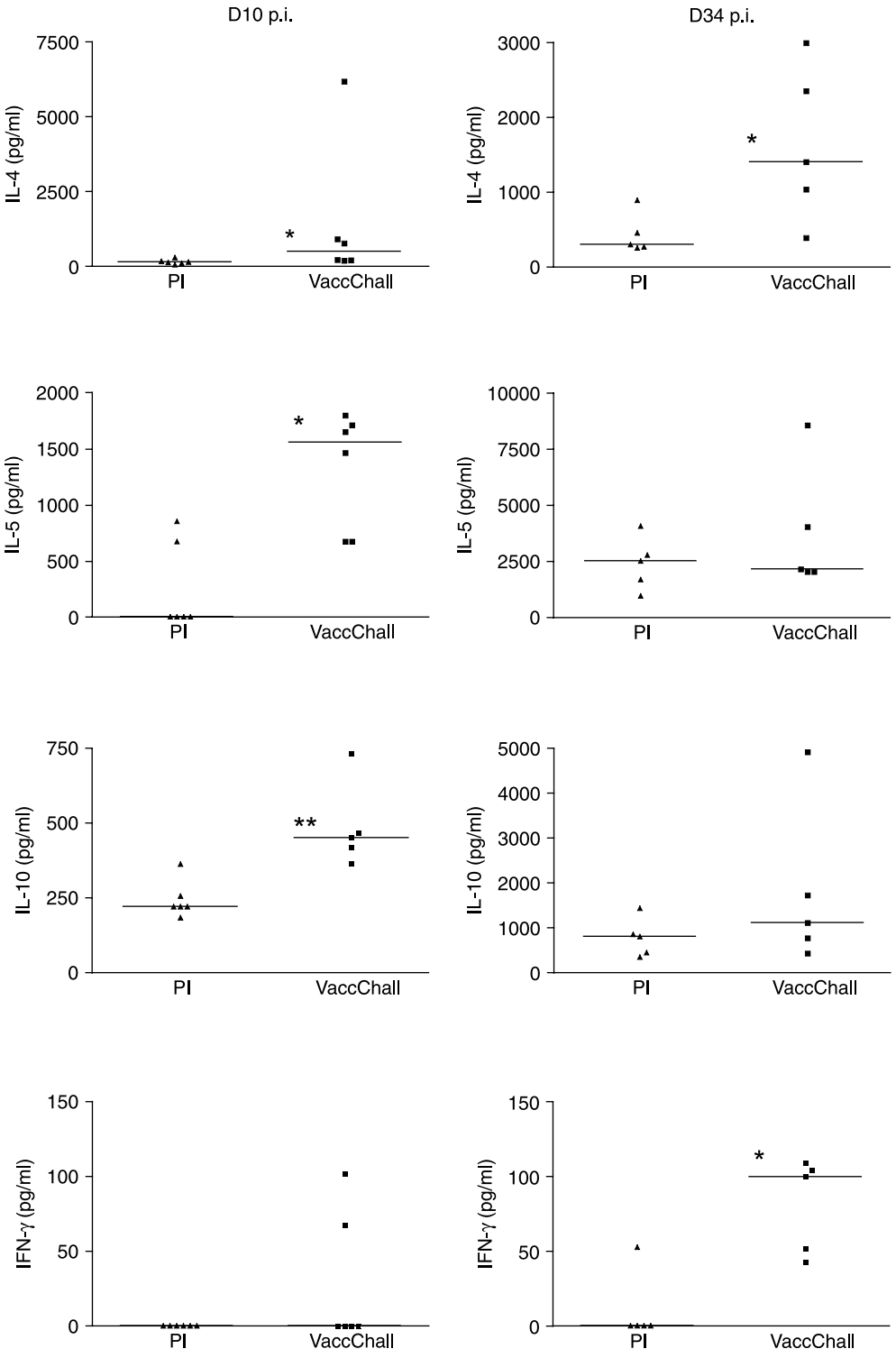

Fig. 4. Cytokine concentrations in the pleural lavage of primary-infected (PI, triangles) and challenged vaccinated (VaccChall, squares) mice at D10 p.i. and D34 p.i. Horizontal bars represent medians. *, $P<0.05$; **, $P<0.01$ (Mann–Whitney U-test).

vaccination protocols (70–75%, Fig. 1A and B) (Le Goff et al., 1997, 2000b; Martin et al., 2000b; 2001), supporting the possibility that protection is overestimated due to the persistence of immune reactions to recent repeated larvae inoculations. However, long-term immunological memory was established as shown by antibody, cytokine and cell recruitment patterns and this led to considerable protection.

