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T-Cell Receptor γδ Lymphocytes and *Eimeria vermiformis* Infection

M. ELAINE ROSE,* PATRICIA HESKETH, LISA ROTHWELL, AND ROBERT A. GRAMZINSKI†

Department of Immunopathology, Institute for Animal Health, Compton, Berkshire, RG20 7NN, United Kingdom

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The role of T-cell receptor γδ T lymphocytes in coccidiosis was examined by determining the course of infection with *Eimeria vermiformis* in BALB/c mice depleted of γδ lymphocytes by treatment with GL3 monoclonal antibody. The replication of the parasite in primary infections was not greatly, or consistently, affected by this treatment, and there was no correlation between the extent of depletion of small intestinal intraepithelial lymphocytes and the number of oocysts produced. The resistance of immunized mice to challenge was not compromised by depletion of intraintestinal epithelial lymphocytes when their depletion was effected at the time of primary infection and/or administration of the challenge inoculum. Thus, T-cell receptor γδ T lymphocytes do not appear to be crucial to the establishment, or the control, of primary infection with *E. vermiformis* and are not principal mediators of the solid immunity to challenge that this infection induces.

The lymphocytes present in the intestinal epithelium (intraintestinal epithelial lymphocytes [i-IEL]) make up a relatively large proportion of the total immune system, but their immunobiology is not well defined (21). As they are the first immune cells to be encountered by a pathogen gaining entry from the intestinal lumen, it is reasonable to suppose that they may be involved in defense against such microbes and also in immune surveillance of malignancy (14). Although their role in these functions is not clear, they have been shown to be capable of antigen-specific and nonspecific cytotoxity (major histocompatibility complex restricted, alloreactive, spontaneous, antibody dependent, and redirected) (16). These properties and their location suggest that i-IEL might participate in resistance to infection with *Eimeria* spp., intracellular protozoan parasites of enterocytes that cause coccidiosis, a disease of considerable economic importance in agriculture, especially the poultry industry. i-IEL could be effective either by lysing infected host cells or by secreting lymphokines in response to signals provided by altered enterocytes or parasite antigens.

Another way in which i-IEL may be involved in defense against some *Eimeria* spp. is by intercepting the initial invasive organisms, the sporozoites, en route to their developmental site, i.e., enterocytes of the crypt. Sporozoites, on release in the lumen of the intestine, first penetrate the surface epithelium, but those of many species embark on development in the less differentiated enterocytes of the crypts. There is some controversy (45) about their transit from surface to crypt, but for some species that parasitize chickens, there is evidence for their transit being within cells of the immune system, including some that bear the CD8 marker (41) and that morphologically resemble granulated and ungranulated i-IEL (20). According to some authors, sporozoites in chickens that have been immunized by previous infection appear to be retained within these cells, thereby failing to reach the enterocytes in which they normally develop (27, 33, 42).

* Corresponding author. Mailing address: Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom. Phone: 44 (1635) 577293. Fax: 44 (1635) 577263.

† Present address: Naval Medical Research Institute, Malaria Program, Rockville, MD 20852.

In an attempt to analyze the role of intestinal lymphocytes of various phenotypes in the establishment and/or control of eimerian infections, we have initially examined the effect of depletion of the T-cell receptor (TCR) γδ population of the i-IEL of the mouse on the course of infection with *Eimeria vermiformis*. This protozoon begins its development in crypt enterocytes of the distal third of the murine small intestine and resembles *E. maxima* of the chicken in its ability to induce a prompt and very effective immunity in the host. The γδ subset of the heterogeneous i-IEL population was chosen for investigation because it forms, in mice (10) as well as in chickens (2), a high proportion of the total and, according to recent findings (13, 17, 25), may be able to recognize antigens rapidly, in the absence of classical antigen-presenting molecules (18). Additionally, Findly et al. (8) have reported fluctuations in the numbers of these cells, and in their TCR γ gene usage, during the course of infection with *E. vermiformis* in the mouse.

Mice were BALB/c females (Harlan Olac, Bicester, United Kingdom, or Charles River, Margate, United Kingdom), 6 to 7 weeks of age at the beginning of the experiment, that were kept in isolation, free from extraneous infection with coccidia (35). The origin of *E. vermiformis*, methods of maintenance, and means to evaluate infection by estimating the daily output of oocysts in the feces of individually caged mice have been fully described (35).

