Cellular immune responses to the murine nematode parasite *Trichuris muris*
I. DIFFERENTIAL CYTOKINE PRODUCTION DURING ACUTE OR CHRONIC INFECTION

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SUMMARY

A variety of T-cell parameters have been analysed in two H-2 compatible strains of mice, B10.BR and BALB/K, which differ in an absolute fashion in their ability to resist infection with the parasitic nematode *Trichuris muris*: BALB/K mice expel *T. muris* relatively rapidly, whereas B10.BR mice are unable to expel the parasite before the infection reaches patency. Analysis of Th1- and Th2-specific cytokines (IFN-γ and IL-5, respectively) produced by *in vitro* Con A-stimulated mesenteric lymph node cells (MLNC) from infected and normal mice demonstrated that MLNC from resistant BALB/K mice produced high levels of IL-5 and low levels of IFN-γ whilst B10.BR MLNC secreted large amounts of IFN-γ in the relative absence of IL-5. As an *in vivo* correlate of *in vitro* IL-5 production, peripheral and tissue eosinophilia were quantified during the course of infection in the two strains of mice. No peripheral eosinophilia was observed in BALB/K or B10.BR individuals. However, a considerable intestinal eosinophilia was seen in the high IL-5-producing BALB/K mice compared to normal levels. Differences observed in cytokine profiles were not due to differential changes in the numbers of T cells within the MLN. Indeed, FACS analysis revealed a decrease in the relative percentage of CD4$^+$ and CD8$^+$ T cells in both strains of mice post-infection. Our results suggest that resistance to *T. muris* involves the preferential induction of Th cells which secrete IL-5, whilst cells of a different Th subset (IFN-γ producing) predominate in chronically infected mice. As such, this represents the first description of a correlation between the reciprocal activation of Th cell subsets in relation to acute or chronic intestinal infection with the same parasite in the same host species.

INTRODUCTION

Resistance to the large intestinal nematode parasite *Trichuris muris* varies considerably between different strains of mice.[1,2] Some mouse strains expel the worm relatively rapidly (NIH, BALB/K), others do so more slowly (C57BL/10, B10.G), whilst some fail to expel the worm before the infection reaches patency (Day 32 post-infection, p.i.). Such strains are defined as non-responder strains (B10.BR, AKR/J). The marked strain variation in resistance enables the mechanisms of immunity operating during both an acute and a chronic infection to be studied in the same host species.

Infections in athymic nude mice readily proceed to patency (K. J. Else and R. K. Grencis, unpublished data) and adoptive transfer experiments with MLNC[3] have demonstrated that T

Abbreviations: FACS, fluorescence-activated cell sorter; FCS, foetal calf serum; mAb, monoclonal antibody; MLNC, mesenteric lymph node cells; pi, post-infection; sIg, surface immunoglobulin; SN, supernatant; TcR, T-cell receptor.

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cells play a central role in protective immunity, although the exact mechanisms involved are unknown. A detailed analysis of the humoral immune responses to *T. muris* has revealed differences in T-cell regulated isotype profiles. Some good responder strains develop high level IgG1 responses and some non-responder strains produce a good IgG2a response in the relative absence of IgG1.[4] However, neither IgG1 or IgG2a levels were absolute correlates of responder status, with high levels of antibody being detected in the sera well after expulsion of the parasite from the gut. To assess in a more direct way, the mechanisms by which T cells regulate *T. muris* infection, through the generation of protective immunity or a state of non-responsiveness, we have examined a variety of T-cell parameters in responder (BALB/K) and non-responder (B10.BR) mouse strains in the nodes draining the site of infection (mesenteric lymph nodes, MLN). Changes in the relative percentage of CD4$^+$, CD8$^+$, $\alpha\beta$- and $\gamma\delta$-expressing T cells, and cells bearing surface immunoglobulin (sIg), in the MLN were analysed by fluorescence-activated cell scoring (FACS). In addition, with the growing evidence for differential induction of helper T-cell subsets during nematode helminth infections,[5,6] assays for Th1-type and Th2-type specific cytokines (IFN-γ and IL-5, respect-

ively) were performed on *in vitro* mitogen-stimulated MLN cell supernatants (SN). Peripheral and intestinal tissue eosinophilia were also quantified as an *in vivo* correlate of *in vitro* cytokine production. Our results suggest that there is a selective expansion of IL-5-producing (Th2-type) cells in resistant strains of mice, whereas in strains of mice unable to expel *T. muris* IFN-γ-producing (Th1-type) cells predominate.