Our data also suggest that similar mechanisms are likely to be involved in long-term protection as in short-term protocols, with protection limited to early stages of infection. Protection

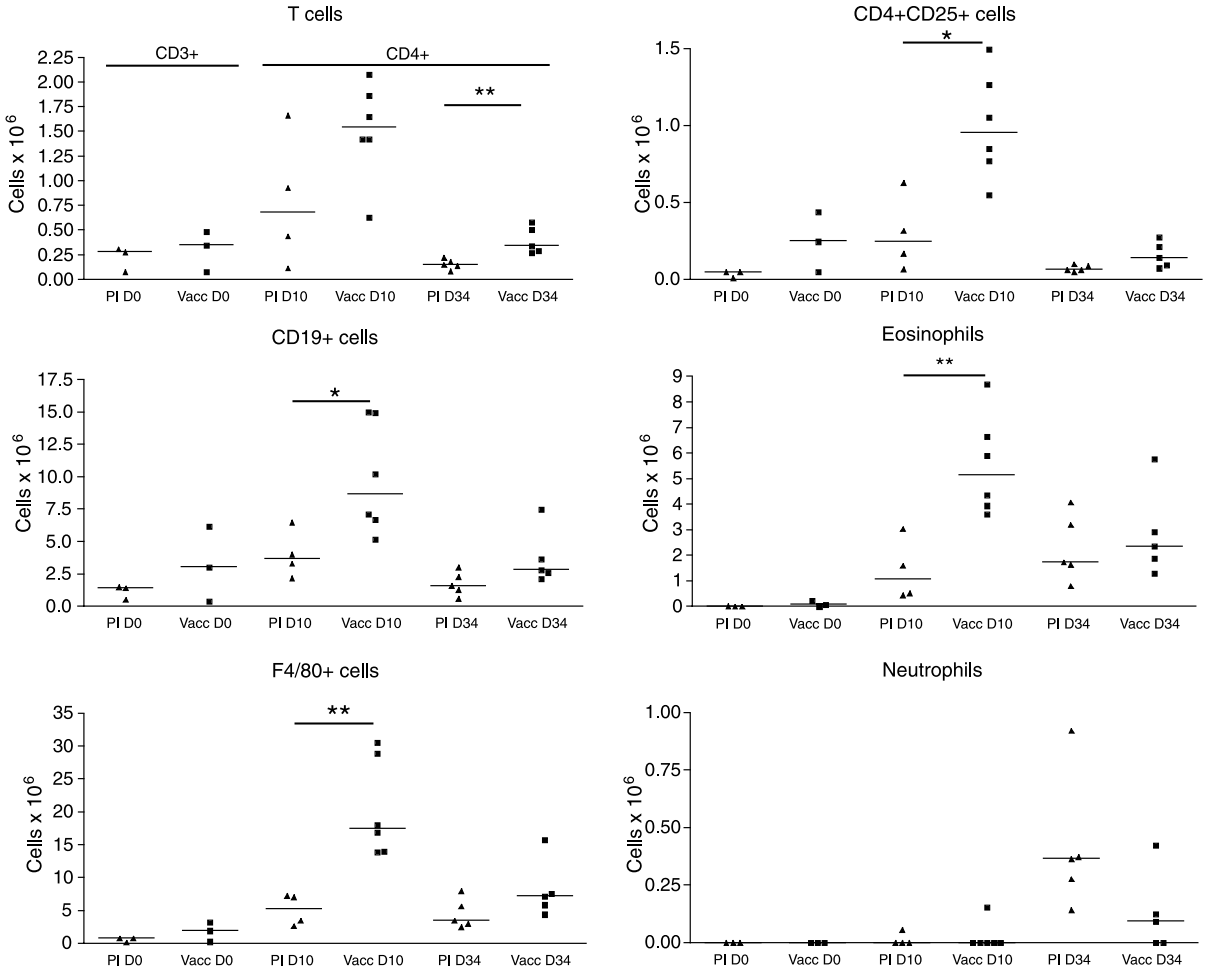

Fig. 5. Accumulation of pleural exudate cells before challenge (D0), at D10 p.i. and at D34 p.i. in primary-infected (PI, triangles) and vaccinated (Vacc, squares) mice. Anti-CD3 staining of T cells is shown for naïve (PI D0) and vaccinated only (Vacc D0) mice and anti-CD4 staining for T cells of mice from later times points. Horizontal bars express medians. *, $P<0.05$; **, $P<0.01$ (Kruskal–Wallis H-test with Dunn's multiple comparison post-test).

likely occurred during the larvae's migration, as we did not find dead filariae in the pleural cavity, nor did we detect any reduction in survival between D10 and D34 p.i. (Fig. 1 A and B). This is consistent with the findings of short-term vaccination studies (Le Goff et al., 1997, 2000b; Martin et al., 2000b; 2001). Within 60 h after challenge, specific IgG (Fig. 2) and cytokines IL-6 first (Fig. 3E), followed by IL-5 (Fig. 3F) were detected in the serum of vaccinated mice, which are hallmarks of vaccine-mediated protection (Lange et al., 1994; Taylor et al., 1994; Martin et al., 2000b; 2001; Ungeheuer et al., 2000). Further, we found that eosinophils remained in the s.c. tissue of vaccinated mice 5 months after their immunisation (Table 1), although around sevenfold less than in short-term protocols (Le Goff et al., 2000b; Martin et al., 2001). Finally, L3-specific IL-5 was produced in the lymph nodes of vaccinated challenged mice 60 h p.i. (Fig. 3B), suggesting that the infective larva is the prime target of the vaccine-mediated immune response.

