Age-dependent Requirement for γδ T Cells in the Primary but Not Secondary Protective Immune Response against an Intestinal Parasite

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Abstract

Between weaning (3 wk of age) and adulthood (7 wk of age), mice develop increased resistance to infection with *Eimeria vermiformis*, an abundant intestinal parasite that causes coccidiosis. This development of resistance was perturbed in T cell receptor (TCR)$\delta^{-/-}$ mice, which at 4 wk of age remained largely susceptible to infection and prone to infection-associated dehydration. These phenotypes were rescued by the repopulation of γδ cells after adoptive transfer of lymphoid progenitors into newborn recipients. Because αβ T cells are necessary and sufficient for the protection of adult mice against *E. vermiformis*, the requirement for γδ cells in young mice shows a qualitative difference between the cellular immune responses operating at different ages. An important contribution toward primary immune protection in young hosts may have provided a strong selective pressure for the evolutionary conservation of γδ cells. This notwithstanding, the development of effective, pathogen-specific immunity in young mice requires αβ T cells, just as it does in adult mice.

Key words: neonatal immunology • mucosal immunology • infection • adoptive transfer • knockout mice

Introduction

The neonatal period in humans and mice is characterized by heightened susceptibility to numerous microbial infections. In 2001, >5 million children worldwide died of infectious diseases to which more robust immune responses might have provided protection. Hence, attempts to improve vaccination and antimicrobial therapies for children and neonates are critically important. Ideally, such efforts can be set against an improved understanding of the basic cellular immune mechanisms that operate in young children and animals (1, 2).

Although T and B cell development begins in the fetus (3, 4), most mammals initially rely on passively acquired immunoglobulin transmitted transplacentally and/or via breastmilk. By adulthood, diverse repertoires of αβ T and B cells establish efficient primary clearance of pathogens, and a reservoir of immunological memory toward rechallenge. By contrast, the transition from dependence on maternal factors to the immune autonomy of adults is relatively poorly understood.

For many years, immunodeficiency in young animals was associated with the "neonatal tolerance" model based on observations by Billingham et al. that neonatal T lymphocytes react to antigen encounter by becoming tolerized, rather than activated (5). Subsequently, three separate groups demonstrated that neonatal T cells can make adult-type responses under particular conditions of antigen presentation and cytokine milieu (6–8). Despite this, the physiologic situation in vivo remains quite unclear. Compelling data demonstrate that naive T cells enter and are tolerized by the tissues of neonatal mice (9), and young mice and humans rarely mount antipathogen responses, particularly Th1 responses, with adult efficacy (10, 11). Thus, conditions provoking conventional immune responsiveness seem de facto to be

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Abbreviations used in this paper: CMF, Click's medium with 5% fetal calf serum; DAI, days after infection; E14, embryonic day 14; IEL, intraepithelial lymphocyte; SFB, segmented filamentous bacteria.

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suboptimal in the young animal. Consistent with this, weaknesses in the αβ T cell response have been attributed to intrinsic defects in the T cells themselves (e.g., poor cytokine responsiveness and "helper" or cytolytic activity; references 12–16) and to defects in the antigen processing and presentation systems on which αβ T cells depend (6, 17–19). Therefore, the question arises as to which immune response mechanisms are functionally available to young animals.

One candidate is the γδ cell compartment. In all species in which their development has been extensively studied, γδ cells are highly represented in young animals (20–25). Many of the early-emerging γδ cells localize to epithelial surfaces, such as that of the gut (26), where they may be well placed to encounter the first waves of pathogens entering the body. Moreover, although γδ cells are clearly pleiotropic (27), they are commonly Th1 biased (28, 29), and their unconventional antigen specificities do not rely on MHC-restricted APCs that may function sub-optimally in young animals (30–32).

γδ cells can contribute to the primary clearance of pulmonary infections (33, 34). Furthermore, even in the many cases where the development of immunity seems largely intact in γδ T cell–deficient mice (25), an immunoprotective potential of γδ cells is evident in the observations that mice lacking γδ cells and αβ T cells are often more susceptible to infection than mice lacking αβ T cells alone (35, 36). For example, although the combined actions of $CD4^+$ αβ T cells and IFNγ largely account for the primary response of adult mice to *Eimeria vermiformis*, a natural protozoan pathogen of intestinal epithelial cells (37–39), mice lacking γδ T cells as well as αβ T cells are markedly more susceptible to primary infection than those lacking only αβ T cells (40). This partial protection provided by γδ cells can be neutralized by anti-IFNγ antibodies (40).

