An αβ T-cell-independent immunoprotective response towards gut coccidia is supported by γδ cells

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SUMMARY

Although γδ cells are commonly hypothesized to provide a 'first line of defence', γδ-cell-deficient mice are generally only marginally more susceptible to pathogens. Because γδ cells are enriched within epithelia, it is important to resolve whether immunoprotective capacity towards epithelial-tropic pathogens is absent from the γδ-cell compartment, or whether such activity is present but simply redundant with that of αβ T cells. In this work, following infection of the intestinal epithelium of αβ T-cell-deficient mice with the coccidian parasite, *Eimeria vermiformis*, γδ cells were shown to support the rapid activation of other lymphoid cells and to confer a transferable antipathogen effect that could be eradicated by neutralization of interferon-γ. However, unlike αβ T cells, these effects of γδ cells showed no evidence of functional immunological memory. These results are directly relevant to coccidiosis, an economically significant disease of livestock, and should have general relevance to infections involving αβ T-cell deficiencies, e.g. cryptosporidiosis in patients with acquired immune deficiency syndrome (AIDS).

INTRODUCTION

The subdivision of the T-lymphocyte compartment into subsets bearing either an αβ or a γδ T-cell receptor (TCR) appears to have been conserved for at least 450 million years.[1] Although many studies have shown that both cell types are capable of producing various cytokines and mediating cytolysis,[2–5] the question of how each cell type contributes to particular immune responses remains unresolved.[1,6,7] This is particularly true for natural infections of epithelial cells, for while TCR-γδ^{+} cells are often rare in the systemic circulation (1–2% of peripheral T cells) they are commonly highly represented among intraepithelial lymphocytes (IELs).[8–11] In that context, they have been proposed to mount a 'first line of defence'[12] in response to recognition of stress-induced major histocompatibility complex (MHC) 'sentinel antigens' expressed by infected epithelial cells.[12,13]

As an example of a natural epithelial-tropic infection, we have studied coccidiosis caused by *Eimeria vermiformis* (phylum: Apicomplexa), an intracellular protozoan pathogen, largely (if not completely) restricted to the intestinal epithelium where γδ cells are enriched. Different species of *Eimerian* parasitize a broad range of vertebrate hosts in which the immune response is measured primarily by each of two criteria: attenuation of primary infection; and the development of

immunity to secondary challenge. Following primary infection, the immune system reduces the number of oocysts released into the faeces and the time-period over which oocyst release occurs (patent period). Nevertheless, *E. vermiformis* is intrinsically self-limiting, and an immunodeficient mouse will become cleared of the pathogen within ≈ 3 weeks. This aspect allows clean segregation of the responses to primary inocula and to subsequent secondary inocula, even in immunocompromised hosts. In immunocompetent mice, immunity to secondary infection is extremely efficient, and essentially no infectious oocysts are released into the faeces, even after administration of inocula 1000 times greater than given in the primary challenge (ref. 14; A. L. Smith & A. C. Hayday, unpublished).

Thymus-dependent responses are largely responsible for the host defences that follow either primary or secondary challenge.[15,16] In attempts to clarify such responses, it was recently shown that both primary and secondary infections are exacerbated in αβ T-cell-deficient mice,[17] just as such mice are defective in specific immunity towards most pathogens so far utilized.[18–22] By contrast, γδ-cell-deficient mice were not obviously defective in either primary or secondary responses.[17]

Despite these results, three observations indicate that γδ cells are somehow involved in the response to *Eimeria*. First, there are increases in γδ[+] IELs during infection either of mice[23] or chickens.[24] Second, γδ cells promote germinal centre formation in αβ T-cell-deficient mice repeatedly infected with *E. vermiformis*.[25] Third, *Eimeria*-infected γδ-cell-deficient mice show an enhanced αβ T-cell-dependent immunopathology,[17] reflecting an immunoregulatory role for γδ cells that has by

Table 1. Primary and secondary infection of T-cell receptor (TCR)-β$^{-/-}$ and TCR-(β × δ)$^{-/-}$ mice with *Eimeria vermiformis*

