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Co-infected C57BL/6 mice mount appropriately polarized and compartmentalized cytokine responses to *Litomosoides sigmodontis* and *Leishmania major* but disease progression is altered

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SUMMARY

This study examines the capacity of the mammalian host to fully compartmentalize the response to infection with type 1 vs. type 2 inducing organisms that infect different sites in the body. For this purpose, C57BL/6 mice were infected with the rodent filarial nematode Litomosoides sigmodontis *followed by footpad infection with the protozoan parasite* Leishmania major. *In this host, nematode infection is established in the thoracic cavity but no microfilariae circulate in the bloodstream. We utilized quantitative ELISPOT analysis of IL-4 and IFN-γ producing cells to assess cytokine bias and response magnitude in the lymph nodes draining the sites of infection as well as more systemic responses in the spleen and serum. Contrary to other systems where co-infection has a major impact on bias, cytokine ratios were unaltered in either local lymph node. The most notable effect of co-infection was an unexpected increase in the magnitude of the IFN-γ response to* L. major *in mice previously infected with* L. sigmodontis. *Further, lesion development was significantly delayed in these mice. Thus, despite the ability of the immune system to appropriately compartmentalize the immune response, interactions between responses at distinct infection sites can alter disease progression.*

Keywords *co-infection, filariasis, nematode, protozoan, T helper cell subsets*

Abbreviations**:** ANOVA, analysis of variance

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INTRODUCTION

A striking feature of the adaptive immune response is the ability to compartmentalize and direct the immune response toward infected tissue through a system of regional lymph nodes and specific homing receptors. One might thus predict that the immune system should readily cope with co-infecting pathogens at distinct sites. We addressed this prediction using *Litomosoides sigmodontis* and *Leishmania major*, which infect distinct sites in the body (thoracic cavity vs. footpad) and do not disseminate beyond the local lymph nodes in C57BL/6 mice (1,2).

Litomosoides sigmodontis is a filarial nematode of cotton rats that can establish full development in the BALB/c mouse, and thus provides a valuable model for the study of human filarial infections (3,4). The identification of both susceptible (BALB/c) and relatively resistant (C57BL/6) strains has provided the opportunity to unravel the parameters that determine disease susceptibility in filarial infection. In both BALB/c and C57BL/6 mice, the infective larvae migrate from the subcutaneous tissue via the lymphatics to the thoracic cavity. Although both strains mount strong Th2 responses to infection (5), in C57BL/6 mice infection is cleared between day 40 and day 60 and the adult nematode never reaches sexual maturity (6). This killing is dependent on IL-4, as in IL-4-deficient C57BL/6 mice, parasites follow a development pattern identical to BALB/c mice, with development of the parasite to adulthood and circulation of microfilariae in the bloodstream (5).

The intracellular parasite *L. major* displays a similar susceptibility/resistance pattern between mouse strains. BALB/c mice are fully susceptible to infection and develop progressive leishmaniasis following subcutaneous inoculation. *Leishmania major* is also able to establish infection in the resistant C57BL/6 mice, but the subcutaneous lesion that develops at the site of inoculation begins to spontaneously resolve by day 40 (7) and the mice are resistant to further infection. Despite the increasing complexity in our understanding of

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the type 1/type 2 paradigm in *L. major* infection, a critical role for type 1 cytokines, especially IFN-γ and its associated anti-microbial effects, has been repeatedly demonstrated for disease resolution (8).

Using these two model systems we can create a defined but fascinating challenge for the immune system. To control *L. sigmodontis* it must mount an effective type 2 response, whereas to control *L. major*, the same host must mount a type 1 response. In each case, regulatory pathways, which can promote infection in the susceptible BALB/c mice (9,10), may not develop in C57BL/6 mice. Interestingly, in *L. major* infection of C57BL/6 mice, an early IL-4 response is observed but fails to be sustained, and a Th1 response develops (11,12). So it may be that a pre-existing helminth infection, even at a distinct site, would compromise the subsequent development of the type 1 response by tipping the balance towards maintenance of the type 2 response. We thus chose to ask whether a pre-existing IL-4 response induced by *L. sigmodontis* could alter the course of *L. major* infection.

MATERIALS AND METHODS

Mice

All experiments used C57BL/6 male mice 4–6 weeks in age that were bred on site in the animal facilities at King's Buildings, University of Edinburgh. Mice were housed in individually ventilated cages and given food and water *ad libitum* during the course of these experiments.

Infection protocol

The life cycle of *L. sigmodontis* (Chandler, 1931) was maintained by cyclical passage of parasites through *Meriones unguiculatus* jirds and the mite species *Ornithonyssus bacoti* as previously described (13). Animals were infected with 25 *L. sigmodontis* L3's in the right lumbar area or with 1×10^6 *L. major* LRC-L137 V121 (14) stationary promastigotes subcutaneously in the left footpad. Co-infected animals were infected with *L. major* at 20 days post *L. sigmodontis* infection. Animals were sacrificed at 40 days post *L. major* infection (60 days post *L. sigmodontis* infection). Footpad lesions were measured using digital callipers, and calculated by subtracting the size of the uninfected right footpad from the size of the infected left footpad of each mouse at each time point.

