Trichuris muris: CD4+ T cell-mediated protection in reconstituted SCID mice

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SUMMARY

Resistance to the murine intestinal nematode *Trichuris muris* requires the development of a strong Th2 response. In a reconstituted SCID mouse model, CD4+ Th2 cells can mediate resistance to infection in the absence of antibody (Else & Grencis, 1996). The data presented here address the issue of how CD4+ T cells mediate this protective immunity within the SCID host. These studies demonstrate that timing and cell dose are critical if transfer is to result in resistance, with a minimum of 5×10^6 immune donor cells required to confer immunity. Furthermore, this CD4-mediated protective immunity only operates against the larval stages of the parasite. When the molecules necessary for activated CD4+ T cell migration to the GALT are inhibited with a cocktail of anti-integrin/addressin antibodies (anti-β7, anti-MAdCAM-1 and anti-αE), the resistance conferred by immune donor cells is completely abrogated. This implies that the effector mechanism acts locally at the level of the gut. CD4+ mediated cytotoxicity, directed against the epithelial cells inhabited by the parasite, could represent a novel, locally acting effector mechanism. However, Fas and Fas ligand-deficient mice, which are unable to mount CD4-mediated cytotoxic responses, readily expel *T. muris* indicating that the mechanism by which CD4+ T cells mediate protective immunity is unlikely to involve killing of infected gut epithelial cells.

Key words: nematode, *Trichuris muris*, protection, CD4, SCID mouse.

INTRODUCTION

Infection of inbred mice with the intestinal nematode *Trichuris muris* can result in either resistance or susceptibility depending on the mouse strain (Else *et al*. 1990). The majority of inbred mice are resistant to *T. muris* and expel the parasite before patency is reached, around day 32 post-infection (Else & Wakelin, 1988). However, a few strains of mouse, such as AKR, are unable to mediate a protective immune response and permit the development of these parasites to fecund adults. Resistance is CD4+ T cell mediated, since animals depleted of the T helper cell population (Koyama, Tamauchi & Ito, 1995), or of an immunodeficient background lacking T cells (e.g. SCID, nude), are unable to expel *T. muris* (Else & Grencis, 1996; Ito, 1991). The ability to expel a *T. muris* infection correlates closely with differences in T helper cell subset development. Resistance is mediated by the ability to develop a Th2-type cytokine response, characterized by the production of IL-4, IL-5, IL-9 and parasite-specific IgG1. Conversely, susceptibility corresponds to the production of Th1 cytokines and parasite-specific IgG2a (Else & Grencis, 1991; Else, Hultner & Grencis, 1992; Else *et al*. 1994). IL-4 is a critical cytokine in resistance, with both anti-IL-4R antibody treatment and studies in IL-4 knockouts demonstrating the susceptible nature of animals infected in the absence of this cytokine (Bancroft, McKenzie & Grencis, 1998; Else & Finkelman, 1998; Else *et al*. 1994). IL-13 has also been shown to be an absolute requirement for resistance in that IL-13 deficient animals are susceptible to infection (Bancroft *et al*. 1998).

Although the characteristics of the immune response mounted by resistant animals have been well documented, the Th2-mediated effector mechanisms which lead to parasite elimination remain undefined. Many of the traditional Type 2 responses associated with helminth infection, such as mastocytosis, eosinophilia and antibody-dependent cellular cytotoxicity, have been shown to be unimportant (Betts & Else, 1999). Indeed, antibody itself appears to be non-essential in that adoptive transfer of highly pure immune CD4+ T cells into SCID mice can transfer immunity in the absence of any antibody (Else & Grencis, 1996).

To investigate further the properties of this CD4+-mediated protection, this study examines the minimum number of immune CD4+ T cells required to confer resistance in SCID mice and the kinetics of worm expulsion. In addition, SCID mice reconstituted with CD4+ cells were treated with saturating levels of anti-integrin antibodies (Picarella

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et al. 1997) to determine the importance of $CD4^+$ entry into the gut-associated lymphoid tissue (GALT) in resistance to infection. Finally, the intracellular niche inhabited by *T. muris* may lend itself to attack via cell-mediated cytotoxic effector mechanisms. Since the majority of CD4-mediated cell killing involves Fas-Fas ligand interactions, the expulsion phenotype of mice deficient in Fas or FasL was examined to determine if CD4-mediated cytotoxicity of infected gut epithelial cells plays a role in $CD4^+$ T cell reconstituted SCID mouse model.