The uncovering of a protective role for γδ cells in the experimentally orchestrated absence of αβ T cells suggested that there may be physiologic instances in which γδ T cells in part compensate for deficiencies in the αβ T cell compartment. One such case may be the young animal. An indication of this was provided by Waters and Harp's studies of *Cryptosporidium parvum*, a human pathogen related to *E. vermiformis* (41). In brief, most T cell–intact mice challenged orally at 7 d of age cleared infection by 3 wk. By contrast, $TCR\delta^{-/-}$ mice shed detectable oocysts until 7 wk of age, whereas $TCR\alpha^{-/-}$ mice developed chronic infection (41). Strikingly, it appeared that αβ cell–deficient mice (that contain γδ cells) initially controlled infection better than γδ cell–deficient mice. Although these findings suggested a nonredundant involvement of γδ cells in responses to infection in early life, the experiments were limited in scope, and the authors could not rule out that there were other differences (e.g., IFNγ levels, between the strains used). In particular, they did not assess whether the reconstitution of genetically identical $TCR\delta^{-/-}$ mice with γδ cells would revert the transient immunodeficiency. Added to this, the use of $TCR\alpha^{-/-}$ mice was a confounding variable, as these animals contain T cells bearing a "β-only" TCR (42, 43).

Therefore, to test directly the hypothesis that γδ cells make a critical contribution to immunological protection in young mice, a set of infections with *E. vermiformis* was undertaken. In the wild, eimerian coccidiosis of the gut is commonly contracted during early life, primarily through exposure to low but chronic inocula of parasites in contaminated feeding areas. In the laboratory, gavage administration of different stocks of parasites, passaged in vivo, can be used to mimic natural infection. We show here, across a broad spectrum of infectivity, that γδ cells are essential for the effective primary protection of young mice shortly after weaning, and that, as a result, the age-related development of increasing immunocompetence toward *E. vermiformis* is temporally perturbed in the $TCR\delta^{-/-}$ strain. This exposes a clear difference between the cell-mediated immune responses of young mice as opposed to adults, and argues for a strong selective pressure for the evolutionary conservation of γδ cells. At the same time, the development of pathogen-specific immunological memory in young mice was fully dependent on αβ T cells, as it is in adults.

Materials and Methods

Mice

Mice, bred locally or obtained from The Jackson Laboratories, were maintained under specific pathogen-free conditions. For comparisons of the susceptibility of different strains to infections, breedings of the different genotypes of mice were set up synchronously, so that precisely age-matched animals could be infected at the same time.

Parasite and Oocyst Enumeration

E. vermiformis (maintained in our laboratory) was passaged in mice, and oocysts were purified and sporulated. After microscopically scoring stocks for sporulation, mice were infected with either 10^2 or $1–2 \times 10^3$ *E. vermiformis* oocysts in sterile water via oral gavage. For the duration of infection, mice were caged singly on wire racks above sand from which feces were collected daily. Oocysts were enumerated in McMaster counting chambers after salt flotation. To assess fluid loss during infection, mice were weighed daily.

Preparation of Cells

Splenocytes. Mice were killed and spleens were placed in sterile media (Click's medium with 5% fetal calf serum [CMF]) or PBS on ice. Tissues were mashed between two frosted slides to obtain a single cell suspension. Red blood cells were removed by incubation in lysis buffer (Sigma-Aldrich). Residual cells were washed and counted before use.