The hamster anti-murine γδ TCR monoclonal antibodies (MAb) used for in vivo depletion were secreted by the GL3 hybridoma (11) and grown either in *scid bg/bg* mice (4) or in vitro (Tecnomouse hollow fiber cell culture system; Integra Parasciences, Ltd., St. Albans, United Kingdom). Ascites or culture supernates were concentrated by precipitation with $(NH4)_2SO_4$, extensively dialyzed against phosphate-buffered saline (PBS, pH 7.2), and examined for reactivity with isolated i-IEL before use. For depletion in vivo, 1 mg was injected intraperitoneally (i.p.) and the phenotypes of isolated i-IEL were examined 3 days later and compared with those found in control, untreated mice.

For preparing suspensions of i-IEL (5), the small intestines were flushed with calcium- and magnesium-free Hanks medium (pH 7.2) containing 2% fetal calf serum, and after being trimmed of fat, having Peyer's patches removed, and being

TABLE 1. Schedule for treatment of mice by inoculation of *E. vermiformis* oocysts and injection of anti-γδ TCR MAb[a]

Group	Inoculation of oocysts[b] on day:		Injection of MAb[c] on day:	
	0	28	−3	25
1	D[d]	D	D	D
2	D	D	D	ND[e]
3	D	D	ND	D
4	D	D	ND	ND
5	ND	D	D	ND
6	ND	D	ND	D
7	ND	D	ND	ND
8	ND	ND	ND	ND

[a] Phenotypes of i-IEL were examined on days 0, 3, and 31 (three mice per group). Feces of oocyst-inoculated mice (eight mice per group) were examined for oocysts from the seventh day after dosing (i.e., days 7 and 35) until the end of patency (feces negative for oocysts on two consecutive days). In the case of secondary infections, the feces were examined by a sensitive concentration method throughout the patency of the primary controls, and any samples indicating the presence of sufficient oocysts to be quantified were further processed.
[b] Oocysts (10^3) were given orally.
[c] One milligram of GL3 MAb was given i.p.
[d] D, done.
[e] ND, not done.

opened, they were cut into segments of approximately 1 cm. These were washed and then incubated twice, with stirring, in calcium- and magnesium-free Hanks medium plus 10% fetal calf serum plus 0.1 mM EDTA for 20 min at 37°C. The pooled supernates were centrifuged, and the pelleted cells were resuspended in RPMI 1640 medium and passed over nylon wool columns. Cells in the filtrate were recovered and then centrifuged (1,500 × *g* for 20 min at 4°C) through a 44/67.5% Percoll gradient. Cells at the interface were collected and washed in cold RPMI before analysis by flow cytometry (FACScan; Becton Dickinson). For this, i-IEL at concentrations of 10^6 to 10^7/ml in RPMI 1640 were incubated in microtiter plates for 20 to 30 min with predetermined concentrations of the following labelled commercial antibodies (made up in 1/25 normal mouse serum): rat monoclonal anti-murine phycoerythrin-conjugated anti-Thy 1.2, anti-$CD8^+$, and anti-$CD4^+$ (Boehringer Mannheim) and fluorescein isothiocyanate-conjugated anti-γδ TCR and anti-αβ TCR (Pharmingen); fluorescein isothiocyanate-conjugated anti-murine immunoglobulin G (Southern Biotechnology Associates) and anti-hamster immunoglobulin G (Vector Laboratories) were polyclonal goat antisera. After two washes with PBS containing 0.2% bovine serum albumin and 0.01% NaN_3, the cells were resuspended in PBS–1% paraformaldehyde for flow cytometric analysis, with mononuclear cells gated by forward and side scatter parameters.

Three replicate experiments were carried out with BALB/c mice, with the aim of determining the effect of depletion of TCR γδ lymphocytes on (i) the course of primary infection with *E. vermiformis* and (ii) susceptibility to a second inoculum of infective oocysts (one infection with this parasite renders normal, immunocompetent mice resistant to reinfection). The protocol is summarized in Table 1.