Exp. no.	Group	Strain of mouse: (β$^{-/-}$ lacks αβ T cells; β × δ$^{-/-}$ lacks αβ and γδ T cells)	Primary infection*			Secondary infection*		
			No. of mice (deaths)	Oocyst output (× 10⁶)†	Patent period (in days)†	No. of mice (deaths)	Oocyst output (× 10⁶)†	Patent period (in days)†
1	A	TCR-β$^{-/-}$	6 (0)	*58·6 ± 6·3*	*14·0 ± 0·6*	5 (0)	*41·2 ± 5·9*	*13·3 ± 0·3*
	B	TCR-(β × δ)$^{-/-}$	6 (0)	**86·3 ± 10·8**	*13·4 ± 0·2*	5 (1)	**74·5 ± 10·7**	*13·8 ± 0·3*
	C	TCR-(β × δ)$^{+/-}$	6 (0)	9·5 ± 2·3	6·5 ± 0·2	6 (0)	0·0 ± 0·0	0·0 ± 0·0
		Primary infection controls‡						
	D	TCR-β$^{-/-}$				6 (0)	*38·9 ± 3·7*	*13·0 ± 0·4*
	E	TCR-(β × δ)$^{-/-}$				7 (2)	**81·8 ± 9·4**	*13·8 ± 0·4*
	F	C57.BL/6				5 (0)	9·2 ± 2·7	7·4 ± 0·2
2	A	TCR-β$^{-/-}$	4 (0)	*44·7 ± 10·7*	*14·4 ± 0·4*	Not done		
	B	TCR-(β × δ)$^{-/-}$	4 (0)	**78·0 ± 8·1**	*13·8 ± 1·5*	Not done		
	C	C57.BL/6	4 (0)	8·8 ± 0·6	7·3 ± 1·5	Not done		
3	A	TCR-(β × δ)$^{-/-}$	7 (1)	*134·0 ± 13·1*	*16·5 ± 0·6*	6 (0)	**117·7 ± 19·2**	*14·2 ± 0·4*
	B	C57.BL/6	5 (0)	9·2 ± 1·1	8·0 ± 0·3	Not done		
	C	TCR-(β × δ)$^{-/-}$	Primary infection control			5 (1)	**63·0 ± 24·4**	*13·8 ± 0·3*

*Secondary infections with 10³ sporulated oocysts were initiated 4–7 weeks postprimary infection. Data is representative of two further experiments that are not presented.

†Data from the oocyst output and the patent period are expressed as mean ± SE. Within experiments, different font types indicate significant differences ($P < 0.05$): italic denotes significantly greater susceptibility than C57.BL/6 mice; bold italic denotes significantly greater susceptibility than αβ T-cell-deficient mice.

‡Primary infection controls were naive mice infected in parallel with the secondary infections shown in the right-hand columns.

now been implicated in numerous immune responses to infectious agents or to self.[17,26–30]

However, as is the case for αβ T cells, immunoregulation may be neither the only nor the major role of γδ cells. Indeed, treatment of mice with anti-TCR-γδ antibodies exacerbates the early phases of certain bacterial, viral and protozoal infections,[29,31–34] with similar, more extensive results being obtained from TCR-δ$^{-/-}$ mice.[18,19,35–37] For these reasons, the potential immunoprotective effects of γδ cells towards an epithelial cell pathogen, delivered via the natural route, warranted further clarification. In particular, we wanted to know whether immunoprotective capacity was absent from the γδ-cell pool, or whether any such activity that existed was simply redundant with the αβ T-cell compartment.

MATERIALS AND METHODS

Animals

TCR-β$^{-/-}$ and TCR-(β × δ) mice were crossed onto a C57.BL/6 background (>10 generations) and bred and maintained in specific pathogen-free (SPF) isolators, in an accredited facility. C57.BL/6 and some TCR-β$^{-/-}$ mice were originally purchased from Jackson Laboratory (Bar Harbor, ME). Mice were given an invariant diet (Hamster chow 3500, Agway, Waverly, NY) and water *ad libitum*. Animals were 6–10 weeks of age at primary infection and secondary infections were initiated 4–6 weeks thereafter.

Parasites and oocyst enumeration

E. vermiformis (a kind gift of Prof. K. S. Todd, University of Illinois, Urbana–Champaign, IL) were maintained by passage *in vivo*, with oocysts purified and sporulated as described previously.[38] Sporulation was scored microscopically and mice were given 10² or 10³ sporulated oocysts in 100 μl of water by oral gavage. At the beginning of patency (7 days postinfection

[p.i.]), mice were individually caged and maintained over autoclaved sand from which the faeces were collected at 24-hr periods, until no oocysts could be detected. Oocysts were released from disaggregated faeces by salt flotation and counted in girded McMaster chambers.

Preparation of lymphocytes

Spleens and mesenteric lymph nodes were removed from freshly killed mice using sterile technique, and lymphocyte suspensions were prepared by gently disrupting diced organs through a steel mesh into sterile phosphate-buffered saline (PBS), pH 7·2, containing 2% fetal calf serum (FCS), and maintained on ice. Erythrocytes were removed from spleen preparations by 'flash lysis' according to a standard technique.[39] Small intestinal intraepithelial lymphocytes (IEL) were prepared as described previously.[23] Lymphocytes were washed, resuspended and counted microscopically. Viability always exceeded 90%, according to Trypan Blue exclusion.