Intraepithelial Lymphocytes (IELs). The method used was based on that published by Goodman and Lefrancois (44). In brief, the small intestine was removed and flushed with ice cold CMF to remove intestinal contents. Peyer's patches were cut out and removed to a separate container. The intestine was cut longitudinally, scraped lightly to remove mucus, and cut into 1-cm pieces that were rinsed several times in cold CMF to remove debris and placed into a clean 50-ml conical tube with 20 ml CMF-1

mM DTE. The tube was shaken at 37° for 20 min to disrupt the epithelial layer. The gut pieces were vortexed briefly and allowed to settle. The cell-rich supernatant was collected to a clean tube and the shaking process was repeated. After collection of the second supernatant (containing both IELs and enterocytes), the cells were pelleted (600 *g*, 10 min) and resuspended in ice-cold RPMI 1640 media (5 ml per gut). This solution was passed over a nylon wool column to remove contaminating enterocytes. The resulting effluent was collected and spun down (600 *g*, 10 min) to pellet the cells. Finally, the pellet was resuspended in 24 ml of 44% Percoll and layered on top of 5 ml of 67% Percoll. The resulting gradients were spun at 600 *g* for 20 min, and the intraepithelial lymphocytes were harvested from the interface. Cells were washed extensively in a large volume of RPMI 1640 medium and counted before use.

Reconstitution of Neonatal Mice with Fetal Liver Cells

Pregnant mice were killed at gestational day 14 by anesthetic overdose (Halothane™; Roche Labs) and fetuses were removed. Fetal livers were dissected and pooled in sterile Click's medium on ice. Liver tissue was disrupted by mashing between sterile frosted slides and passed through a 20-gauge needle to obtain a single cell suspension. The whole suspension was spun (600 *g*, 5 min) to pellet the cells. Red blood cells were lysed, and the residual cells were washed, counted, and resuspended at a concentration of 10^7 cells/30 μl. Recipient mice were 1–3 d old at the time of transfer, and each recipient was given 10^7 cells by intraperitoneal injection. Total volume of the injection was 30 μl. Mock-reconstituted control mice received a 30-μl injection of either buffer alone or an irrelevant cell type.

Flow Cytometry Analysis

Between 5×10^4 and 10^6 freshly isolated cells were preincubated with an antibody to block nonspecific labeling of FcRII/III (Fc Block™; BD Biosciences). Cells were washed and reacted with fluorescently tagged monoclonal antibodies for 15 min in 100 μl of staining buffer (1× PBS with 2% FCS) followed by three washes in 1× PBS. Cells were analyzed immediately (FACScan™ flow cytometer) or fixed in 2% formaldehyde (pH 7.0 in PBS). Data were analyzed using CELLQuest™ software. Antibodies used in these studies were obtained from BD Biosciences.

Molecular Analysis

Cells were harvested from chosen tissues into cell lysis buffer plus β-mercaptoethanol (RNAeasy mini-kit; QIAGEN), from which RNA was ultimately eluted in 30–60 μl of sterile water and stored at −80°C until use. Reverse transcriptase coupled polymerase chain reaction (RT-PCR) was undertaken exactly as described previously (45), using the primers as follows: IFNγ, 5′-CATTGAAAGCCTAGAAAGTCTG-3′, reverse, 5′-CTCATGAATGCATCCTTTTTCG-3′; and HPRT, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′, reverse, 5′-GAGGGTAGGCTGGCCTATAGGCT-3′.

Statistics

Statistical analysis was performed using the Wilcoxon-Rank Sum test for unpaired data, from which a two-tailed p-value was obtained. Differences between tested groups were considered significant when $P < 0.05$. Statistical tests were made using InStat™ software for statistical analysis. Except where otherwise noted, error bars on graphs reflect ±SEM. Graphs with error bars were generated using Prism™ graphing software.

Results

The Capacity of T Cell–intact Mice to Resolve Primary E. vermiformis Infection Increases with Age. Young T cell–intact C57.BL/6 mice infected between 21 d of age (1 d after weaning) and 40 d of age (after the onset of sexual matu-