Mice were divided into two main treatment sets, one (groups 1 to 4) being given a primary inoculation of oocysts (day 0) and the other (groups 5 to 8) being kept coccidium free to act as primary infection controls when a second inoculum was given, on day 28, to the previously infected animals. Subgroups were treated with anti-γδ TCR MAb 3 days before oocysts were inoculated, i.e., on day −3 and/or day 25, as shown in Table 1. The efficacy of the MAb was confirmed by fluorescence-activated cell sorter analysis of i-IEL on day 0 (day of infection) and on days 3 and 31 (corresponding to midinfection in oocyst-inoculated mice). Each treatment group contained eight mice for measurement of infection and three mice for monitoring the phenotypes of i-IEL. The provision of groups of primary infection controls for the challenge inoculum of oocysts enabled us to evaluate the effect of γδ TCR depletion on the course of primary infection twice within each experiment (albeit in hosts of different ages). Inoculations of *E. vermiformis* (10^3 sporulated oocysts in 0.2 ml of PBS) were given by gavage, and the subsequent infections were measured by determining the daily output of oocysts in the feces from the seventh day after dosing (days 7 and 35). All data were analyzed by Student's *t* test.

Depletion of TCR γδ i-IEL in the course of the experiments. The results of phenotypic analysis of lymphocyte subsets in isolated i-IEL, obtained for the different treatment groups in each of the three experiments, were pooled, and the mean values are presented in Fig. 1. There were no major differences caused by the treatments, other than the decreases in percentages of cells positive for TCR γδ in response to the injection of the MAb. Since sampling was limited to one time point after infection or reinfection (i.e., 3 days postinoculation of oocysts), no analysis of the effect of infection on the phenotypes of i-IEL was attempted.

The mean value for the percentage of TCR γδ i-IEL cells in uninjected mice ± the standard error of the mean (SEM) was 13.9% ± 0.6%, compared with 0.9% ± 0.1% for injected mice ($P < 0.0001$). (All values were included, irrespective of time or number of injections and presence or absence of infection.) Peripheral and mesenteric lymph nodes were also depleted of TCR γδ lymphocytes by treatment (nodes were examined in preliminary experiments on day 14 after antibody injection [data not given]). The values obtained for this category of i-IEL in individual experiments and in the different treatment groups, together with the replication of the parasite, are given in Tables 2 and 3.

These results, confirming those obtained in the preliminary testing in vivo of the antibody preparations (not given), showed that the numbers of TCR γδ i-IEL were significantly reduced in MAb-injected mice sampled during the course of the infections. This effect of MAb treatment was long-lasting, as shown by results of the analyses done on day 31: the percentage of TCR γδ cells in the groups injected on day −3 ± the SEM was 1.9% ± 0.5%, compared with 14.4% ± 1.0% in the uninjected mice (pooled data, $P < 0.0001$) (Tables 2 and 3). It is likely, therefore, that the depletion demonstrated at day 3 postinoculation (i.e., approximately midway through the developmental cycle) lasted throughout the replicative (asexual) phase of the parasite, normally 5 to 6 days in BALB/c mice (34).

Infections with *E. vermiformis*. Each of the three experiments yielded two sets of data (Table 2) for primary infections, i.e., one for the groups inoculated with oocysts on day 0 and another for those inoculated on day 28 (controls for the challenge inoculum given to previously infected mice).

In six of the nine comparisons (Table 2) that could be made between MAb-treated and untreated mice, oocyst production was greater in the treated mice than in the controls, but the differences were small in two cases (experiment 1, the group infected on day 28 and treated with MAb on day −3, and experiment 2, the group infected on day 0) and statistically significant ($P < 0.05$) in only two cases ($P = 0.07$ in experiment 1, the groups infected on day 0). In the remaining three comparisons, treated mice produced fewer oocysts than the controls, but the differences were not statistically significant ($P > 0.05$). In all nine comparisons, however, the percentages of