As expected, the immune response of vaccinated challenged mice was faster and stronger than in primary-infected animals, with an increased production of cytokines directly linked to the control of filarial infections (Le Goff et al., 2002; Saeftel et al., 2003; Volkmann et al., 2003). Although 6 h p.i., transcription of Th2 type cytokines IL-4, IL-13 and IL-5 as well as Th1-type cytokine IFN-γ was increased in the lymph nodes near the site of entry of the challenge L3, at 60 h p.i., restimulation of cells from the lymph nodes that drain the site of inoculation produced only Th2-type cytokines (Fig. 3A–D). This suggests that Th1 cytokine secretion was inhibited post-transcriptionally, as observed with *Brugia pahangi* in mice (Osborne and Devaney, 1998; Devaney and Osborne, 2000). A dominant Th2 phenotype in vaccinated mice was supported by high levels of IgG1 and high concentrations of IL-4 and IL-5 in the pleural lavage (Figs. 2 and 4) within the first 10 days after challenge inoculation. Immunological memory was further demonstrated by antibody responses to ALT. Ls-ALT-specific antibodies were not detected at the time of challenge in vaccinated mice, but the antibody concentration increased significantly by 10 days in contrast to primary-infected mice. The ALT-specific response partially receded by day 34 p.i. although filariae were present at that time point. This is likely to be a consequence of the L3-specific secretion of some ALT family members by filarial nematodes as shown in *Brugia malayi* (Gregory et al., 2000).

Although a marked Th2 trend of cytokine production was found in the pleural lavage of vaccinated challenged mice at D10 (Fig. 4) by D34 p.i., amounts of IL-5 and IL-10 had declined to the same level as primary-infected mice, while IFN-γ reached significantly higher concentrations, lending support to the previously described mixed phenotype of late infections in this model (Hoerauf and Brattig, 2002; Babayan et al., 2003, 2005). At this time point, B cell, eosinophil and macrophage numbers in the vaccinated mice were equivalent to those found in primary-infected mice (Fig. 5) despite the presence of parasites in the pleural cavity (Fig. 1). Although the lower numbers of nematodes in the vaccinated mice might explain that reduction (Babayan et al., 2005), one may argue that strong down-regulatory mechanisms, perhaps enhanced in vaccinated mice by the amplitude of their early immune reaction, may also be a cause. The latter is supported by the higher number of CD4+CD25+T cells and macrophages found in the cavity of vaccinated mice at D10 p.i. (Fig. 5), which have established roles in the immune down-regulation occurring in filarial infections (Devaney and Osborne, 2000; Loke et al., 2000; Cooper et al., 2001; Maizels and Yazdanbakhsh, 2003; Steel and Nutman, 2003; Maizels et al., 2004; Taylor et al., 2005). In the light of our present study, regulatory mechanisms are likely to be a handicap for the establishment of complete vaccine-mediated protection. Indeed, Taylor et al. have shown that depleting T regulatory cells allows susceptible mice to reduce their filarial parasite numbers during primary infections (Taylor et al., 2005).

Filarial nematodes like other parasitic helminths have developed many mechanisms to avoid being eliminated by the host before achieving patency (Maizels et al., 2004). Increasing evidence suggests the nature and amplitude of the early immune response has a strong effect on the development of *L. sigmodontis*. For example, we have shown that filarial nematode development is retarded in mice that respond poorly during the first days of infection but is accelerated when the larvae encounter a strong early immune response (Babayan et al., 2003) and that accelerated growth is correlated with high concentrations of IL-5 soon after the challenge (Martin et al., 2000a,b; Babayan et al., 2003, 2005). Consistent with this, in the present study the larvae's development was significantly accelerated in vaccinated mice (Fig. 1C and D) and at D34 p.i., only in the vaccinated mice had they all reached the adult stage. This may reflect an adaptation of these filarial nematodes to their mammalian hosts' immune reactions in order to maximise their fitness, as seen in other groups of nematodes (Guinnee et al., 2003; Fenton et al., 2004).

Our results show that the early events affecting the L3 are key to the subsequent evolution of the infection and of the immune response throughout, as seen in primary infections of susceptible (Wanji et al., 1990; Bain et al., 1994; Maréchal et al., 1996) or of resistant hosts (Maréchal et al., 1996; Babayan et al., 2003) or vaccinated hosts (Le Goff et al., 1997, 2000b). Likewise, in multi-inoculated hosts the recovery rate of *B. pahangi* from cats (Denham et al., 1983) or of *Monanema martini* from natural rodent hosts (Wanji et al., 1994) drops very soon after the inoculation and then remains stable until patency. In our study, once *L. sigmodontis* L3 larvae had reached the pleural cavity, the natural evolution of the host's immune response had little to no effect on their survival until the adult stage at which stage regulatory mechanisms may be at play. It may therefore be necessary to develop vaccines that target not only the infective L3 stage but target later stages as well (Wenk and Wegerhof, 1976).

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