MATERIALS AND METHODS

Animals

C.B-17 *scid/scid* (SCID) mice were bred and maintained in micro-isolator cages in the animal facility at the University of Manchester. Original breeding pairs were obtained from Charles River. All bedding, food and water were kept sterile with all handling and experimental manipulations carried out in a laminar flow hood. All experiments involved animals 6–12 weeks of age and SCID mice were routinely screened for the presence of total serum immunoglobulin before and after infection. Mice with serum immunoglobulin levels higher than 5 μg/ml were considered 'leaky' (Else & Grencis, 1996). Sera taken from reconstituted SCID animals were also tested for parasite-specific IgG post-infection and were routinely negative confirming the absence of B cells in the donor population. Male donor BALB/c mice were obtained from Harlan (Bicester, UK) and infected between 6 and 8 weeks of age. The *gld* (FasL deficient) and *lpr* (Fas deficient) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) on a C57BL/6 background and the relevant age-matched controls purchased from Harlan (UK).

Parasite

The maintenance of *T. muris* and collection of eggs and antigen have been previously described (Bancroft *et al*. 1998; Wakelin, 1967). Mice were infected with approximately 150–200 embryonated eggs via oral gavage and killed at various time-points post-infection. Worm burdens in the caecum and large intestine were assessed as previously described (Else *et al*. 1990).

Donor cell preparation and reconstitution

Mesenteric, inguinal and axillary LN were removed from naïve donor mice, or immune donor mice at day 21 post-infection, and pooled before sterile disassociation in Hanks balanced salt solution (Hanks) supplemented with 2% FCS (Life Technologies, Paisley, Scotland). Cells were washed, counted and the $CD4^+$ subset purified by negative selection as previously described (Else & Grencis, 1996). Briefly, whole LN cell suspensions were incubated with rat anti-mouse B220 and rat anti-mouse CD8 antibodies (Pharmingen) prior to separation with Biomag anti-rat Ig magnetic beads (Metachem Diagnostics, Northampton, UK). A second selection step removed any cells expressing MHC Class II by labelling cells with mouse anti-mouse $I\text{-}A^d$ antibody (Pharmingen) followed by incubation with anti-mouse Ig particles (Biomag). After final washing and counting, cells were resuspended in Hanks, at an appropriate concentration to give the required number of cells in a volume of 200 μl, and injected i.v. via the tail vein. Control mice received an i.v. injection of Hanks alone. Donor cell purity was verified by FACS prior to transfer.

FACS

The purity of the donor cells, and the presence of transferred cells in the SCID organs was verified by FACS analysis for CD4, CD8 and B220 cell markers using fluorescein isothiocyanate-conjugated rat anti-mouse antibodies against the above molecules (CD4 and CD8 from Sera Lab, B220 from Pharmingen). A fluorescein isothiocyanate-conjugated rat isotype control antibody (Pharmingen) was used as a negative control. Donor populations were typically 96–98% $CD4^+$ and contained no CD8 or B220 positive cells. Similarly, FACS carried out on SCID mesenteric LN (MLN) populations post-reconstitution failed to detect any CD8 or B cell populations confirming donor cell purity.

Antibody treatment

Homing of $CD4^+$ T cells to the GALT was blocked *in vivo* using a cocktail of anti-integrin monoclonal antibodies consisting of anti-β7 (FIB504.84), anti-MAdCAM 1 (MECA 367) and anti-αE (M290). These antibodies are routinely used for blocking *in vivo* and are non-depleting (Picarella *et al*. 1997; Peter Kilshaw, personal communication). Cell lines were obtained from ADCC (FIB504.84, MECA 367) and the M290 was kindly donated by Dr Peter Kilshaw (Babraham Institute, Babraham, UK). Supernatants were collected and the antibodies purified by chromatography over a Protein G column (Pharmacia). Each antibody was given at a dose of 200 μg in a cocktail intraperitoneally (i.p.) on alternate days from 2 days prior to reconstitution until killing. In addition, purified $CD4^+$ T cells were injected resuspended in the same concentration of antibody cocktail. Control animals were given 600 μg of rat Ig i.p. at the same time-points. In all cases, sera from treated animals showed a level of rat Ig in the circulation of greater than 500 μg/ml 1 week after cessation of antibody treatment (data not shown).