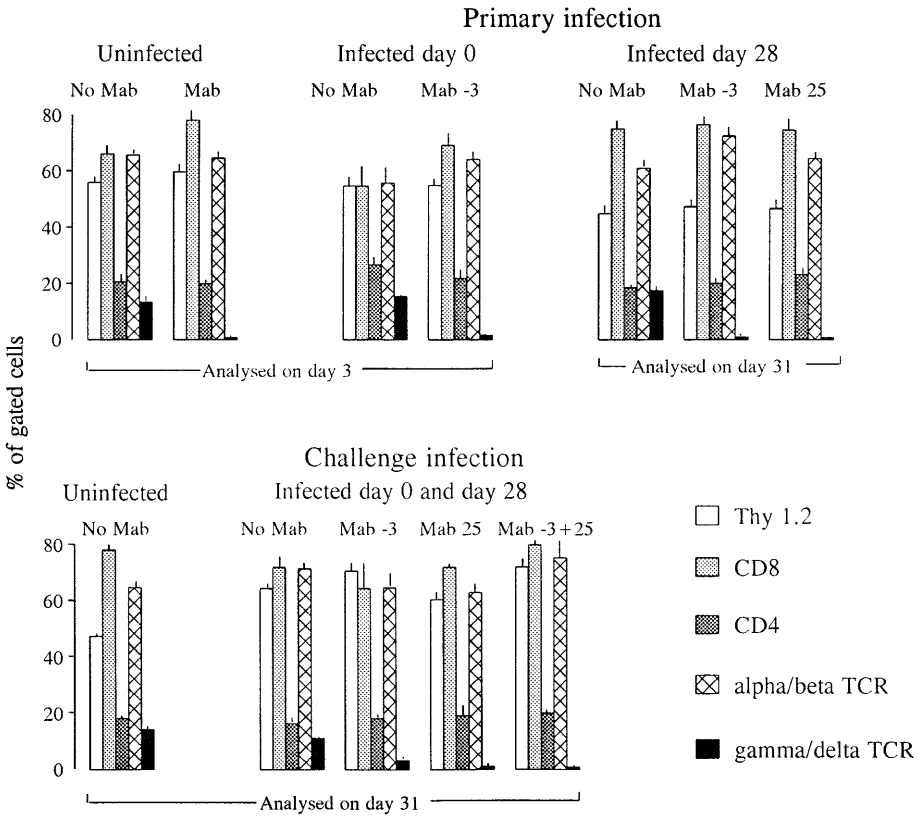

FIG. 1. Phenotypes of isolated i-IEL in BALB/c mice, treated or not treated with GL3 hamster anti-murine TCR γδ MAb and infected or not infected with *E. vermiformis*. Mice were dosed with oocysts (10^3) on day 0 and/or day 28 and injected with MAb (1 mg i.p.) on day −3 and/or day 25. The intestines were sampled on day 3 or 31, approximately corresponding to the midpoint of the developmental cycle in infected mice. The values are means ± the SEM, the results of three replicate experiments (numbers of mice represented vary from 5 to 10).

TCR γδ i-IEL, as measured in the mice sampled on the third day of infection, were reduced to similar levels.

Thus, the depletion of TCR γδ cells had neither a consistent nor a profound effect on the replication of the parasite in BALB/c mice and there was no correlation between the extent of depletion and changes in the numbers of oocysts produced. Additionally, there was no demonstrable effect of depletion upon the duration of patency (data not given), a reliable indicator of compromise of the immune response in infections with *E. vermiformis* (30).

In a single experiment similar to those described above and carried out with the more susceptible NIH strain of mouse, in which the animals were injected with MAb on day −3 and infected on day 0, oocyst production was enhanced in the MAb-treated mice (the mean total number for 10^6 oocysts per mouse ± the SEM was 116.6 ± 5.9 [11 mice] compared with 81.1 ± 5.8 [7 mice] for the control mice, $P < 0.001$; no data are available for the TCR γδ composition of isolated i-IEL cells).

To investigate the effect of depletion of TCR γδ cells, at the time of priming and/or challenge, on resistance to reinfection, BALB/c mice that had experienced an infection initiated on day 0 were given a challenge inoculum of 10^3 oocysts on day 28. In normal circumstances this challenge is resisted and few, if any, oocysts are subsequently detected in the feces, even when examined by a sensitive concentration method. The results, together with data for the percentages of TCR γδ i-IEL, are shown in Table 3. Values obtained for the control mice contemporaneously given the inoculum of oocysts as a primary infection, i.e., mice dosed on day 28, are in Table 2.