Preparation of parasite antigens

Mixed adult *L. sigmodontis* were homogenized in sterile PBS, centrifuged at 1000 ***g*** and the pellet discarded. *Leishmania major* stationary promastigotes were suspended in sterile PBS and lysed by freezing and thawing three times. Both extracts were syringe-filtered through a 20 μm membrane and stored at −20°C until required.

ELISPOT

ELISPOT plates (Millipore) were coated with 50 μL/well of capture antibody (11B11 anti-IL-4 and R4-6A2 anti-IFN-γ (both Pharmingen)) diluted to 15 μg/mL in sterile carbonate buffer and incubated overnight at 4°C. Plates were blocked with 150 μL/well of 2% skimmed milk powder (Marvel) for at least 2 h at 37°C. The spleen and lymph nodes were removed from each mouse and aseptically pressed through a cell sieve (BDH). Red blood cells were removed from the splenocyte preparations using red blood cell lysis buffer (Sigma). Cells were resuspended in Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (all from Gibco) and 0·5% mouse serum (Sigma). Fifty μL/well of cell suspensions containing 5×10^5 cells (splenocytes) or 3×10^5 cells (lymph node cells) were plated in duplicate. Fifty μL of parasite antigen (20 μg/mL) or supplemented DMEM was added to each well. The plates were incubated for 72 h at 37°C +5% CO_2.

After incubation 50 μL of biotinylated antibody was added to each well and incubated for 2 h at 37°C (XMG1·2 at 1 μg/mL for IFN-γ and BVD6-24G2 at 0·1 μg/mL for IL-4 – both Pharmingen). Fifty μL Extravidin alkaline phosphatase (Sigma) diluted 1 in 25 000 was added to each well and incubated for a further 30 min. To detect spots 100 μL of syringe-filtered (0·45 μm) BCIP/NBT substrate (Moss Substrates, Inc.) was added to each well. Plates were read by an ImmunoSpot analyser (Cellular Technology Ltd) and analysed using ImmunoSpot software. This software calculates the number of spots per well, in addition to the average spot size per well. By multiplying both measurements together it is possible to also compare an estimate of cytokine area per well. We found no major differences between spot number and cytokine area for any of the groups. Therefore the ELISPOT data are represented by spot number. For thoracic lymph nodes, spot numbers were adjusted for 5×10^5 cells per well for comparison with splenic cell numbers.

Antibody ELISA

ELISA plates (Nunc) were coated with 100 μL parasite extract diluted to 5 μg/mL in 0·05 M carbonate buffer. Plates were blocked with 100 μL/well of 1% skimmed milk powder (Marvel) for 90 min at 37°C. Test sera were diluted 1 : 200 (IgG1 measurements) or 1 : 100 (IgG2a measurements) in PBS with 0·5% Tween (PBST). Fifty μL of each sample was added to each well and plated in duplicate. Plates were left for 90 min at 37°C. Antibody levels were detected with 50 μL

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of peroxidase-conjugated goat anti-mouse IgG1 or goat anti-mouse IgG2a (both Southern Biotechnology Associates Ltd) diluted 1 : 6000 or 1 : 200, respectively, in PBST. After a further 90 minute incubation step at 37°C, plates were developed with 50 μL of 2,2′-azinodi(ethylbenzthiazoline-6-sulphonate) (ABTS, Kirkegaarde and Perry Laboratories, Inc.) and read at 405 nm.

Statistical analysis

Five mice were included in each group for each experiment. Two experiments were carried out and the data analysed together, removing experimental effects using parametric analysis of variance (ANOVA) (Minitab Inc). The number of cells secreting IFN-γ against *L. major* in the popliteal lymph nodes was logarithmically transformed prior to analysis to conform to the assumptions of parametric testing. Media-induced responses in the popliteal lymph nodes, and all data obtained from splenocytes, were square root transformed to allow parametric testing. Ratios of cytokines were calculated by dividing number of IFN-γ-producing spots by the number of IL-4-producing spots for each animal. For intuitive ease, the natural logs of ratios have been graphed, with bars above 0 indicating a predominant type 1 response and bars below 0 indicating a predominant type 2 response. The natural log of the antigen-specific ratios (Figure 1) was analysed. No experiment–by-treatment interaction effects were observed in any of these analyses. Cytokine responses of naïve animals were not included in any of the analyses, although the data are shown graphically for comparison. To calculate the overall *L. major*-induced pathology, the area under the footpad swelling curve was calculated for each infected animal and group differences were compared using ANOVA to control for experimental effects.