Adoptive transfer

For adoptive transfer, cells were resuspended in PBS (pH 7·2) containing 2% FCS, and injected intraperitoneally into recipient mice. Controls received PBS (pH 7·2), containing 2% FCS, with no cells. Recipient mice were challenged with *E. vermiformis* 24–48 hr after adoptive transfer, as described by Rose *et al.*[38,40]

Flow cytometry

Cells were washed and resuspended at 5×10^6/ml in staining buffer at 4° (PBS pH 7·2, 2% FCS, 0·01% sodium azide). Antibodies used for fluorescence-activated cell sorter (FACS) analysis were anti-CD3, anti-γδ, anti-α$_{IEL}$, anti-CD8α and anti-CD8β (all purchased from Pharmingen Inc., San Diego, CA). Briefly, 5×10^5 cells in 100 μl of staining buffer were stained on ice for 30 min, washed and fixed in 1% paraformaldehyde

before analysis on a FACScan (Becton-Dickinson, Palo Alto, CA) using CELLQUEST software.

Depletion of interferon-γ (IFN-γ) in vivo
Fifty micrograms of rat anti-IFN-γ (XMG1.2; Endogen, Woburn, MA) was injected intravenously in 100 μl of sterile PBS (pH 7·2) via the orbital plexus 3 hr prior to initiation of infection. Control animals received 50 μg of rat immunoglobulin G (IgG). This concentration of XMG 1.2 has previously been used for *in vivo* depletion of IFN-γ.[41]

RESULTS

Infection of T-cell-deficient strains

To determine if γδ cells conferred a reproducible, protective effect against *Eimeria*, which was independent of that provided by αβ T cells, infections of C57.BL/6.TCR-β^{−/−} mice (that contain γδ cells) were compared with those of C57.BL/6.TCR-(β × δ)[−/−] mice (that contain no T cells). C57.BL/6 mice were included as controls. Immunoprotection was assessed by measuring the scale and duration of oocyst output following primary and secondary infections (Table 1). To accommodate inevitable variations in the reproductive efficiency of parasites harvested from passage *in vivo*, and to enable the appropriate comparisons to be made, all secondary infections were accompanied with parallel primary infections of mice of the same strain (Table 1; right-hand columns Exp. 1, groups D, E and F; Exp. 3, group C). All individual experiments were internally controlled, and direct comparisons were never made between one experiment and another.

As anticipated from previous analyses of αβ T-cell-deficient mice,[17] oocyst production during primary infection of T-cell-deficient TCR-β^{−/−} or TCR-(β × δ)[−/−] mice greatly exceeded that of immunologically intact mice [either (β × δ)[+/−] heterozygotes or C57.BL/6]. Likewise, while immunologically intact mice exhibited complete immunity to reinfection (Table 1; Exp. 1 group C), all αβ T-cell-deficient mice remained fully susceptible to reinfection (Table 1; Exp. 1, groups A and B; Exp. 3, group A). However, TCR-γδ-intact, TCR-β^{−/−} mice were significantly less susceptible to infection than completely T-cell-deficient TCR-(β × δ)[−/−] mice (Table 1, Exp. 1, groups A and B, D and E; Exp. 2, groups A and B; *P* < 0·05). Increased susceptibility of mice with no T cells was further illustrated by deaths (≈ 10% of mice) that occurred uniquely in TCR-(β × δ)[−/−] mice, between 8 and 9 days p.i., corresponding to peak parasite numbers in the intestine (Table 1). Indeed, TCR-(β × δ)[−/−] mice showed the highest susceptibility to infection of any of numerous mutant mouse strains tested (A. L. Smith & A. C. Hayday, unpublished). Compared with controls, the duration of oocyst output (patent period) was greater in TCR-β^{−/−} and TCR-(β × δ)[−/−] mice, but was not reproducibly different between the two mutant strains.

Although these data indicate that γδ cells can contribute some anti-eimerian function, the magnitude of oocyst yield from infected TCR-β^{−/−} mice did not differ significantly between primary and secondary infections, suggesting that γδ cells in mice receiving a primary infection mediated a protective effect as efficiently as γδ cells from pre-exposed mice. This issue is explored further below. Likewise, the highly susceptible

TCR-(β × δ)[−/−] mice were equally susceptible to primary and secondary infection.

### Cellularity of TCR-β^{−/−} mesenteric lymph nodes (MLN) during infection

Mesenteric lymph node (MLN) cells of intact animals show marked expansion during infection with *E. vermiformis*.[23,42] To determine whether this activation of lymphoid cells can be provoked by responding γδ cells, the numbers and phenotype of MLN cells were compared during infections of TCR-β^{−/−} and TCR-(β × δ)[−/−] mice. There was an average of 3·52 ± 0·44 × 10⁶ (mean ± SE) MLN cells in uninfected TCR-β^{−/−} mice. This number increased progressively more than 10-fold to 42·53 ± 6·41 × 10⁶ at 12 days p.i., before returning to resting levels by 21 days p.i. (Fig. 1). During the course of infection, $CD3^+$ $TCR\text{-}\gamma\delta^+$ lymphocytes composed 13·7 ± 1·1% of MLN cells, and remained largely negative for both CD4 and CD8. The remaining lymphocytes were predominantly $CD3^-$ $B220^+$ B cells (data not shown). In contrast, there was no reproducible increase in the cellularity of the MLNs in TCR-(β × δ)[−/−] mice (uninfected mice 3·48 ± 0·67 × 10⁶; mice 12 days p.i. 3·03 ± 0·27 × 10⁶). Therefore, the MLN cell expansion in infected TCR-β^{−/−} mice, which is mostly B cells, is dependent on direct and/or indirect effects of γδ cells.