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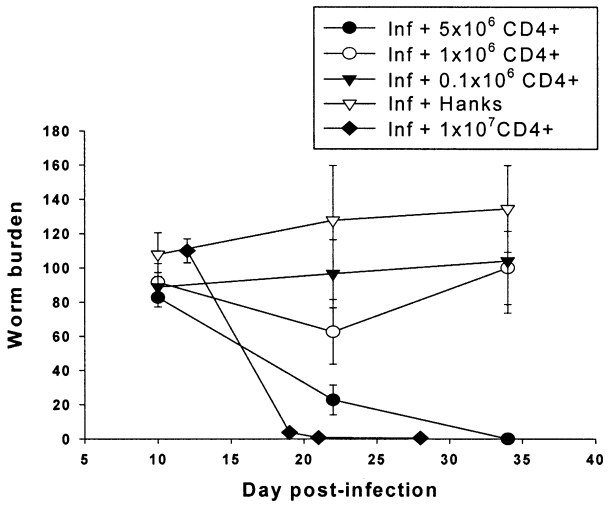

Fig. 1. Worm burdens in SCID mice reconstituted with increasing numbers (from $0{\cdot}1 \times 10^6$ to 1×10^7) of pure $CD4^+$ cells 1 day prior to infection with 150 *Trichuris muris* eggs. A control group receiving Hanks medium alone without cells is also illustrated. Values shown are the mean for 4 or 5 animals ±S.E.M.

Antibody and cytokine ELISAs

Serum was collected from each experimental animal by cardiac bleed at autopsy. Total and parasite-specific immunoglobulin in the serum was detected by capture with goat anti-mouse Ig (Dako) or *T. muris* excretory–secretory (E–S) antigen (5 μg/ml) followed by detection with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Dako) and substrate as previously described (Else & Grencis, 1996). Parasite-specific IgG1 and IgG2a were analysed in sera from Fas/FasL-deficient animals as previously described (Bancroft *et al.* 1998). Detection of IFNγ, IL-4, IL-5, IL-9 and IL-12 in supernatants from 5×10^6 reconstituted SCID MLN cells cultured *in vitro* with 50 μg/ml excretory–secretory (E–S) antigen was carried out as previously described (Bancroft *et al.* 1998).

Statistics

Where relevant, statistical differences between groups of animals were calculated using a Mann–Whitney *U*-test where $P < 0{\cdot}05$ was considered significant. The significance of the data is depicted on the figures by star notation with *** representative of $P < 0{\cdot}005$; *$P < 0{\cdot}05$.

RESULTS

It has been previously established that reconstitution with 1×10^7 immune BALB/c $CD4^+$ T cells can confer resistance to *T. muris* in immunodeficient SCID mice (Else & Grencis, 1990). However, the kinetics and limits of this model have not been addressed. The number of immune cells required to induce immunity was investigated by reconstitution of SCID mice with varying numbers of $CD4^+$ cells from $0{\cdot}1 \times 10^6$ to 1×10^7 or with Hanks balanced salt solution (Hanks) as injection controls. It can be seen from Fig. 1 that immunity was not transferable with less than 5×10^6 immune BALB/c $CD4^+$ cells when mice are reconstituted 1 day prior to infection. Thus, SCID mice reconstituted with 1×10^7 or 5×10^6 immune BALB/c $CD4^+$ cells expelled the parasite, harbouring significantly lower worm burdens on days 19 and 22 post-infection respectively (both $P < 0{\cdot}05$) compared to control infected, unreconstituted SCID mice injected with Hanks alone. The typical kinetics of parasite expulsion obtained following reconstitution with 1×10^7 immune cells can also be seen in Fig. 1, with the majority of parasites lost by day 19. This profile mimics the expulsion kinetics observed in resistant immunocompetent BALB/c animals following infection with *T. muris* (Else & Wakelin, 1988). Furthermore, the immune response mounted in the reconstituted SCID mouse results in the production of a Th2-biased cytokine profile (Table 1) as routinely observed in immunocompetent resistant animals (Else & Grencis, 1991). Thus, on day 21 post-infection, MLN cells from SCID mice given 1×10^7 immune cells produce IL-4, very high levels of IL-5 and IL-9, lower levels of IFN-γ, and negligible IL-12 after *in vitro* restimulation. $CD4^+$ T cells derived from normal BALB/c mice also confer resistance to infection in the absence of antibody, as shown in Fig. 2. Here, 2×10^7 naïve $CD4^+$ T cells conferred immunity to infection, in contrast to the control SCID mice injected with Hanks, which harboured high worm burdens on day 34 post-infection. However, this model is less robust than reconstitution with immune donor cells and requires higher cell numbers. Thus, to address the question of how the $CD4^+$ T cell transfers immunity to the SCID mouse in the absence of antibody, focus centred on the immune $CD4^+$ T cell, primed in an immunocompetent environment.