MATERIALS AND METHODS

Animals

B10.BR and BALB/K male mice were purchased from Harlan Olac Ltd, Bicester, Oxon, U.K. and infected when 6–8 weeks old.

Parasite

The maintenance of *T. muris* and the method used for infection were as described by Wakelin.[7] Mice were killed at various time points p.i. and worm burdens assessed as described by Else *et al.*[2]

Cell supernatants

MLNs were removed from infected or normal mice and dissociated in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) containing 10% foetal calf serum (FCS) and antibiotics at 100 IU/ml penicillin and 100 μg/ml streptomycin. After washing three times, cells were resuspended in RPMI-1640 medium (Gibco) containing FCS and antibiotics as above plus 2 mM L-glutamine and 7.5×10^{-5} M monothioglycerol. Viable cell concentrations were adjusted to give 5×10^6 cells/ml and dispensed into cluster plates (Nunc, Roskilde, Denmark) with a maximum of 5×10^6 cells/well. Concanavalin A (Con A) at a predetermined optimal final concentration of 2.5 μg/ml or medium were added and SN harvested at 24 and 48 hr, aliquoted and stored at −80° prior to use.

Fluorescence-activated cell sorting (FACS)

CD4+ and CD8+ T cells were analysed using rat monoclonal antibodies (mAb) (anti-L3T4; anti-Ly2; S. Cobbold) followed by a FITC-conjugated anti-subclass specific antibody (Norig, Seralab).[8] Cells bearing sIg were detected using a biotinylated rabbit anti-mouse Ig (Dakopatts, High Wycombe, Bucks, U.K.) in combination with phycoerythrin-streptavidin (Seralab, Crawley Down, Sussex, U.K.). mAb specific for the αβ-[9] or γδ-[10] T-cell receptor (TcR) were conjugated to biotin, and used together with phycoerythrin-streptavidin. Labelled cells were analysed using a FACScan equipped with the Consort 30 program (Becton-Dickinson, Mountain View, CA). Gates were set so as to exclude non-viable cells and adjusted for γδ TcR analysis so that only cells with high forward and side-scatter were examined. This enhanced the detection of the small γδ-TcR-positive population and hence enabled a clearer discrimination between strains in percentages of γδ-TcR-positive cells. Data are presented as percentage positive cells following subtraction of background staining with the fluorescent conjugate alone.

Lymphokine analysis

IL-5 was measured in MLNC SN by sandwich ELISA as described by Mosmann & Fong[11] using a pair of rat anti-mIL-5 mAb (5P8.23, TRFK-4; T. Mosmann). Amounts of IL-5 in test SN were quantified by reference to commercially available murine recombinant IL-5 standards. Interferon-gamma IFN-γ

was also measured by sandwich ELISA using the two anti-mIFN-γ mAb R4-6A2 (E. Havell)[12] and XMG1.2 (T. Mosmann) (both at 10 μg/ml) and the methodology described by Mosmann & Fong[11] for the mouse IL-5 ELISA. A cell SN from CHO cells transfected with the gene for recombinant murine IFN-γ (211A)[13] previously quantified for IFN-γ content using a viral replication bioassay, was used to generate a standard curve from which levels of IFN-γ in test SN were established. For both the IL-5 and IFN-γ ELISAs test SN were considered positive if greater than the mean +3 standard deviations of 16 control wells, to which all reagents except the test SN had been added. Using this criteria the limit of detectability was 8 U/ml for IL-5 and 6 U/ml for IFN-γ.

Eosinophil staining

Blood smears from individual mice taken throughout the course of infection were fixed in absolute methyl alcohol before staining with Giemsa's stain for 40 min. Slides were rinsed in distilled water, dried and mounted. The number of eosinophils in a total of 100 cells per slide were counted.

At autopsy the caecum tip was removed from individual mice, opened, and fixed in 10% neutral buffered formalin, prior to processing and embedding in paraffin wax. 3 μ sections were cut and stained for eosinophils using chromotrope 2R. Briefly, after taking sections to water, staining with Mayer's haematoxylin and blueing in tap water, sections were stained with 0.5% chromotrope 2R in 1% phenol for 30 min. After washing, sections were dehydrated, cleared and mounted and the number of eosinophils in 20 caecal/crypt units enumerated in the epithelium and lamina propria.[14]

Statistical analysis

Significant differences between experimental groups in worm burden, cytokine production and eosinophilia were calculated using the Mann–Whitney U-test. A value greater than $P = 0.05$ was considered non-significant.