Adoptive transfer of MLN cells from naive and infected TCR-β^{−/−} mice

To confirm that the difference in susceptibilities of TCR-β^{−/−} and TCR-(β × δ)[−/−] mice were attributable to the differences in their lymphoid populations, 5 × 10⁷ MLN cells from TCR-β^{−/−} mice were adoptively transferred to T-cell-deficient TCR-(β × δ)[−/−] recipients. Control mice received PBS, pH 7·2. This regimen, applied to conventional strains, has previously been used to demonstrate transferable immunological protection afforded by $CD4^+$ T cells.[38,40] In those studies, MLN cells were more effective than spleen cells.[40]
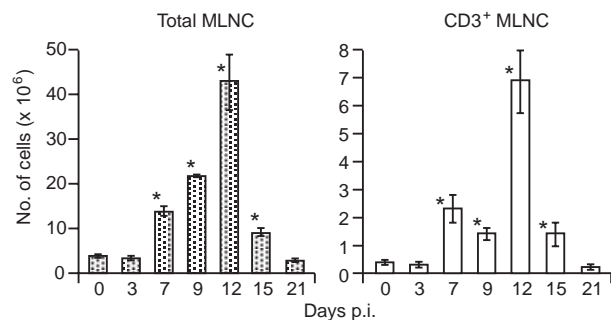
Figure 1. Cellularity of mesenteric lymph nodes (MLNs) from T-cell receptor (TCR)-β[−/−] mice infected with *Eimeria vermiformis*. MLN cells were harvested, at the time-points indicated on the figure, from TCR-β^{−/−} mice infected with 1000 sporulated oocysts. $CD3^+$ cells were enumerated using flow cytometry with fluorescein isothiocyanate (FITC)-conjugated anti-CD3. Results are expressed as mean ± SE (*n* = 3 at each time-point). *Significant difference versus uninfected mice analysed in parallel (*P* < 0·05). p.i., postinfection.
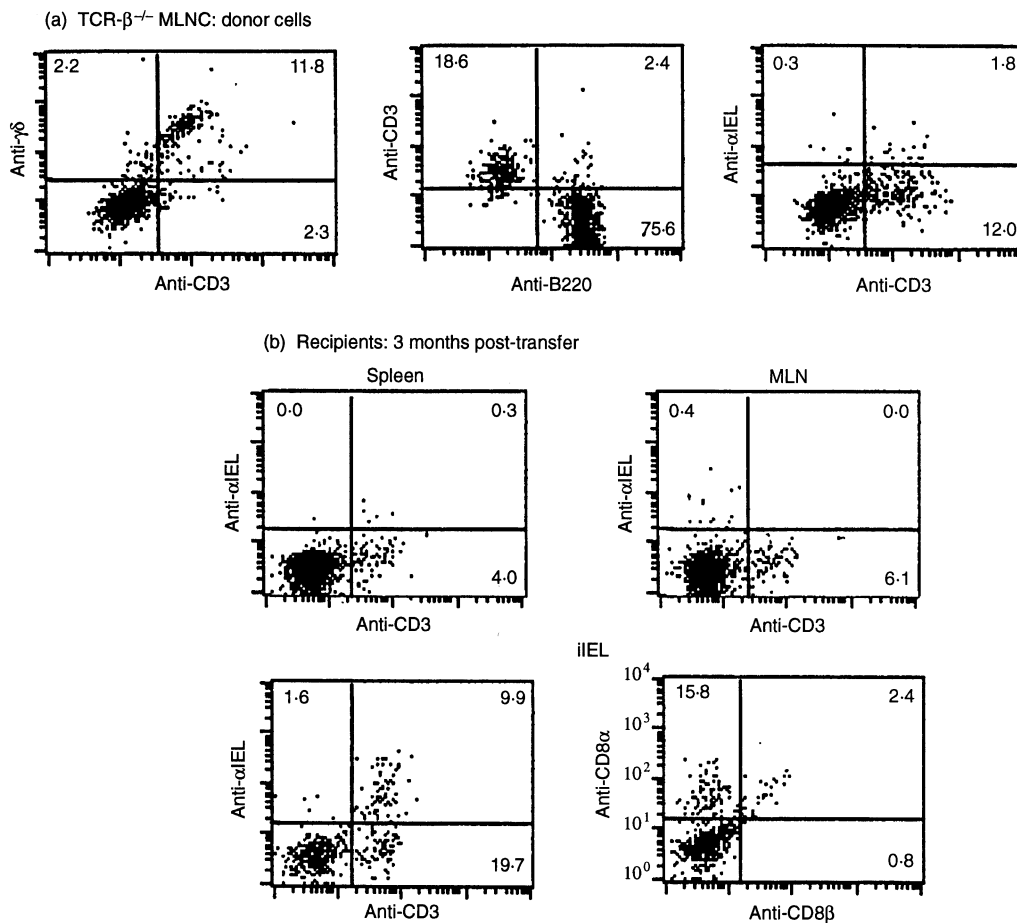
Figure 2. Reconstitution of T-cell receptor (TCR)-$(\beta \times \delta)^{-/-}$ mice with mesenteric lymph node (MLN) cells from TCR-$\beta^{-/-}$ mice: phenotypic analysis. (a) TCR-$\beta^{-/-}$ MLN cells; donor cells. (b) Recipients, 3 months post-transfer. Representative flow cytometry plots from at least four individual mice are presented. Antibodies used were phycoerythrin (PE) -conjugated anti-$\gamma\delta$, -anti-CD3, -anti-B220, -anti-α_{IEL}, and -anti-CD8-α, and fluorescein isothiocyanate-conjugated anti-CD3 and -anti-CD8β. iIEL, intestinal intraepithelial lymphocytes.