Further to the number of cells required to generate immunity, the timing of reconstitution was investigated, to determine if the effector mechanism operated against larval (as in immunocompetent resistant strains) or adult stages of the parasite. Whilst 1×10^7 immune donor cells given 1 day prior to infection induced almost total clearance of parasites by day 21 post-infection (Fig. 3, CD4 d-1), the same number of immune cells given at day 34 post-infection (Fig. 3, CD4 d34) failed to expel a resident adult worm population by day 22 post-reconstitution. Hence, the $CD4^+$-mediated effector mechanism generated in SCID mice is anti-larval and not anti-adult. The differences in worm burden between the 2 control groups receiving Hanks alone represents the establishing infection of the 2 separate infections. This difference is not critical since it has

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Table 1. Mesenteric LN cells from SCID mice reconstituted with 1×10^7 immune $CD4^+$ T cells secrete Type 2 cytokines when restimulated *in vitro*

(Mice were autopsied on day 21 post-infection, MLN cells pooled within each group and cultured in the presence of parasite antigen as described in the Materials and Methods section. Cytokine levels in MLN cell supernatants are shown for control mice, receiving Hanks medium alone prior to infection, (Hanks), and mice reconstituted with 1×10^7 $CD4^+$ (immune CD4) prior to infection.)

Treatment	IFNγ U/ml	IL-4 U/ml	IL-5 U/ml	IL-9 U/ml	IL-12 pg/ml
Hanks	2·0	0·3	320	41	1825
Immune CD4	147	2·0	> 500	> 1000	80

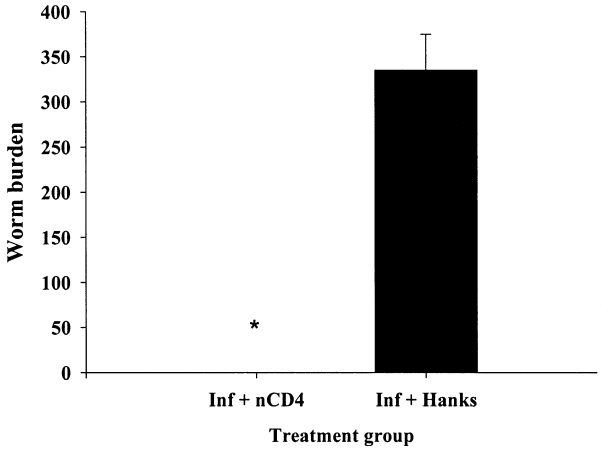

Fig. 2. Worm burdens in SCID mice reconstituted with 2×10^7 pure $CD4^+$ T cells from naïve donors 1 day prior to infection (Inf+nCD4). Control mice received Hanks before infection (Inf+Hanks). Values shown are the mean for 4 or 5 mice ±S.E.M. at day 34 post-infection, with significant differences as denoted in the Materials and Methods section.