RESULTS

Experiment 1

B10.BR and BALB/K mice were infected with 400 *T. muris* eggs on Day 0 and killed in groups of usually five individuals at various time-points p.i. MLNC were pooled per strain per time-point and SN generated as outlined above.

Figure 1a shows the worm burdens recovered from mice on Days 13, 20 and 34 p.i. The number of larvae established on Day 13 p.i. did not differ significantly between the two strains studied ($P > 0.05$). The worm burdens recovered from BALB/K mice on Day 20 p.i. were significantly lower ($P < 0.005$) than seen on Day 13 p.i. and by Day 34 p.i. expulsion was complete. The majority of B10.BR mice failed to expel the parasite, Day 34 p.i. worm burdens not being significantly different to Day 13 values ($P > 0.05$). However, one individual in this group had a very low worm burden on Day 34 p.i. Although atypical, this has been reported previously for other B10 congenics.[1]

Lymphokine analysis

Twenty-four- and 48-hr Con A SN from MLNC derived from infected and normal mice were assayed for IL-5 and IFN-γ by ELISA. Optimal levels of IFN-γ were detected in the 24 hr SN, whilst 48 hr SN contained higher levels of IL-5. Results are
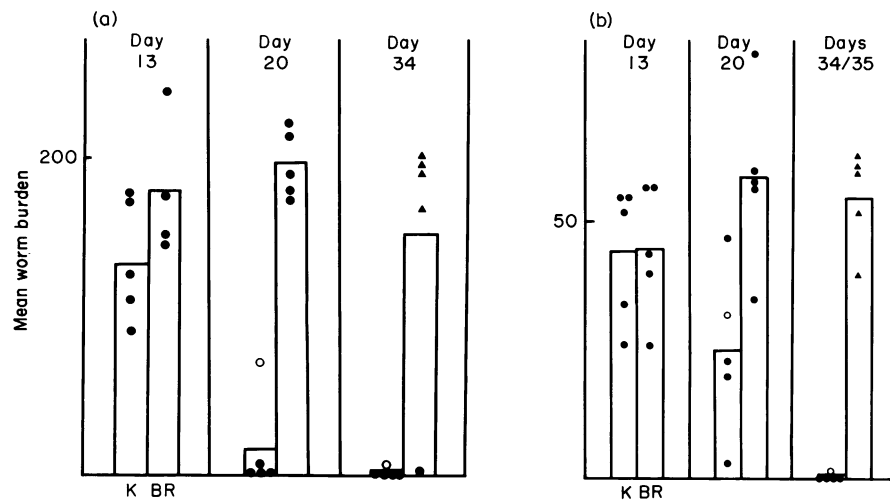
Figure 1. (a) Worm burdens recovered from B10.BR (BR) and BALB/K (K) mice used in Exp. 1 on Days 13, 20 and 34 p.i. Bar graphs represent the mean worm recovery with individual worm burdens indicated by symbols (●). The presence of mature adult worms on Day 34 is indicated by (▲); (○) indicates the presence of at least some immature stunted worms. (b) Worm burdens recovered from B10.BR and BALB/K mice used in Exp. 2, on Days 13, 20 and 34 p.i. for BALB/K mice or 35 p.i. for B10.BR individuals. Bar graphs represent the mean worm recovery for each strain with individual worm burdens indicated by symbols (●). Individuals harbouring a patent infection (▲); some immature stunted worms present (○).

Table 1.

	Day 13	Day 20	Day 34	Normal
(a)				
BR IL-5	31	57	0	16
BR IFN-γ	143	221	21	0
K IL-5	64	>250	52	23
K IFN-γ	7	24	15	7

	Day 13	Day 20	Days 34/35	Normal
(b)				
BR IL-5	0±0	3·4±4·7	16·8±23·7	9·5±15·7
BR IFN-γ	40·4±36·5	269·0±71·4	140·0±35·8	39·0±51·9
K IL-5	22·8±21·0	54·0±24·2	65·0	24·1±30·5
K IFN-γ	5·6±7·7	70·0±36·0	14·0	18·0±19·3

(a) Levels of IL-5 and IFN-γ secreted by *in vitro* Con A-stimulated MLNC from infected and normal B10.BR (BR) and BALB/K (K) mice used in Exp. 1. Values (in U/ml) were obtained from pooled supernatants from five individuals per strain per time-point.