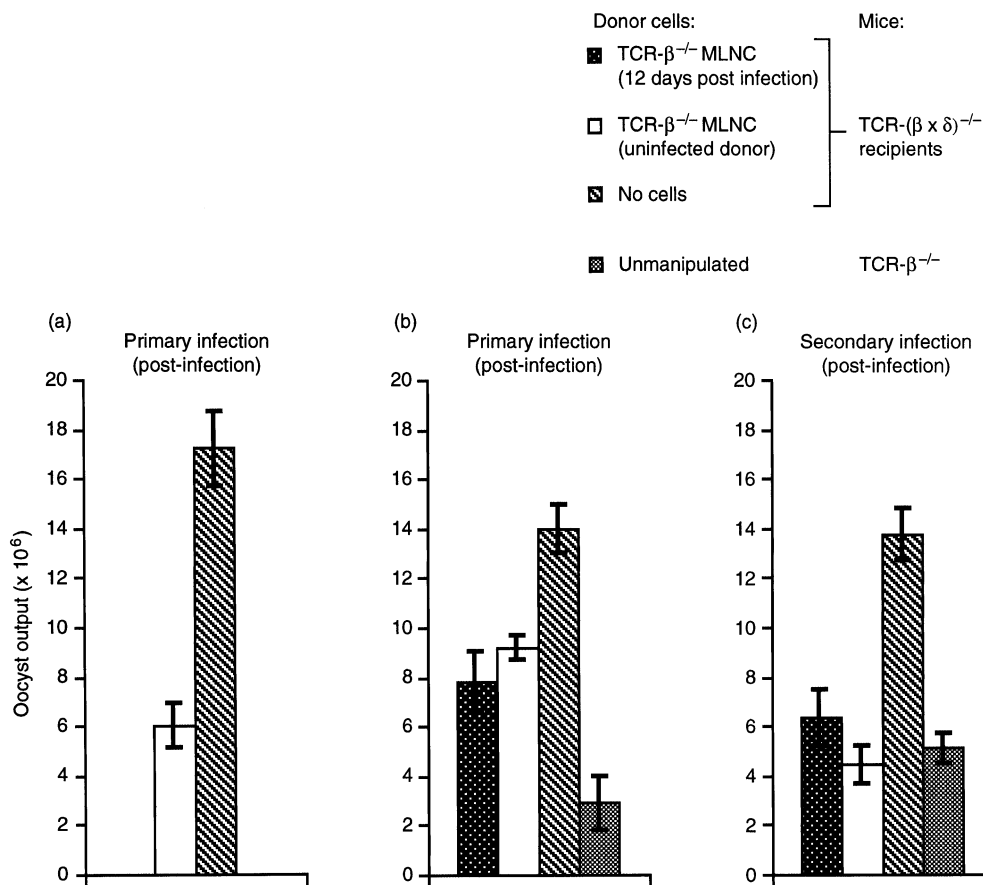
Successful transfer and repopulation of recipients with $\gamma\delta$ cells was determined by flow cytometry. Because unmanipulated TCR-$(\beta \times \delta)^{-/-}$ mice harbour no CD3$^+$ cells, the requirement for a congenic marker to determine T-cell transfer is obviated. Substantial numbers of CD3$^+$ TCR-$\gamma\delta^+$ cells were detected in TCR-$(\beta \times \delta)^{-/-}$ recipients within 1 day of transfer, and T cells became established long term among splenic, MLN and intestinal IEL populations; representative plots are shown in Fig. 2. Interestingly, while $\gamma\delta$ cells in the spleen and MLN remained CD4$^-$ CD8$^-$, a significant proportion of $\gamma\delta^+$ IEL acquired the CD8$\alpha\alpha$ homodimer (Fig. 2b), which is commonly expressed by $\gamma\delta$ IELs of normal[43,44] and TCR-$\beta^{-/-}$ mice (our unpublished data; ref. 45). Furthermore, > 30% of TCR-$\gamma\delta^+$ IEL (but neither splenic nor MLN cells) displayed the integrin $\alpha_{IEL}\beta_7$, which is ordinarily expressed by $\approx 90\%$ of IEL on which it promotes association with E-cadherin on the gut epithelium.[46–49] Nevertheless, the fact that not all gut-associated $\gamma\delta$ cells expressed $\alpha_{IEL}\beta_7$ is apparently consistent with the finding that $\gamma\delta$ cells are less dependent on $\alpha_{IEL}\beta_7$ than are CD8$^+$ $\alpha\beta$ T cells.[49] In summary, $\gamma\delta^+$ MLN cell inocula reconstituted diverse gut-associated lymphoid compartments of T-cell-deficient mice.