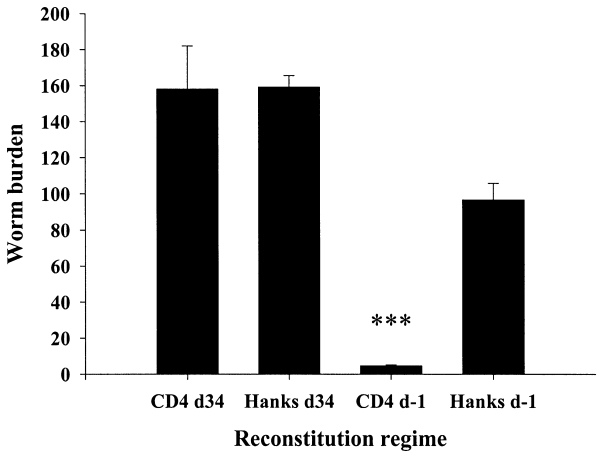

Fig. 3. Worm burdens in SCID mice reconstituted either 1 day prior to-(CD4 d-1) or 34 days post- (CD4 d34) infection. Control groups received Hanks medium alone at the time of reconstitution. The worm burdens were assessed at day 22 post-reconstitution, at day 56- or day 21 post-infection, for the presence of adult or day 21 larvae respectively. Values shown are the mean of 5 mice ±S.E.M. with significant differences between reconstituted and control groups denoted as described in the Materials and Methods section.

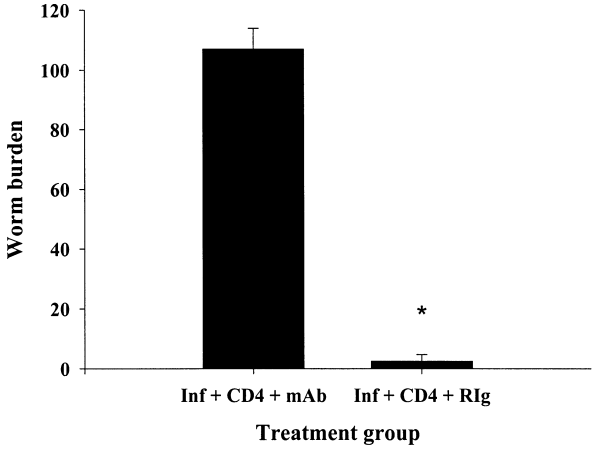

Fig. 4. Worm burdens in SCID mice reconstituted with 1×10^7 immune $CD4^+$ donor cells, 1 day prior to infection, in the presence of an anti-integrin antibody cocktail (Inf+CD4+mAb) or control rat Ig (Inf+CD4+R Ig). Values shown are the mean±S.E.M. of 4–5 mice per group at day 21 post-infection with statistical significance denoted as described in the Materials and Methods section.

been previously confirmed that 1×10^7 immune $CD4^+$ cells can clear an established larval infection of up to 200 worms (data not shown).

The importance of cell trafficking to the MLN in the generation of a protective immune response was addressed by inhibiting the migration of immune donor cells with a cocktail of anti-integrin/addressin monoclonal antibodies previously shown to inhibit $CD4^+$ cell entry into the GALT *in vivo* (Picarella *et al*. 1997). Homing to the intestine has been shown to depend upon the $\alpha 4\beta 7$ integrin and the addressin MAdCAM-1 (Bargatze, Jutila & Butcher, 1995; Brandtzeag, Farstad & Haraldsen, 1999). Thus, anti-$\beta 7$ and anti-MAdCAM-1 monoclonal antibodies were administered to impair homing of primed $CD4^+$ to the GALT (Berlin *et al*. 1993; Streeter *et al*. 1988). In addition an anti-αE monoclonal antibody was incorporated to prevent migration within the epithelium (Kilshaw & Murant, 1990, 1991).

The complete abrogation of resistance in the anti-integrin/addressin antibody-treated group is depicted in Fig. 4. Mice which received immune donor cells in conjunction with control antibody had only a few remaining worms by day 21 post-infection. In contrast, the group which received donor cells in conjunction with anti-integrin/addressin antibodies had in excess of 100 parasites present at this time-point. Furthermore, donor cell entry into the MLN of mice treated with the anti-integrin antibodies was reduced, although not