(b) Levels of IL-5 and IFN-γ produced by *in vitro* Con A-stimulated MLNC from infected and normal B10.BR (BR) and BALB/K (K) mice used in Exp. 2 on Days 13, 20 and 34 (BALB/K) or 35 (B10.BR). Values (in U/ml) represent mean ± SD for five infected individuals per strain per time-point except Day 34 BALB/K values where cells were pooled from five individuals. Normal values represent the mean ± SD for at least 10 animals except for the B10.BR IFN-γ mean, where SNs from only six mice were assayed.

presented in Table 1a for IFN-γ (24 hr SN) and IL-5 (48 hr SN). IL-5 levels rose post-infection in SN from BALB/K mice to levels greater than 250 U/ml on Day 20, compared to normal levels of around 23 U/ml. In contrast, SN derived from B10.BR MLNC contained only low amounts of IL-5 which fell to below background levels by Day 34. IFN-γ profiles for the two strains

of mice showed the reverse pattern with high levels of IFN-γ being detected in B10.BR MLNC SNS, particularly on Days 13 and 20 p.i. BALB/K MLNC, however, produced very little IFN-γ. Normal MLNC from BALB/K mice produced low amounts of IFN-γ when stimulated with Con A. No detectable IFN-γ was produced by normal B10.BR MLNC.

Experiment 2

The results from Exp. 1 suggested that during an acute *T. muris* infection, as seen in BALB/K mice, there is a selective expansion of IL-5-secreting cells whilst IFN-γ-producing cells predominate in chronically infected mice. To substantiate our findings we infected a second group of B10.BR and BALB/K mice to generate MLNC SN from individual mice for lymphokine analysis. A lower level of infection was used to provide information on cytokine production in relation to antigenic exposure. FACS analysis was also performed on pooled MLNC per strain per time-point. In addition individual blood smears were made during the course of infection to assess peripheral eosinophilia and tissue from the caecum was taken at autopsy for analysis of local intestinal eosinophilia.

Worm expulsion

Worm burdens recovered from mice on Days 13, 20 and 34 p.i. for BALB/K mice, and Days 13, 20 and 35 p.i. for B10.BR mice, are shown in Fig. 1b. As seen on Day 13, the level of infection was lower than in experiment 1. No significant difference in worm numbers between strains was observed on Day 13 ($P > 0.05$). As before, by Day 20 p.i., BALB/K mice had significantly fewer worms than on Day 13 p.i. ($P < 0.05$), with almost no worms remaining on Day 34. In contrast worm burdens harboured by B10.BR mice on Day 35 were not significantly different to Day 13 levels ($P > 0.05$).

Table 2.

Day	αβ			γδ			CD4			CD8			sIg			αβ:sIg		
	13	20	34/35	13	20	34/35	13	20	34/35	13	20	34/35	13	20	34/35	13	20	34/35
BRi	61·1	52·5	39·2	3·1	3·5	3·1	35·1	25·1	20·0	27·0	17·0	13·0	N/D	29·0	31·0	N/D	1·8	1·3
BRn	63·5	61·8	N/D	0·8	2·4	N/D	33·1	33·1	N/D	28·4	22·3	N/D	N/D	18·4	20·8	N/D	3·4	N/D
Ki	73·3	70·6	58·1	0·0	0·0	0·0	48·1	42·9	40·8	26·0	20·3	21·3	N/D	19·3	21·0	N/D	3·7	2·8
Kn	76·3	72·5	68·6	0·9	0·8	0·0	47·8	44·8	46·8	25·9	24·2	24·0	N/D	16·8	16·2	N/D	4·3	4·2

Relative percentages of αβ- and γδ-TcR-positive cells recovered from the MLN of infected and normal B10.BR (BRi, BRn) and BALB/K (Ki, Kn) mice throughout the course of infection. Cells were pooled from five individuals per strain per time-point. mAb to the various surface markers were revealed by FITC-conjugated anti-rat IgG1 antibody (CD4, CD8) or biotinylated and stained with phycoerythrin-streptavidin (αβ-, γδ-TcR, sIg). Samples were analysed on a FACScan (Becton-Dickinson). Gates were set to exclude non-viable cells and for γδ-TcR analysis gates were adjusted so that just cells with high forward and side-scatter were analysed. This was done to enhance the detection of a very small cell population and enable clearer differences between strains to be seen. Data are presented as percentage positive cells following subtraction of background staining with the fluorescent conjugate alone.

Table 3.