Table I. *Summary of Infection Experiments*

			Mice per group		Total oocysts shed × 10^6 ± SEM		
Expt	Dose	Age	Wild type	TCRδ$^{-/-}$	Wild type	TCRδ$^{-/-}$	p-value[a]
1[b]	1,000	21 d	7	10	49.01 ± 4.85	50.84 ± 1.15	NSD
	1,000	28 d	7	3	6.06 ± 0.86	25.98 ± 6.59	0.02
	1,000	40 d	4	3	1.65 ± 0.44	4.34 ± 0.60	0.06
2	100	21 d	4	4	12.25 ± 0.68	11.45 ± 1.90	NSD
3	1,000	24 d	4	4	32.30 ± 2.82	40.03 ± 6.23	NSD
4[c]	100	30 d	8	11	0.62 ± 0.15	2.33 ± 0.39	0.001
5	1,000	30 d	6	7	18.66 ± 2.33	31.07 ± 2.41	0.005
				4 [IFNγ$^{-/-}$]		23.69 ± 3.13 [IFNγ$^{-/-}$]	NSD
6	1,000	30 d	4	4	2.96 ± 0.78	10.45 ± 0.9	0.03
7	2,000	10 wk	6	8	15.5	12.60	NSD
Total			50	54			

[a]Within experiments, statistical comparisons between groups were made using the Wilcoxon-Rank Sum Test. Differences were considered significant when $P < 0.05$. No comparisons were made between experiments because of variability in parasite stocks.
[b]Identical stocks, depicted in Fig. 1 A.
[c]Shown in Fig. 1 C.