RESULTS

Local immune responses to *L. major* and *L. sigmodontis* remained highly polarized and compartmentalized following co-infection

We set out to compare the parasite-specific immune responses mounted by singly infected vs. co-infected animals and assess any immunological interactions that occurred in animals co-infected at distinct anatomical sites. For this purpose, C57BL/6 mice were infected with *L. sigmodontis*, and at 20 days post-infection, when the adult parasites are resident within the thoracic cavity, *L. major* was inoculated into the footpad. Day 20 was chosen as a time point at which a full Th2 response had developed in the C57BL/6 mouse but prior to significant death of *L. sigmodontis* parasites (6). At 40 days post *L. major* infection, when lesions normally begin to heal (7),

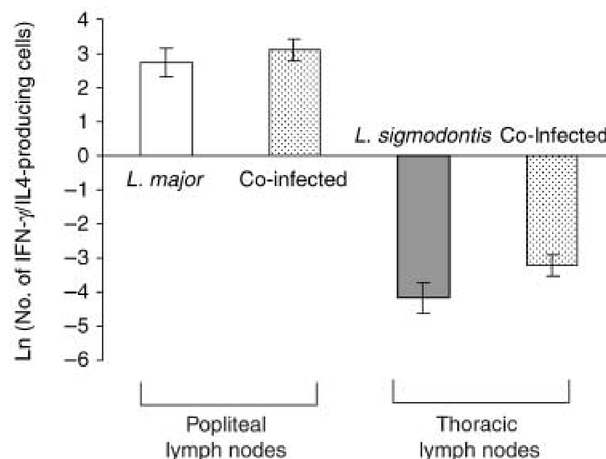

Figure 1 The ratio of IFN-γ/IL-4-secreting cells in the lymph nodes draining the sites of infection at 40 days post-*L. major* infection (60 days post-*L. sigmodontis* infection). Graphs represent the average natural log of the number of cells secreting IFN-γ divided by the number of cells secreting IL-4 for each group of animals. Thus values greater than 0 show a type 1-dominated response and values less than 0 represent a type 2-dominated response. Ratios for the antigen-specific response to each parasite in each compartment are shown. Animals singly infected with *L. major* and *L. sigmodontis* are represented by the white and grey solid bars, respectively. The hatched bars represent the co-infected animals. Error bars represent the SEM. Both graphs represent the combined values for two different experiments each containing five mice per group.

we measured IL-4 and IFN-γ recall responses in the popliteal lymph nodes draining the *L. major* infection site and the thoracic lymph nodes draining the *L. sigmodontis* infection site.

We chose to measure the number of IL-4- and IFN-γ-producing cells in singly vs. co-infected animals by the ELISPOT method. Although the demands of this method (on reagents and cell number) limited the number of cytokines we could test, it provided a highly quantitative assessment of these signature cytokines in terms of both cytokine bias and magnitude of the immune response. We first assessed the ratio of IFN-γ- to IL-4-producing cells in each mouse and found that as expected, *L. major* infection induced a predominant IFN-γ response, whereas *L. sigmodontis* induced a response dominated by IL-4-producing cells. This analysis of IFN-γ- to IL-4-producing cell ratios also demonstrated that the bias of the immune response was not altered by co-infection (ANOVA, $P > 0{\cdot}05$ in both compartments) (Figure 1).

The magnitude of the immune response to *L. major* in the draining lymph node is altered by co-infection

We also compared the number of cytokine producing cells per treatment group to see if co-infection altered the magnitude of the immune response and found that a pre-

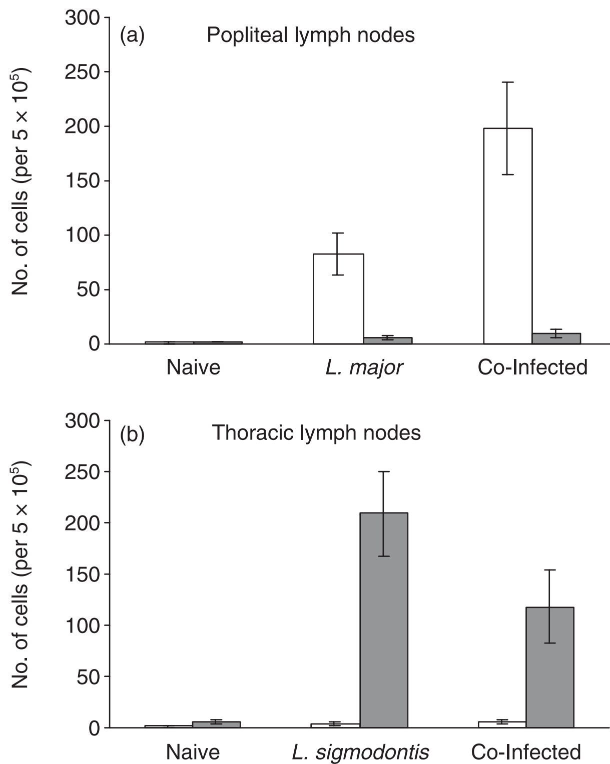

Figure 2 Antigen-specific IL-4 and IFN-γ responses in singly and co-infected mice to *L. major* and *L. sigmodontis* at day 40 post-infection with *L. major*/day 60 post-infection with *L. sigmodontis*. The number of cells per 5×10^5 lymph node cells secreting IFN-γ (white bars) and IL-4 (grey bars) against *L. major* in the popliteal lymph nodes (a) and against *L. sigmodontis* in the thoracic lymph nodes (b) are shown. Data shown include the combined values of two different experiments each containing five mice per group. The error bars show the SEM.