	Eosinophils/20 caecal crypt units			
	Day 13	Day 20	Day 34/35	Normal
BR	8·6±2·97	21·4±11·8	18·8±6·8	5·0±3·52
K	4·6±3·05	34·6±13·4	43·0±41·0	6·3±4·2

Mean number (±SD) of eosinophils/20 caecal crypt units in infected and normal B10.BR (BR) and BALB/K (K) mice, on Days 13, 20 and 34 (BALB/K) mice, or 13, 20 and 35 (B10.BR). Normal values represent the mean ±SD for at least 12 animals.

FACS analysis

The relative percentages of CD4$^+$, CD8$^+$, αβ- and γδ-TcR-bearing T cells, and sIg expressing MLN cells from infected and normal BALB/K and B10.BR mice are shown in Table 2. In both strains a gradual decrease in percentage CD4$^+$ and CD8$^+$ cells was observed p.i. relative to normal levels. This decrease was reflected in levels of αβ-TcR-positive T cells, and was greater in the non-responder B10.BR strain of mouse.

Coincident with this decrease was an increase in the relative percentage of sIg-bearing cells in the MLN of infected mice. This increase was greatest in B10.BR mice resulting in an αβ:sIg ratio of only 1·26:1·0 on Day 35 compared to a normal B10.BR ratio of 3·4:1·0 (Day 20) and a corresponding infected BALB/K value of almost 3:1 (Day 34). The percentage of T cells bearing the γδ-TcR was also evaluated by FACS. As seen in Table 2, around 3% γδ-TcR-positive cells were consistently detected within the large, granular cell population of infected B10.BR MLNS, unlike MLN from infected BALB/K mice. However, no change in this value occurred during infection and normal B10.BR MLNC also contained around 2% γδ T cells (Day 20).

Lymphokine analysis

Mean levels of IFN-γ and IL-5 produced by MLNC from infected and normal mice after *in vitro* Con A stimulation are shown in Table 1b. As before, 24-hr SN were assayed for IFN-γ and 48-hr SN for IL-5.

Levels of IL-5 were in general lower than seen in Exp. 1, this probably being related to the lower worm burdens established. However, MLNC from infected BALB/K mice again produced significantly more IL-5 than those from infected B10.BR mice ($P < 0.05$). Indeed levels of IL-5 produced by infected B10.BR mice did not rise significantly above normal B10.BR levels at any time-point p.i. Levels of IL-5 produced by MLNC from infected BALB/K individuals on Day 20 showed an inverse relationship with worm burden. Thus the two highest IL-5-producing individuals harboured the fewest worms, whilst no decrease in worm burden from Day 13 levels was seen in the individual producing the least IL-5. MLNC from normal B10.BR and BALB/K mice produced IL-5 after Con A stimulation. However, levels of IL-5 produced by infected BALB/K MLNC, particularly on Day 20 p.i., were significantly higher than normal levels ($P < 0.05$), the large SD seen in normal BALB/K mice relative to the mean reflecting the presence of 2/10 individuals with raised IL-5 levels. Analysis of the MLNC SN for IFN-γ revealed a similar pattern to that seen in Exp. 1. MLNC from infected B10.BR individuals produced very significantly higher levels of IFN-γ, compared to normal mice on Days 20 ($P < 0.005$) and 35 ($P < 0.01$) and compared to infected BALB/K mice on Days 13 ($P < 0.05$) and 20 ($P < 0.005$).

Although levels of IFN-γ significantly greater than normal levels ($P < 0.01$) were produced by infected BALB/K MLNC on Day 20, levels were considerably lower than seen for infected B10.BR mice at this time point.

Peripheral and tissue eosinophilia

Blood smears taken from individual mice throughout the course of infection were stained for the presence of eosinophils. Numbers of eosinophils were low in both infected BALB/K and B10.BR mice and remained at levels similar to normal levels throughout the course of infection (data not shown).

Using chromotrope 2R to stain locally for eosinophils in the caecum, a distinct tissue eosinophilia was detected in infected BALB/K mice on Days 20 and 34 (Table 3), numbers being significantly above normal BALB/K values ($P < 0.001$ and

$P < 0.01$, respectively). Although infected B10.BR mice on Day 20 also had levels of eosinophils in the gut above normal levels, the eosinophilia seen in infected BALB/K mice at this time was significantly greater ($P < 0.05$).