To test the immunoprotective capacity of transferred MLN cells, all mice were infected with 100 sporulated oocysts. A reduction from 1000 oocysts was made to reduce the lethality in infected TCR-$(\beta \times \delta)^{-/-}$ mice (see Table 1). The data in Fig. 3(a) show clearly that, compared with PBS-treated controls, TCR-$\beta^{-/-}$ MLN cells could reduce oocyst output in recipient TCR-$(\beta \times \delta)^{-/-}$ mice by 30–60% ($P < 0.05$), similar to the differences between unmanipulated TCR-$\beta^{-/-}$ mice and TCR-$(\beta \times \delta)^{-/-}$ mice, confirming that those differences are attributable to differences in the lymphoid compartments of the two strains.

Adoptive transfer analyses of immunological memory

MLN cells displayed measurable increases in number during the 2 weeks of the primary immune response to *E. vermiformis* infection (Fig. 1). To test whether this reflected enhanced immune responsiveness to the pathogen, further transfer experiments were set up in which the immunoprotective efficacies of TCR-$\beta^{-/-}$ MLN cells from naive mice and from mice 12 days p.i., respectively, were compared. Both MLN cell preparations conferred significant and comparable degrees of protection (35·8–44·3%) to recipient TCR-$(\beta \times \delta)^{-/-}$ animals (Fig. 3b).
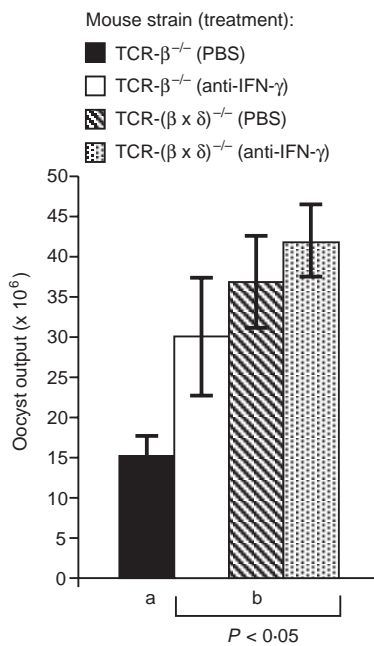
Donor cells: Mice:

▨ TCR-β$^{-/-}$ MLNC
(12 days post infection)

☐ TCR-β$^{-/-}$ MLNC   TCR-(β × δ)$^{-/-}$
(uninfected donor)   recipients

▨ No cells

▨ Unmanipulated   TCR-β$^{-/-}$

Figure 3. Adoptive transfer of T-cell receptor (TCR)-γδ T cells. Comparison of the efficacy of naive and responding (12 days postinfection) TCR-β$^{-/-}$ mesenteric lymph nodes (MLNs) as donor cells. (a) TCR-(β × δ)$^{-/-}$ mice received cells from naive TCR-β$^{-/-}$ mice (open bar); (b) and (c) TCR-(β × δ)$^{-/-}$ mice received cells from naive TCR-β$^{-/-}$ mice (open bars) or from infected mice (stippled bars), and were challenged once (panel b), rested, and then rechallenged (panel c). Results are expressed as mean ± SE from groups of four to 10 individually housed animals. Oocyst counts from adoptive-transfer recipients were comparable and statistically significantly different from counts from TCR-(β × δ)$^{-/-}$ mice that received phosphate-buffered saline (PBS) but no cells ($P<0.05$). Fifty million TCR-β$^{-/-}$ MLN cells were adoptively transferred into TCR-(β × δ)$^{-/-}$ mice (both strains on a C57.BL/6 background). Primary infection was initiated with 100 sporulated oocysts 24–48 hr post-transfer, and secondary infection was initiated (with 100 oocysts) 4 weeks later.