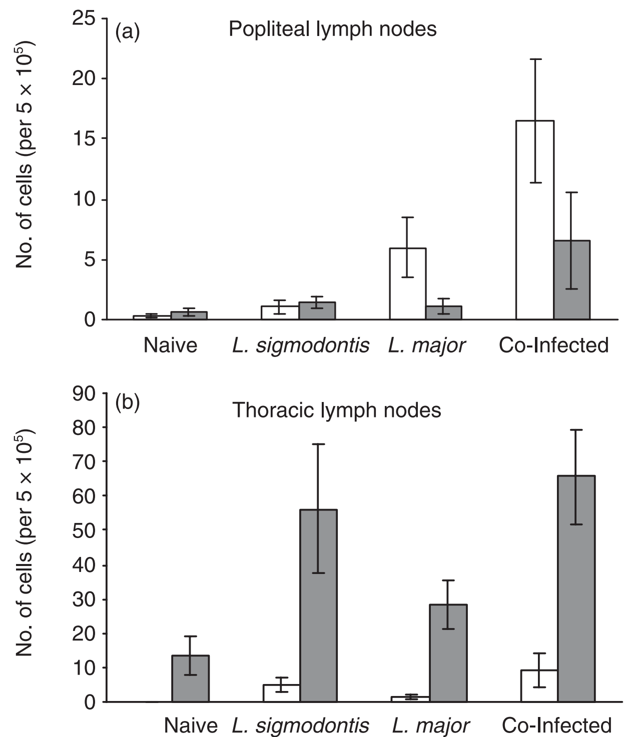

Figure 3 Baseline IL-4 and IFN-γ responses in mice singly and co-infected with *L. major* and *L. sigmodontis* in the lymph nodes at 40 days post-*L. major* infection/60 days post-*L. sigmodontis*. The response in the popliteal lymph nodes (a) and the thoracic lymph nodes (b) is shown. The white bars represent the number of cells secreting IFN-γ and the grey bars represent the number of cells secreting IL-4 per 5×10^5 lymph node cells. In both graphs, the error bars represent the SEM of data from two independent experiments.

existing filarial infection enhanced the immune response mounted against *L. major.* In the popliteal lymph nodes we observed a significant increase in the number of cells secreting IFN-γ in response to *L. major* in co-infected animals compared with animals singly infected with *L. major* (ANOVA, $P < 0{\cdot}05$) (Figure 2a). This enhancement of the anti-*L. major* type 1 response by a pre-existing type 2 response was surprising because type 2 responses are thought to antagonize the development of a type 1 response (15). Infection with *L. major* at 20 days post-*L. sigmodontis* infection had a lesser impact on the established immune response mounted against *L. sigmodontis*. There was a decrease in the number of cells secreting IL-4 against *L. sigmodontis* in the thoracic lymph nodes of co-infected animals, compared with animals singly infected with *L. sigmodontis*, but this did not reach statistical significance (ANOVA, $P = 0{\cdot}069$) (Figure 2b). Thus in both lymph node populations, the anti-parasite immune response measured was altered in magnitude in comparison with singly infected animals. However the immune response was still highly polarized towards type 2 in the *L. sigmodontis* compartment and towards type 1 in the *L. major* compartment. The significant increase in *L. major*-specific IFN-γ production, for example, was accompanied by small but proportional shift in IL-4, accounting for the unaltered ratios in Figure 1.

We also evaluated the lymph node responses in the absence of antigen to assess whether there were baseline changes in immune responsiveness. As observed for the antigen-specific *L. major* response, there were a greater number of cells secreting IFN-γ in the popliteal lymph nodes of co-infected animals compared with animals singly infected with *L. major* (ANOVA, $P < 0{\cdot}01$) (Figure 3a). However, there was also a population of IL-4-secreting cells in co-infected animals that was not present in animals singly infected with *L. major* (ANOVA, $P < 0{\cdot}05$) (Figure 3a). Analysis of nonspecific background responses was thus able to demonstrate that *L. sigmodontis* infection did influence the number of IL-4-producing cells in the lymph nodes draining *L. major*

infection, which was not apparent when looking at parasite-specific responses. Consistent with the antigen-specific data, however, this reflects the overall enhancement of cytokine responses in co-infected mice, because relative amounts of the two cytokines were unaltered. In contrast to the popliteal lymph node, the number of cells spontaneously secreting IL-4 or IFN-γ in the thoracic lymph node draining *L. sigmodontis* infection was unaffected by co-infection with *L. major* (ANOVA, $P > 0{\cdot}05$) (Figure 3b). Overall, the evaluation of both the background and antigen-specific responses demonstrated that even following co-infection the response was correctly polarized against each parasite and that the predominant effect of co-infection was to alter the magnitude of that immune response. The most significant of these was the enhancement of the IFN-γ response to *L. major* resulting from the presence of a pre-existing *L. sigmodontis* infection.