DISCUSSION

We have analysed a variety of T-cell parameters in two H-2-compatible strains of mice which differ in an absolute fashion in their ability to mount a protective immune response to the intestinal nematode parasite T. muris: BALB/K mice are very resistant to infection whilst B10.BR mice fail to expel the parasite before the infection reaches patency.[1] Analysis of the lymphokine profiles of in vitro Con A-stimulated MLNC from non-responder B10.BR and resistant BALB/K mice provided interesting data. Resistance to infection correlated with the production of high levels of IL-5 and negligible IFN-γ, whilst non-responder status was associated with the production of large amounts of IFN-γ and little IL-5. This IFN-γ was bioactive when tested in an IFN-γ bioassay (data not shown).

These profiles are consistent with the differential induction of the two Th cell subsets proposed by Mosmann et al.[15] to give predominantly IFN-γ-secreting Th1 cells in non-responder mice and mainly IL-5-producing Th2 cells in resistant strains. Th1 and Th2 cells, defined primarily by their patterns of lymphokine production, differ markedly in function:[5] Th1 cells produce IFN-γ, IL-2 and lymphotoxin and mediate mainly inflammatory-type responses, whilst Th2 cells, through their secretion of IL-4 and IL-5, are intimately associated with antibody production and strong allergic responses. Originally identified using long-term cell clones, where the process of selection could favour survival of only certain Th phenotypes, evidence is accumulating that, at least in the mouse, Th1 and Th2 cells do occur in vivo where immunization protocols or parasitic infection can dramatically affect the type of clone obtained in vitro.[16]

We have used in vitro Con A stimulation of MLNC from infected mice to define the presence of cells precommitted to the Th1 or Th2 phenotype following their exposure to parasitic infection. Such an approach, originally used to identify the helper T-cell subtypes in long-term T-cell clones[15,17] has also been used by Street & Mosmann to show the enhanced ability of spleen cells to synthesise IL-4 and IL-5 after activation by Nippostrongylus brasiliensis infection.[16]

Another study of cytokine production during intestinal helminth infection has also provided evidence for the preferential induction of Th1 or Th2 cells in vivo. Pond, Wassom & Hayes[6] have reported that for Trichinella spiralis resistance and susceptibility correlates with the preferential activation of Th1 and Th2 cells, respectively. However, the data presented here are the first description of a correlation between the reciprocal activation of Th cell subsets in relation to acute or chronic intestinal helminth infection.

Although demonstrating the importance of IL-5-secreting Th2-type cells in resistance to T. muris, our results do not directly implicate IL-5 in the protective immune response. However, the in vivo tissue eosinophil response to T. muris correlated well with IL-5 production in vitro. This suggests that IL-5 is indeed released in vivo, IL-5 being active in the differentiation of eosinophils[18] and the major mediator in helminth-induced eosinophilia.[19] Given the importance of IL-5 in the regulation of IgA expression[20,21] and the fact that the only

anti-T. muris mAb known to transfer protective immunity are of the IgA isotype,[22] it is possible that Th2-type cells may also influence resistance to T. muris via enhancing local specific IgA production.

The differential cytokine production profiles observed between the responder and non-responder mice did not simply reflect alteration in proportions of T cells within the MLN (Table 2), both strains exhibiting similar trends. In addition, examination of TcR usage by FACS analysis with a variety of Vβ chain-specific monoclonal antibodies have failed to reveal any correlation between Vβ receptor expression and responder status (K. J. Else and R. K. Grencis, unpublished data). The data, therefore, do support the hypothesis of differential T-cell cytokine gene activation during acute or chronic parasitic infection. Indeed, the phenomenon of 'polarization' of the T-cell response towards either Th1 or Th2 has been demonstrated at the clonal level for a protozoan parasite which exhibits a spectrum of disease manifestations (acute or chronic, Leishmania).[23,24] The outstanding question which remains to be answered, therefore, is how the two Th subsets are differentially induced. Current opinion is for the existence of precursor T cells which progress through a Th0 state before becoming finally committed to a Th1 or Th2 phenotype, according to environmental stimuli.[5] These stimuli include the type of antigen, the type of antigen-presenting cell (APC), the density of antigen on the APC surface and interactions with other T-cell populations.[5,25,26] A further level of complexity is introduced by reports that different Th subsets utilize different TcR-associated signal transduction mechanisms.[27] Clearly an understanding of the mechanism operating to tightly control the ratio of Th-cell subsets produced during an immune response is critical in our understanding of how responder and non-responder phenotypes are generated during parasitic infection. Future studies will address this problem and examine the mechanisms by which Th2-type cells influence resistance to T. muris infection.

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