TABLE 2. Replication of *E. vermiformis* in primary infection and percentages of TCR γδ i-IEL in MAb-treated mice and controls

Expt	Day of MAb treatment[a]	Day of infection[b]	Total no. of *E. vermiformis* oocysts produced (10^6) (SEM)[c]	% of TCR γδ cells gated (SEM)[d]
1	−3	0	2.4 (0.5)	1.3 (0.6)*[e]
	None	0	1.2 (0.3)	15.7 (0.9)*
	−3	28	1.9 (0.3)	0.4 (0.1)*
	25	28	2.9 (0.5)*	0.9 (0.2)*
	None	28	1.2 (0.4)	16.5 (3.0)
2	−3	0	2.7 (0.6)	2.3 (0.5)*
	None	0	2.4 (0.4)	19.8 (2.8)
	−3	28	2.6 (0.6)	1.8 (0.6)*
	25	28	5.8 (1.9)	2.3 (0.2)*
	None	28	3.9 (0.7)	21.5 (2.2)
3	−3	0	4.3 (0.7)*	1.0 (0.2)*
	None	0	2.6 (0.3)	11.5 (0.3)
	−3	28	2.0 (0.8)	0.5 (0.1)*
	25	28	0.8 (0.1)	0.6 (0.2)*
	None	28	3.6 (1.4)	13.6 (1.1)

[a] MAb treatment consisted of a single injection of 1 mg in 200 μl of PBS given i.p.
[b] Infection was initiated by giving 10^3 sporulated oocysts orally.
[c] Values are means (SEM) for groups of eight mice.
[d] Values are means for groups of three mice. Mice were sampled on day 3 or 31 according to whether they were infected on day 0 or 28.
[e] Values marked with an asterisk differ significantly ($P < 0.05$) from those of relevant untreated controls.

TABLE 3. Resistance of immunized mice to challenge with *E. vermiformis* and percentages of TCR γδ i-IEL in MAb-treated mice and controls

Treatment with MAb[a] on day(s)	No. of mice with oocysts detected in feces after challenge inoculation[b] (no. positive/no. in group) in expt:			% of TCR γδ cells gated (SEM)[c] in expt:		
	1	2	3	1	2	3
−3 and 25	1/8	0/8	3/8	0.4 (0.1)	0.3[d]	1.2 (0.1)
−3	2/8	0/8	0/8	1.1 (0.7)	2.9 (0.7)	4.6 (0.3)
25	2/8	0/8	0/8	0.4 (0.1)	1.6 (0.1)	2.0 (0.5)
None	0/8	0/8	4/8[e]	10.9 (0.7)	11.0 (1.1)	12.5 (1.5)
None	0/8[f]	0/8[f]	0/8[f]	16.7[d]	15.3 (2.0)	12.8 (0.9)

[a] One milligram of MAb in 200 μl of PBS given i.p.
[b] Oocysts (10^3) were given orally on day 28; all mice had previously been infected as the result of an inoculum given on day 0 (results of the inoculation, as well as those of infection in control unimmunized mice, are given in Table 2).
[c] Values are means (SEM) derived from groups of three mice, except where indicated. In each experiment, values for MAb-treated mice were lower than for untreated controls ($P < 0.005$).
[d] One mouse only.
[e] Numbers of oocysts throughout patency totalled 10^3 per mouse.
[f] Mice were not infected and not challenged.

In experiment 1, all three MAb-treated groups contained one or two mice that produced a few oocysts on challenge, whereas there was none in the undepleted group. In experiment 2, all groups, irrespective of treatment (and consequent effect on the percentages of TCR γδ cells in isolated i-IEL), completely resisted the infection, with no oocysts being detected in the feces. In experiment 3, the feces of three mice were positive in only one of the MAb-treated groups, compared with four mice in the control group, of which one produced oocysts in numbers that could be estimated by the dilution method. If the results for the three experiments are pooled, oocysts were produced in response to challenge by 4 of 24 normal mice, and by 8 of 72 MAb-treated mice, with reductions in TCR $\gamma\delta^+$ cells in isolated i-IEL populations ranging from 63 to 97% (mean/group).

Although not uniform, these results do not suggest that the TCR γδ-depleted mice were more susceptible to challenge than controls. Taken together with the results obtained for primary infections in BALB/c mice, it would seem that this subset of T lymphocytes is not crucial for the control of *E. vermiformis.*