Co-infection did not detectably alter responses at the systemic level, as demonstrated by responses in the spleen and parasite-specific serum istoypes

Because anti-parasite splenic immune responses can be measured in both murine *L. sigmodontis* (5) and *L. major* (16) infection, we hypothesized that the spleen could be a possible site of immunological interaction that may lead to the observed augmented anti-*L. major* response in the popliteal lymph nodes of co-infected mice. Consistent with the immune responses measured in the popliteal lymph nodes (Figure 2a), co-infected animals appeared to have more IFN-γ-secreting anti-*L. major* specific cells in the spleen compared with animals singly infected with *L. major* (Figure 4a). This trend was not significant however (ANOVA, $P > 0{\cdot}05$), indicating that the effect of co-infection on the splenic anti-*L. major* immune response was limited. There was also no significant difference in the number of anti-*L. sigmodontis* IL-4-secreting splenocytes between co-infected animals and animals singly infected with *L. sigmodontis* (ANOVA, $P > 0{\cdot}05$). However, again similarly to the local lymph nodes (Figure 2b), there was a decrease (albeit non-significant: ANOVA, $P > 0{\cdot}05$) in the co-infected animals compared with animals singly infected with *L. sigmodontis*. The spleen thus mirrored the draining lymph nodes with regard to changes in parasite-specific responses on co-infection, but the effects were less pronounced and did not reach statistical significance.

Antibody isotypes provide a useful marker of overall Th1/Th2 bias and can reflect a more cumulative effect rather than the snapshot provided by cytokine analysis. We thus measured parasite-specific antibody isotypes in the serum, using IgG2a as a measure of type 1 responses, and IgG1 as an indicator of type 2 responses. Although we saw no significant differences between co-infected animals and animals singly infected with *L. major* in the amount of anti-*L. major* IgG1 or IgG2a (ANOVA, $P > 0{\cdot}05$ in both cases) (Figure 5a), a slight reduction in IgG1 was seen in co-infected animals. This could reflect the enhanced IFN-γ observed in this group because IFN-γ can have inhibitory effects on isotype switching to IgG1 (17). Likewise there were no significant differences in the levels of either IgG1 or IgG2a mounted against adult *L. sigmodontis* at day 60 post *L. sigmodontis* infection, whether or not *L. major* was present (Figure 5b) (ANOVA, both $P > 0{\cdot}05$). The antigen-specific IgG1/IgG2a antibody ratios thus supported the cytokine data and demonstrated that co-infected animals were able to mount the appropriate response to each infecting parasite, with little evidence for significant interactions between the immune responses mounted against *L. major* and *L. sigmodontis*.

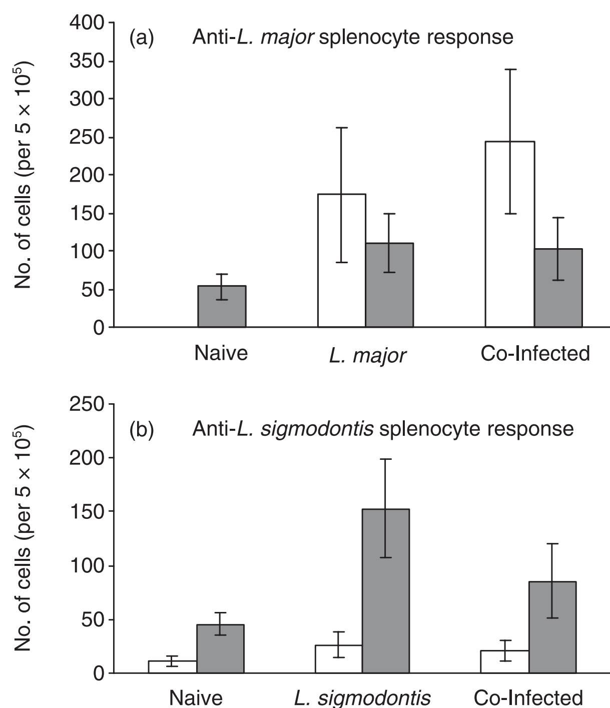

Figure 4 Splenic IL-4 and IFN-γ responses to *L. major* and *L. sigmodontis* during co-infection. Results are shown at day 40 post-infection with *L. major*/60 days post-infection with *L. sigmodontis.* The number of cells secreting IFN-γ (white bars) and IL-4 (grey bars) against *L. major* (a) and *L. sigmodontis* (b) are shown. The graphs represent the combined values of two different experiments each with five animals per group. The error bars represent the SEM.

The presence of filarial nematode infection is coupled to a delay in *L. major*-induced lesion development

Our immunological analysis demonstrated that in the first 40 days following infection with *L. major*, a pre-existing