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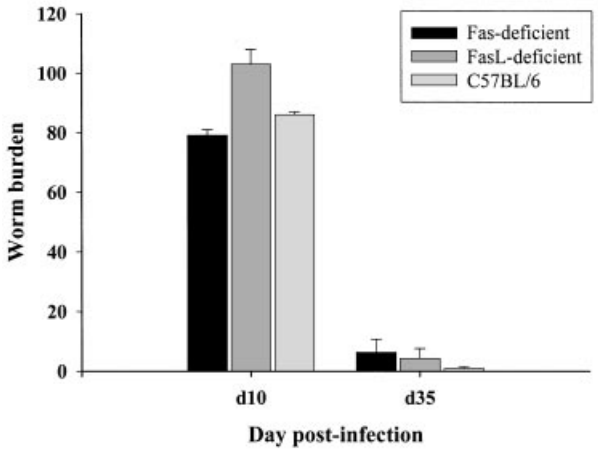

Fig. 5. Worm burdens in Fas-(lpr) and FasL-(gld) deficient mice and the relevant WT control animals at days 10 and 35 post-infection. Values shown are the mean ± S.E.M. of 5 mice per group at each time-point.

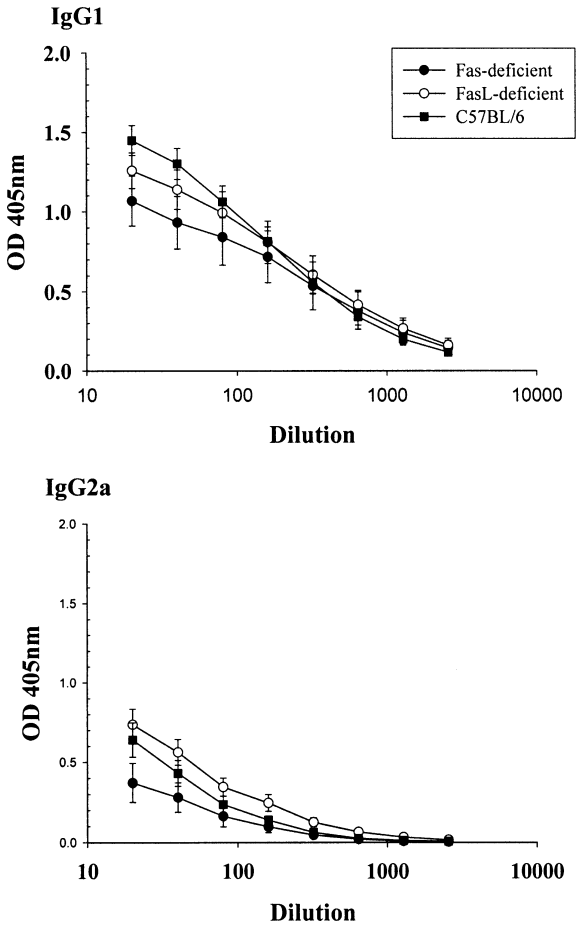

Fig. 6. Parasite-specific IgG1 and IgG2a in sera from Fas- and FasL-deficient mice and the relevant WT control animals at day 35 post-infection. Values shown are the mean ± S.E.M. of 5 mice per group.

ablated (12·2 ± 2·9% [mean ± S.E.M.] $CD4^+$ cells present in the MLN of reconstituted SCID mice infected and treated with anti-integrin/addressin antibodies, compared to 23·4 ± 5·4% $CD4^+$ cells in the rat Ig control group). Thus an impaired entry of $CD4^+$ T cells to the local draining LN correlates with an inability to expel *T. muris*.

To investigate any role for CD4-mediated cytotoxicity in the resistance mechanism, Fas and FasL mice were infected with *T. muris*. Both strains were resistant to infection (Fig. 5) and expelled the parasite burden by day 35 post-infection, with the few remaining worms at day 35 being stunted and immature, a phenotype typical of the resistant C57BL/6 background. Subsequent experiments revealed that the kinetics of worm expulsion in the Fas and FasL-deficient mice were equivalent to WT animals (data not shown). In addition, Fas and FasL deficient mice produced parasite-specific antibody isotype profiles identical to the resistant WT C57BL/6 strain (Fig. 6).