Thus, the active immunoprotective elements of the inoculum were not obviously enhanced by prior exposure to the pathogen.

To test this further, the adoptive-transfer recipients were rested and rechallenged. The resistance of recipients receiving either inoculum was again comparable. Although it was slightly better than it had been to primary challenge (55·8–67·9%) (Fig. 3c), it was noticeably different to the 99% protection of TCR-(β × δ)$^{-/-}$ recipients afforded by adoptive transfer of TCR-αβ^{+} 12-day p.i. MLN cell inocula (data not shown; ref. 17). Indeed, the resistance to infection in the rechallenged recipients of TCR-β$^{-/-}$ MLN cells was comparable with that of TCR-β$^{-/-}$ mice of the same cohort from which the transfer inocula were derived (Fig. 3c). The comparability of MLN-cell inocula from naïve or previously infected mice is consistent with the observation that γδ cell-bearing TCR-β$^{-/-}$ mice show no enhanced resistance following repeated infection with higher doses of parasite (Table 1).

An IFN-γ-dependent mechanism reduces susceptibility of TCR-β$^{-/-}$ mice to that of TCR-(β × δ)$^{-/-}$ mice

IFN-γ plays an important role in the T-cell-intact animal in limiting infection with *E. vermiformis* (refs 42,50; A. L. Smith & A. Hayday, submitted). Moreover, γδ^{+} cells have been reported to produce IFN-γ under various circumstances.[1,2,4,5,51] Therefore, an antibody-depletion strategy was applied to test whether the relative resistance of TCR-β$^{-/-}$ mice, as compared with TCR-(β × δ)$^{-/-}$ mice, involved IFN-γ. TCR-β$^{-/-}$ mice were infected with 250 oocysts of a particularly effective inoculum. Comparison of oocyst output from anti-IFN-γ-treated and rat IgG-treated C57.BL/6 mice revealed a significant ($P<0.05$) increase in oocyst production, similar to that reported previously,[50] which confirmed the efficacy of the antibody treatment (data not shown). Strikingly, anti-IFN-γ-treatment increased the susceptibility of TCR-β$^{-/-}$ mice to the level seen in TCR-(β × δ)$^{-/-}$ mice, but did not significantly increase the susceptibility of TCR-(β × δ)$^{-/-}$ mice (Fig. 4). Therefore, IFN-γ is an important component of the immunoprotective mechanism present in TCR-β$^{-/-}$ mice.

Mouse strain (treatment):

■ TCR-β⁻/⁻ (PBS)
□ TCR-β⁻/⁻ (anti-IFN-γ)
▨ TCR-(β x δ)⁻/⁻ (PBS)
▦ TCR-(β x δ)⁻/⁻ (anti-IFN-γ)

Figure 4. Exacerbation of infection in T-cell receptor (TCR)-β⁻/⁻ mice by anti-interferon-γ (IFN-γ) treatment. Results are expressed as mean ± SE from groups of five or six individually housed animals; the bar annotated 'a' was significantly different ($P < 0.05$) from the bars annotated with 'b'. Mice were injected intravenously with 50 μg of rat anti-IFN-γ or rat immunoglobulin G (IgG) as indicated, and infected with sporulated oocysts 3 hr later.

DISCUSSION

The present study used complementary genetic and cellular approaches to test the capacity of γδ cells to support an immunoprotective function against *E. vermiformis*, a natural pathogen of the murine small intestinal epithelium and an example of the coccidian parasites that plague birds, reptiles and mammals. The results of both approaches are consistent: TCR-β⁻/⁻ mice that lack αβ T cells but contain γδ cells are more resistant to infection than are TCR-β × δ⁻/⁻ mice that lack γδ cells; and lymph node cells from γδ-intact mice transfer protection to γδ-deficient mice. The importance of γδ cells is further highlighted by the striking, infection-driven stimulation (direct or indirect) of other lymphoid cells, particularly B cells, that is seen in infected TCR-β⁻/⁻ mice but not in γδ-deficient TCR-(β × δ)⁻/⁻ mice.

At the same time, no significant, functional immunological memory is evident in the γδ-driven activities: TCR-β⁻/⁻ mice do not exhibit an enhanced response during secondary infection and adoptive transfers of γδ cells are essentially equally efficacious whether drawn from naive or previously challenged mice. Consistent with this, the patent period in γδ-intact TCR-β⁻/⁻ mice was the same as in T-cell-deficient, TCR-(β × δ)⁻/⁻ mice, indicating that although the γδ-cell-driven activities in TCR-β⁻/⁻ mice consistently reduce parasite growth, they are unable to resolve infection, a function that we have previously attributed to αβ T cells.[17] In these several respects, the actions of γδ cells seem to comply with the characteristics of the innate response, rather than the adaptive αβ-T-cell response.