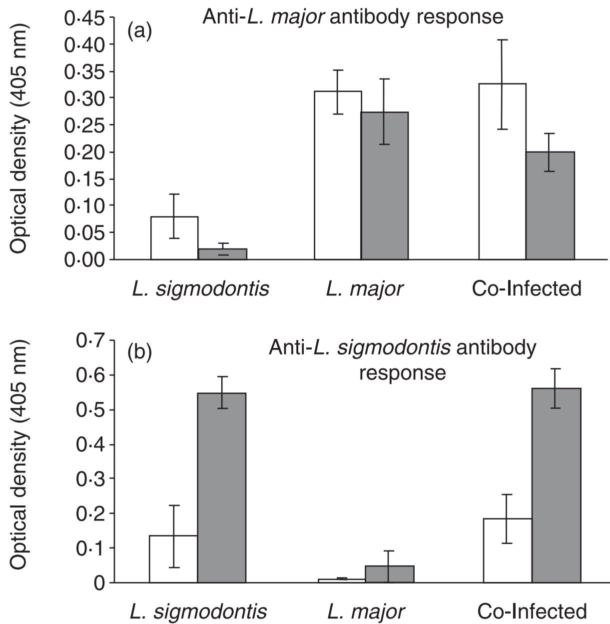

Figure 5 Antibody isotypes generated against *L. major* (a) and *L. sigmodontis* (b) in singly infected and co-infected animals. The graphs show responses measured at day 40 post-infection with *L. major*/60 days post-infection with *L. sigmodontis*. The white bars represent optical density values (405 nm) measured for IgG2a and the grey bars represent optical density values (405 nm) measured for IgG1. The graphs represent the pooled values of two different experiments each with five animals per group. The average of the naïve animals for each experiment and each isotype was subtracted from the response of each individual mouse in the corresponding isotype and experiment. The error bars represent the SEM.

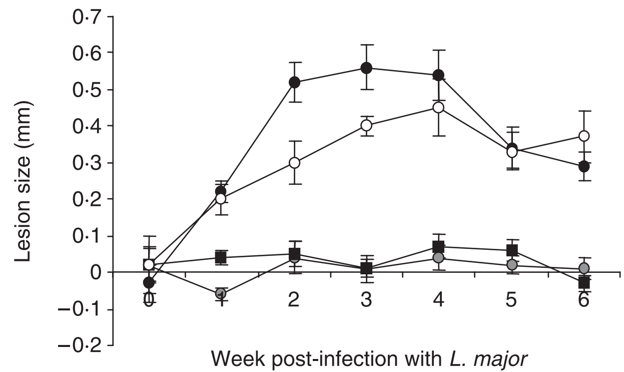

Figure 6 The pathology induced by *L. major* is delayed in mice co-infected with *L. sigmodontis*. The size of the control right footpad was subtracted from the size of the left infected footpad for each mouse. The graph represents the combined data from two different experiments. Each experiment contained five mice in each group. The grey circles represent the naive mice, the black circles represent the animals infected with 3×10^6 *L. major* stationary promastigotes and the open circles represent the co-infected animals. The footpads of the animals singly infected with *L. sigmodontis* are shown by the black squares and are shown as an additional control. Each symbol represents the mean value for each group at each time point. The error bars represent the SEM for 10 animals per group.

L. sigmodontis infection in the thoracic cavity did not alter immune bias but did alter magnitude. This was apparent even at the popliteal LN draining the site of footpad infection. We assessed *L. major*-induced lesion development over this 40-day period as an indicator of whether the established nematode infection could alter the early course of disease progression. Footpad swelling was measured every week from the time of *L. major* infection until the termination of the experiment. We observed a significant delay in the development of footpad lesions in co-infected mice when compared with mice singly infected with *L. major* (Figure 6): 2 weeks post-infection with *L. major* the co-infected animals had smaller footpad swellings than the mice singly infected with *L. major* (ANOVA, $P < 0{\cdot}01$) (Figure 6). Co-infected mice also had less overall pathology during the course of the experiment compared with mice singly infected with *L. major*, as measured by the average area under the curve for each group of mice (2·675 mm^2-weeks for singly infected mice and 2·182 mm^2-weeks for co-infected mice, ANOVA, $P < 0{\cdot}05$) (Figure 6).

Both *L. major* and *L. sigmodontis* parasite numbers were also assessed at the end of the experiments. Although no significant differences were observed, there was a trend toward higher *L. major* parasite numbers in the co-infected mice (data not shown). Of interest, there was also a trend toward reduced *L. sigmodontis* numbers in co-infected mice, suggesting that the incoming *L. major* infection may have accelerated *L. sigmodontis* killing (data not shown).

DISCUSSION

Using the highly quantitative ELISPOT assay for IFN-γ and IL-4, we demonstrated that mice co-infected with a nematode and a protozoan mount highly polarized immune responses. This polarization is most striking at the lymph nodes draining the sites of infection, but is also apparent at a more systemic level in terms of splenocyte responses as well as serum antibody. Although this result is not surprising, based on our knowledge that the immune system can mount independent Th1 and Th2 responses even at the same site (18,19), it is a dramatic reminder that the mammalian immune system has the capacity to appropriately compartmentalize the immune response to simultaneous infection with distinct pathogens (Figure 2).