In view of the efficacy of the MAb in reducing the percentage of γδ TCR^+ cells recovered from the small intestine, it seems unlikely that there would be enough residual cells of this phenotype to mask the effects of treatment, especially as the variability in the results could not be correlated with the extent of depletion. Such a possibility, however, cannot be discounted and deserves more consideration in assessing the results of challenge of immunized mice. However, in no experiment did immunized MAb-treated mice produce countable numbers of oocysts as the result of a challenge inoculum (Table 3). Established immunity to *E. vermiformis*, the basis of which is unknown, has proved very difficult to abrogate. Procedures that enhance primary infection, such as depletion of $CD4^+$ cells or of gamma interferon, are without effect (31, 36), and susceptibility to reinfection with this organism has been described only for nude or *scid* mice (32) and for TCR αβ knockout mice (7a). Experiments with *E. vermiformis* in mice with disruptions or deletions in the gene coding for the γδ TCR should provide more conclusive evidence than was obtained from the MAb-depleted mice used for this study, in that there would be no residuum of unaffected cells. However, even in knockout mice there is a possibility that, owing to the redundancy of the immune response, compensatory mechanisms come into effect (15). Such substitution may also have taken place in the MAb-depleted mice used for this study. For reasons given earlier, our investigations have centered on the γδ TCR population of the i-IEL, but as the effects of MAb injection are likely to have been general, it seems that no lymphocytes of this phenotype exert a major controlling influence on the replication of *E. vermiformis* in primary infections of BALB/c mice or in the expression of resistance to challenge. TCR γδ lymphocytes have been implicated in the response and, in some instances, resistance to intracellular pathogens (12). Where there is evidence for the latter, it seems that the role of these lymphocytes is, in the main, an interactive one with TCR αβ lymphocytes in, for example, listeriosis (19, 24, 25), in which they appear to be effective against primary, but not challenge, infections (39), and in malaria (37, 44). Cloned human TCR γδ lymphocytes have, however, been shown to inhibit the replication of blood-stage malaria parasites in vitro, ostensibly after direct contact with merozoites (6), and to transfer protection adoptively (43). In contrast, a lack of TCR γδ lymphocytes did not affect the replication of another protozoon, *Trypanosoma cruzi*, (23), and the TCR phenotype of i-IEL cytotoxic to lymphocytic choriomeningitis virus is αβ, not γδ (40).

The role of γδ i-IEL may lie not in defense against infection but in regulating the generation and differentiation of intestinal epithelial cells (17), hosts for *E. vermiformis*. In this case it is, perhaps, surprising that the development of the parasite was not affected in the depleted mice. The finer details of the early phase of the developmental cycle of *E. vermiformis* have not been investigated, but it is known that although sporozoites initially invade the villous epithelium, differentiation and growth begin in enterocytes of the crypt; whether there is an association with lymphocytes in the intervening phase, as demonstrated by some authors, for some eimerian species in chickens is not known. The results described here, however, show that depletion of TCR γδ i-IEL in no way impaired the establishment of infection. Similarly, there is no impediment to infection in athymic and *scid* mice (but these mice possess i-IEL that are predominantly TCR $\gamma\delta^+$ in the former [22, 26, 28] and TCR^- $CD8^+$ in the latter [3]). If i-IEL are involved in the migration of sporozoites, it is possible that, as is the case with immunological mechanisms, there is a redundancy in the system that complicates analysis of specific mechanisms. Certainly, in unnatural situations, e.g., in vitro, sporozoites of *E. tenella*, a species thought to utilize leukocytes in vivo, will grow without their intervention and in cells which do not support development in vivo.

Although the results reported here suggest that TCR γδ cells are not of paramount importance either in the establishment or in the control of *E. vermiformis* infection, they may yet participate in these processes in the intact animal. TCR γδ knockout mice would provide a more complete model of depletion that could be compared with the TCR αβ knockout model, referred to earlier. We did not examine the possibility that TCR γδ lymphocytes could be involved in protecting the epithelium from the pathological effects of infection, as suggested by Findly et al. (8). Both potentially tissue-protective and -destructive roles have been proposed for TCR γδ i-IEL. With respect to the former, it has been suggested that they down-regulate inflammatory responses (9, 14) and, by the secretion of tissue-specific growth factors (13), maintain the integrity of epithelia and promote healing. In contrast, reductions in TCR γδ-depleted mice of (i) enteropathy due to graft-versus-host response (38) and (ii) tissue lesions and mortality in Chagas' disease (23) suggest that these cells are implicated

in tissue damage. Infection with *Eimeria* spp. is associated with a variety of pathological effects, including a marked intestinal inflammatory response (1, 7) that is mitigated in the athymic host (29). It would be interesting to compare these effects of infection in TCR γδ-depleted and in normal mice.

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