The experiments presented do not define whether the immunoprotective effects of γδ cells are direct effects, or indirect effects driven via the activation of other effector leucocytes. Indeed, despite a plethora of studies, the same is true for the more potent immunoprotective effects of CD4⁺ αβ⁺ T helper 1 (Th1) cells toward *Eimeria*. In that case, IFN-γ production has been strongly implicated (refs 40,50; A. L. Smith & A. C. Hayday, submitted), as it is in the immunological response of TCR-β⁻/⁻ mice described here. IFN-γ is known to be produced by at least some subsets and clones of γδ cells.[1,2,4,5,51] Furthermore, IFN-γ depletion failed to significantly increase the susceptibility of γδ-deficient TCR-(β × δ)⁻/⁻ mice, a result that is consistent with the previous finding that natural killer (NK) cells and other non-T lymphocytes are not major effectors of IFN-γ-mediated resistance to *E. vermiformis*.[52] Likewise, B cells have previously been shown to make only minor contributions to the anti-*Eimeria* response in either chickens or mice (refs 14,16,38; A. L. Smith & A. C. Hayday, submitted), and so it is unlikely that the promotion of IgG production is a primary role for IFN-γ. Taken together, these data encourage the view that γδ cells might act directly against infected enterocytes, consistent with previous reports that IFN-γ production by IELs can directly induce villus epithelial damage.[53] While it is also possible that γδ cells regulate epithelial recovery via fibroblast growth factor VII (FGFVII) production,[3] the dramatic results of IFN-γ depletion indicate that any effects of FGFVII must be insufficient on their own to account for the immunoprotective contributions of γδ cells.

The adoptive transfer approaches that are shown here to complement the genetic analysis of γδ-cell function, may in future be combined with the use of mice that have genetic disruptions in specific γδ-cell subsets.[54] Such studies will reveal whether the same cells are responsible for the three bioactivities of γδ cells now evident in the *Eimeria* system: namely, immunoprotective effects (Table 1; Figs 3, 4); lymphoid stimulation (Fig. 1; ref. 25); and the suppression of immuno-pathology.[17] Possibly, γδ⁺ IELs are responsible for some phenotypes and systemic γδ T cells for others. This issue is unresolved by the studies presented here because we show that MLN inocula can partially reconstitute the IEL compartment. The definition of relevant subsets of γδ cells will also cast light on the nature of γδ-cell stimulation in this system. It has recently been shown that murine and human γδ cells respond to MHC class I-related antigens expressed by activated cells, including human enterocytes,[1,13] but there has as yet been no implication of these molecules in the response to infection.

The capacity of γδ cells to support some level of protection against the parasite, and at the same time to induce lymphocyte responses, apparently without the development of memory, could reasonably describe the functions of macrophages, NK cells, or other members of the innate immune compartment. This comparison is further provoked by the lack of diversity of the γδ-TCR, characteristic of epithelium-associated γδ cell repertoires,[1,7] and the capacity of the γδ-TCR to engage autologous 'stress-antigens' seemingly unmodified by foreign antigen.[1] Other links between γδ and NK cells are evident in the report that stimulation of bronchoalveolar lavage cells with anti-CD3 and recombinant interleukin-2 (rIL-2) induces γδ T cells to express NK1.1 and to lyse YAC1 cells, the prototypic NK-cell target.[55] Furthermore, independent studies have attributed the mild immunoprotective effects of γδ cells

towards *Listeria* to a collaboration between γδ cells and NK cells.[56,57]

On the other hand, the classification of γδ cells within the adaptive response seems clear. For example, the TCR-δ-chain complementarity-determining region-3 (CDR3) shows greater diversity than observed for any αβ T cells.[1,25,58] Given this, and given the generally accepted view that the activation of lymphocytes with specific receptors is followed by the transition of those lymphocytes into a memory state, it may be that γδ cells engage peripheral autologous 'activation/stress' antigens early in their development, and become activated to a 'constitutive memory' state.[1,13]

It might be argued that the capacity of γδ cells to support lymphoid activation (Fig. 1) and immunoprotection (Figs 3, 4) is largely irrelevant because it is only revealed when mice are deficient in αβ T cells. (αβ T-cell-intact, γδ-cell-deficient mice are no more susceptible than wild-type animals to either primary or secondary infection.[17]) Nonetheless, there are important instances of defective αβ T-cell function, particularly the CD4$^+$ compartment in patients with acquired immune deficiency syndrome (AIDS). Likewise, there may be particular times or anatomical sites in normal, healthy animals when αβ T-cell function is compromised and hence γδ cell-driven immunoprotection would be particularly significant. This will be investigated.

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