At least three possible immunological outcomes of co-infection with helminths and 'type 1' inducing pathogens can be envisaged. The first of these would be a cross-regulatory outcome consistent with the competing roles of IFN-γ and IL-4 in early T cell development. This scenario is

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supported by studies that show a strong impact of helminth infection on the development of subsequent type 1 immune responses (20–22). However, recent data suggest that the ability of helminth parasites to modulate type 1 immune responses may have more to do with the induction of regulatory pathways than with a direct effect of type 2 cytokines (23). Thus a second possibility is that a pre-existing helminth infection induces regulatory pathways that diminish type 1 effector function. Although IL-4 and IFN-γ are the only cytokines we used as read-outs for the anti-parasite immune response, we saw little evidence of type 1/type 2 cross-regulation, nor any indication that type 1 responses were diminished by regulatory activity. The regulatory cell population that allows nematode survival in BALB/c mice (10) may not be induced in C57BL/6 mice.

A third, more rarely considered possibility is that type 1 and type 2 responses act additively or even synergistically to enhance overall immune responsiveness. Although only a single time point was measured in our study, the pre-existing *L. sigmodontis* type 2 response apparently led to an increased IFN-γ response against *L. major* by 40 days post-infection. *Ex vivo* ELISPOT showed that co-infection with *L. sigmodontis* also significantly increased the number of IL-4-secreting cells in the popliteal lymph nodes compared to animals singly infected with *L. major*. Because IL-4 has been shown to promote IL-12 (24,25), it is possible that the presence of IL-4 derived from the *L. sigmodontis* response could result in the observed enhanced anti-*L. major* IFN-γ response by promoting IL-12 production. Enhancement of type 1 responses by IL-4 is not without precedent; IL-4 can potentiate type 1 immune responses against tumours (26) and fungal infection (27) as well as promote type 1-mediated pathology in auto-immune disease (28,29). The apparent synergy of type 1 and 2 cytokines has also been reported recently in the context of *L. sigmodontis* infection, whereby IFN-γ and IL-5 act synergistically in the killing of the adult stage of the parasite (30). This provides a possible explanation for our observation of a decrease in *L. sigmodontis* survival in the face of increased IFN-γ following co-infection.

Despite apparent anatomical segregation of the immune responses, we did observe a delay in *L. major*-induced lesion development. A delay in lesion development has also been reported to occur in co-infection with established *Schistosoma mansoni* infection (31,32), although the delay in the study by Yoshida *et al*. (32) did not reach statistical significance. La Flamme *et al*. measured lesion size every 4 weeks for 16 weeks (31) and demonstrated a delay both in lesion resolution and development. We did not extend our observations as far, choosing instead to concentrate on a weekly analysis of the early period, where we hypothesized that a pre-existing nematode infection may tip the balance. However, data up to 6 weeks post-*L. major* infection look remarkably similar between our study and the *S. mansoni* studies both in terms of a delay in lesion size and a trend towards more *L. major* parasites in the co-infected animals. This, combined with a slightly higher lesion size at 6 weeks in our study (Figure 6), suggests that the long-term outcome of this co-infection would mirror that of the La Flamme study. Interestingly, in that study, enhanced IFN-γ responses at 8 weeks post *L. major* infection were preceded by a lower IFN-γ response at 4 weeks post-infection in co-infected mice, a time point we did not measure. Thus an early delay in IFN-γ production as a result of pre-existing helminth infection may explain higher parasite numbers and delayed lesion size in both studies.

The ability of *S. mansoni* and *L. sigmodontis* to similarly alter the progression of leishmaniasis is somewhat surprising, considering that schistosomes and nematodes come from different phyla and vary widely in their ecology and life cycle patterns. Careful investigation of these similarities may reveal important common features of co-infection dynamics.

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REFERENCES

1 Laskay T, Diefenbach A, Rollinghoff M & Solbach W. Early parasite containment is decisive for resistance to *Leishmania major* infection. *Eur J Immunol* 1995; **25**: 2220–2227.
2 Babayan S, Ungeheuer M-N, Martin C, *et al.* Resistance and susceptibility to filarial infection with *Litomosoides sigmodontis* are associated with early differences in parasite development and in localized immune reaction. *Infection Immunity* 2003; **71**: 6820–6829.
3 Petit G, Diagne M, Maréchal P, Owen D, Taylor D & Bain O. Maturation of the filaria *Litomosoides sigmodontis* in BALB/C mice; comparative susceptibility of nine other inbred strains. *Ann. Parasitol. Hum. Comp* 1992; **67**: 144–150.
4 Hoffman W, Petit G, Schulz-Key H, Taylor D, Bain O & Le Goff L. *Litomosoides sigmodontis* in mice: reappraisal of an old model for filarial research. *Parasitol Today* 1999; **16**: 387–389.
5 Le Goff L, Lamb TJ, Graham AL, Harcus Y & Allen JE. IL-4 is required to prevent filarial nematode development in resistant but not susceptible strains of mice. *Int J Parasitol* 2002; **32**: 1277–1284.
6 Graham AL, Taylor MD, Le Goff L, Lamb TJ, Magennis M & Allen JE. Quantitative appraisal of murine filariasis confirms host strain differences but reveals that BALB/c females are more susceptible than males to *Litomosoides sigmodontis*. *Microbes and Infection* 2005; **7**: 612–618.
7 Matthews DJ, Emson CL, McKenzie GJ, Jolin HE, Blackwell JM & McKenzie AN. IL-13 is a susceptibility factor for *Leishmania major* infection. *J Immunol* 2000; **164**: 1458–1462.