DISCUSSION

The experiments reported above provide further insight into the ability of primed $CD4^+$ T cells to mediate resistance to *Trichuris muris* in the absence of B cells and antibody. Previous studies have demonstrated that highly pure $CD4^+$ T cells from immune mice can mediate expulsion in immunodeficient animals (Else & Grencis, 1996). However the kinetics of worm expulsion in this model system, and the mechanism(s) by which these $CD4^+$ T cells act, have not been explored. We here show that a minimum of 5×10^6 immune $CD4^+$ cells are required to transfer resistance to SCID mice, and that these cells need to be present during the larval stages of infection. Indeed, immune $CD4^+$ cells transferred once the parasites have reached maturity are ineffective at resolving infection. This correlates well with the kinetics of parasite expulsion in resistant strains of mice where the effector response has mediated elimination by days 21–24 post-infection before the adult stage has developed (Else & Wakelin, 1988). Furthermore, the cytokine profile of *in vitro* restimulated MLN cells from reconstituted SCID mice, displays a bias towards the Th2 profile, as routinely observed in resistant strains of mice infected with *T. muris* (Else & Grencis, 1991).

Interestingly, $CD4^+$ T cells do not require priming in the donor mouse in order to mediate worm expulsion upon transfer to the SCID recipient. Thus naïve $CD4^+$ T cells also confer resistance to infection in the absence of antibody, raising questions regarding the priming of CD4 cells and the development of a protective immune response in the absence of B cells. However, the investigations presented here were designed to explore the effector mechanism(s) elicited by Th2-like $CD4^+$ T cells which have been primed in a normal environment, in an attempt to understand how protection is mediated in the absence of the antibody responses so commonly associated with helminth infection.

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To assess whether the protective CD4+ T cells have to act locally at the level of the gut to mediate resistance, groups of mice were administered with a cocktail of anti-β7, anti-MAdCAM-1 and anti-αE antibodies to block CD4+ migration to the GALT *in vivo* (Picarella *et al*. 1997). The treatment of CD4+-reconstituted SCID mice with anti-integrin/addressin antibodies impaired CD4+ T cell entry into the MLN and completely ablated worm expulsion, thus strongly suggesting that the CD4+ T cells do need to migrate to the GALT to transfer immunity. Similar results have been observed in a rat model of intestinal nematode infection, where administration of anti-α4 antibody to block α4β7 *in vivo*, thus inhibiting cell entry to the GALT, has been shown to impair the expression of immunity to *Trichinella spiralis* (Bell & Issekutz, 1993).

Cell-mediated cytotoxicity may represent a novel, locally acting effector mechanism by which the CD4+ T cells confer protective immunity against the larval stages of this intracellular dwelling nematode in the absence of B cells and antibody. CD4+ T lymphocytes are able to kill target cells via a Fas-FasL interaction and epithelial cells are known to express Fas on the cell surface (Moller *et al*. 1994). Although traditionally associated with the CD4+ Th1 phenotype, Th2 cells are also known to express FasL and could operate as an effector mechanism in this model (Suda *et al*. 1995; Watanabe *et al*. 1997). However, the strong protective response mounted by Fas and FasL-deficient mice to *T. muris* infection clearly demonstrates that CD4-mediated cytotoxicity of infected epithelial cells is unlikely to play a role in immunity to this parasite. The deficient strains were thus resistant to infection and behaved identically to the WT controls with respect to timing of expulsion and antibody responses.

The experiments discussed in this report have elucidated essential characteristics of the effector mechanism against *T. muris*. There are now several pieces of the 'resistance' jigsaw in place. These data suggest that the target of this CD4-mediated immunity is the larval stage and that these cells act locally, since treatment with a cocktail of monoclonal antibodies against the integrins important in migration to the GALT prevents worm expulsion. Further, the local action of the effector mechanism is unlikely to involve CD4-mediated cytotoxicity.

The exact effector mechanism by which CD4+ cells can mediate resistance remains unclear. Cytokines may play a direct role at the local level, and it has already been shown that IL-4 and IL-13 are critical for immunity (Bancroft *et al*. 1998). Indeed, IL-4 given as a complex with anti-IL-4 antibody (Finkelman *et al*. 1993) can act locally on the gut epithelium to make the gut an inhospitable environment for some nematodes (Urban *et al*. 1995). However, previous experiments have shown that this IL-4 treatment is not sufficient for the expulsion of *T. muris* larvae from SCID mice (Else, Finkelman and Betts, unpublished data). Other Th2 cytokines, such as IL-9 and IL-13, remain good candidates for the CD4-mediated effector mechanism, and further analyses of these and other cytokines in the SCID mouse model may identify the critical elements which culminate in expulsion of *T. muris*.

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