8 Sacks D & Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* 2002; **2**: 845–858.

9 Noben-Trauth N, Lira R, Nagase H, Paul WE & Sacks DL. The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to *Leishmania major*. *J Immunol* 2003; **170**: 5152–5158.

10 Taylor MD, Le Goff L, Harris A, *et al.* Removal of T regulatory cell activity reverses hyporesponsiveness and leads to clearance of filarial parasites *in vivo*. *J Immunol* 2005; **174**: 4924–4933.

11 Reiner SL, Zheng S, Wang ZE, Stowring L & Locksley RM. *Leishmania* promastigotes evade interleukin 12 induction by macrophages and stimulate a broad range of cytokines from $CD4^+$ T cells during initiation of infection. *J Exp Med* 1994; **179**: 447–456.

12 Scott P, Eaton A, Gause WC, di Zhou X & Hondowicz B. Early IL-4 production does not predict susceptibility to *Leishmania major*. *Exp Parasitol* 1996; **84**: 178–187.

13 Diagne M, Petit G, Liot P, Cabaret J & Bain O. The filaria *Litomosoides galizai* in mites; microfilarial distribution in the host and regulation of the transmission. *Ann Parasitol Hum Comp* 1990; **65**: 193–199.

14 Misslitz A, Mottram JC, Overath P & Aebischer T. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes. *Mol Biochem Parasitol* 2000; **107**: 251–261.

15 Szabo SJ, Sullivan BM, Peng SL & Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol* 2003; **21**: 713–758.

16 Huang FP, Xu D, Esfandiari EO, Sands W, Wei XQ & Liew FY. Mice defective in Fas are highly susceptible to *Leishmania major* infection despite elevated IL-12 synthesis, strong Th1 responses, and enhanced nitric oxide production. *J Immunol* 1998; **160**: 4143–4147.

17 Hasbold J, Hong JS, Kehry MR & Hodgkin PD. Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a. *J Immunol* 1999; **163**: 4175–4181.

18 Ismail N & Bretscher PA. The Th1/Th2 nature of concurrent immune responses to unrelated antigens can be independent. *J Immunol* 1999; **163**: 4842–4850.

19 Cervi L, MacDonald AS, Kane C, Dzierszinski F & Pearce EJ. Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J Immunol* 2004; **172**: 2016–2020.

20 Kullberg MC, Pearce EJ, Hieny SE, Sher A & Berzofsky JA. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *J Immunol* 1992; **148**: 3264–3270.

21 Pearlman E, Kazura JW, Hazlett FE & Boom WH. Modulation of murine cytokine responses to mycobacterial antigens by helminth-induced T-helper-2 cell responses. *J Immunol* 1993; **151**: 4857–4864.

22 Rodriguez M, Terrazas LI, Marquez R & Bojalil R. Susceptibility to *Trypanosoma cruzi* is modified by a previous non-related infection. *Parasite Immunol* 1999; **21**: 177–185.

23 Maizels RM & Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 2003; **3**: 733–744.

24 Kalinski P, Smits HH, Schuitemaker JH, *et al.* IL-4 is a mediator of IL-12p70 induction by human Th2 cells: reversal of polarized Th2 phenotype by dendritic cells. *J Immunol* 2000; **165**: 1877–1881.

25 Bullens DM, Kasran A, Thielemans K, Bakkus M & Ceuppens JL. CD40L-induced IL-12 production is further enhanced by the Th2 cytokines IL-4 and IL-13. *Scand J Immunol* 2001; **53**: 455–463.

26 Schuler T, Qin Z, Ibe S, Noben-Trauth N & Blankenstein T. T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J Exp Med* 1999; **189**: 803–810.

27 Mencacci A, Del Sero G, Cenci E, *et al.* Endogenous interleukin 4 is required for development of protective $CD4^+$ T helper type 1 cell responses to *Candida albicans*. *J Exp Med* 1998; **187**: 307–317.

28 Fort M, Lesley R, Davidson N, *et al.* IL-4 exacerbates disease in a Th1 cell transfer model of colitis. *J Immunol* 2001; **166**: 2793–2800.

29 Ramanathan S, de Kozak Y, Saoudi A, *et al.* Recombinant IL-4 aggravates experimental autoimmune uveoretinitis in rats. *J Immunol* 1996; **157**: 2209–2215.

30 Saeftel M, Arndt M, Specht S, Volkmann L & Hoerauf A. Synergism of gamma interferon and interleukin-5 in the control of murine filariasis. *Infect Immun* 2003; **71**: 6978–6985.

31 La Flamme AC, Scott P & Pearce EJ. Schistosomiasis delays lesion resolution during *Leishmania major* infection by impairing parasite killing by macrophages. *Parasite Immunol* 2002; **24**: 339–344.

32 Yoshida A, Maruyama H, Yabu Y, Amano T, Kobayakawa T & Ohta N. Immune response against protozoal and nematodal infection in mice with underlying *Schistosoma mansoni* infection. *Parasitol Int* 1999; **48**: 73–79.

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