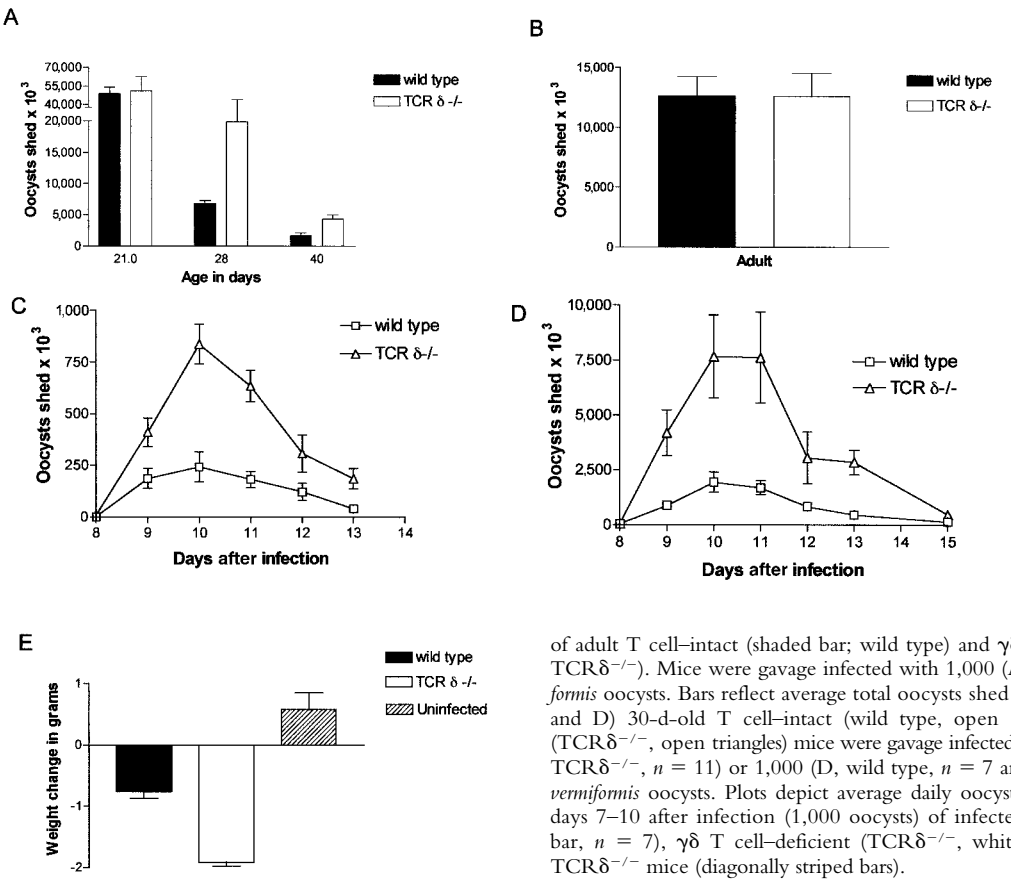

Figure 1. Age-dependent gain of immunocompetence is delayed in mice lacking γδ T cells. (A) Infection of T cell–intact (shaded bars, wild type) and γδ T cell–deficient (white bars, TCRδ$^{-/-}$) mice at 21 d old (wild type, $n = 7$; TCRδ$^{-/-}$, $n = 10$); 28 d old (wild type, $n = 4$; TCRδ$^{-/-}$, $n = 3$); and 40 d old (wild type, $n = 4$; TCRδ$^{-/-}$, $n = 3$). (B) Infection of adult T cell–intact (shaded bar; wild type) and γδ T cell–deficient mice (white bar, TCRδ$^{-/-}$). Mice were gavage infected with 1,000 (A) or 2,000 (B) sporulated *E. vermiformis* oocysts. Bars reflect average total oocysts shed over the patent period ±SEM. (C and D) 30-d-old T cell–intact (wild type, open squares) and γδ T cell–deficient (TCRδ$^{-/-}$, open triangles) mice were gavage infected with 100 (C, wild type, $n = 8$ and TCRδ$^{-/-}$, $n = 11$) or 1,000 (D, wild type, $n = 7$ and TCRδ$^{-/-}$, $n = 3$) sporulated *E. vermiformis* oocysts. Plots depict average daily oocyst yields. (E) Weight change during days 7–10 after infection (1,000 oocysts) of infected T cell–intact (wild type, shaded bar, $n = 7$), γδ T cell–deficient (TCRδ$^{-/-}$, white bar, $n = 3$), and mock-infected TCRδ$^{-/-}$ mice (diagonally striped bars).

rity) gained immunological resistance to *E. vermiformis* as measured by a declining yield of infectious oocysts after infection with a uniform dose (1,000 oocysts) of a single stock (Fig. 1 A and Table I, Experiment 1). This development of immunocompetence did not proceed normally in young TCRδ$^{-/-}$ mice, which remained significantly immunocompromised when infected at ~28–30 d of age (Fig. 1 A and Table I, Experiment 1). Although 40-d-old TCRδ$^{-/-}$ mice also showed increased susceptibility, the difference was no longer statistically significant (Fig. 1 A and Table I). Moreover, consistent with previous experiments conducted in our laboratory (46, 47), 10-wk-old adult TCRδ$^{-/-}$ mice displayed no enhanced susceptibility to primary infection with *E. vermiformis* (Fig. 1 B and Table I). These experiments point to a "window of susceptibility" between 28 and 40 d of age in which γδ cells provide the young mouse with an essential, nonredundant contribution to primary protection. After this, αβ T cells are necessary and sufficient to provide protection (37, 46, 47). Because the difference in susceptibility between wild-type and TCRδ$^{-/-}$ mice was greatest at 28–30 d of age, additional experiments focused on 30-d-old mice.

Dependence on γδ Cells for Primary Protection Is Independent of Pathogen Dose or Virulence of Inoculum. The infectivity and dose of pathogen encountered in the wild varies considerably. To mimic natural infection as closely as possible, sets of mice were infected with different doses of several different stocks of *E. vermiformis* derived by passage through different live hosts. The range of infectivity of these stocks is reflected in the broad spectrum of oocyst yields from infected C57.BL/6 mice (Table I). Nonetheless, the oocyst yields within individual experiments showed low standard error, permitting a clear comparison with the yields from γδ cell–deficient C57.BL/6 mice. The results show that young TCRδ$^{-/-}$ mice infected at around 4 wk of age were invariably compromised relative to control mice, regardless of virulence or dose (Table I). Although this susceptibility was first evident in mice infected at 24 d of age (Table I, Experiment 3), the differences at that time point, like those at 40 d, were not statistically significant.

Analysis of daily oocyst yield across the patent period is presented in Fig. 1 (C and D) for representative low and high dose infections, respectively. Among young TCRδ$^{-/-}$ mice, the margin of susceptibility was consistently greatest between days 9 and 13 after infection (9–13 days after infection [DAI]), with full resolution of the infection occurring at about the same time as control mice (usually 15–17 dpi). In other systems, the role of γδ cells has likewise been reported to be most crucial in the early phase of the T cell response (48, 49). By contrast, the effects of αβ T cell deficiency reported in adult mice extend beyond 13 DAI (47).

Susceptibility of Young TCRδ$^{-/-}$ Mice Is Confirmed by Fluid Loss during Infection. An established clinical manifestation of coccidiosis is a reproducible fluid loss after the start

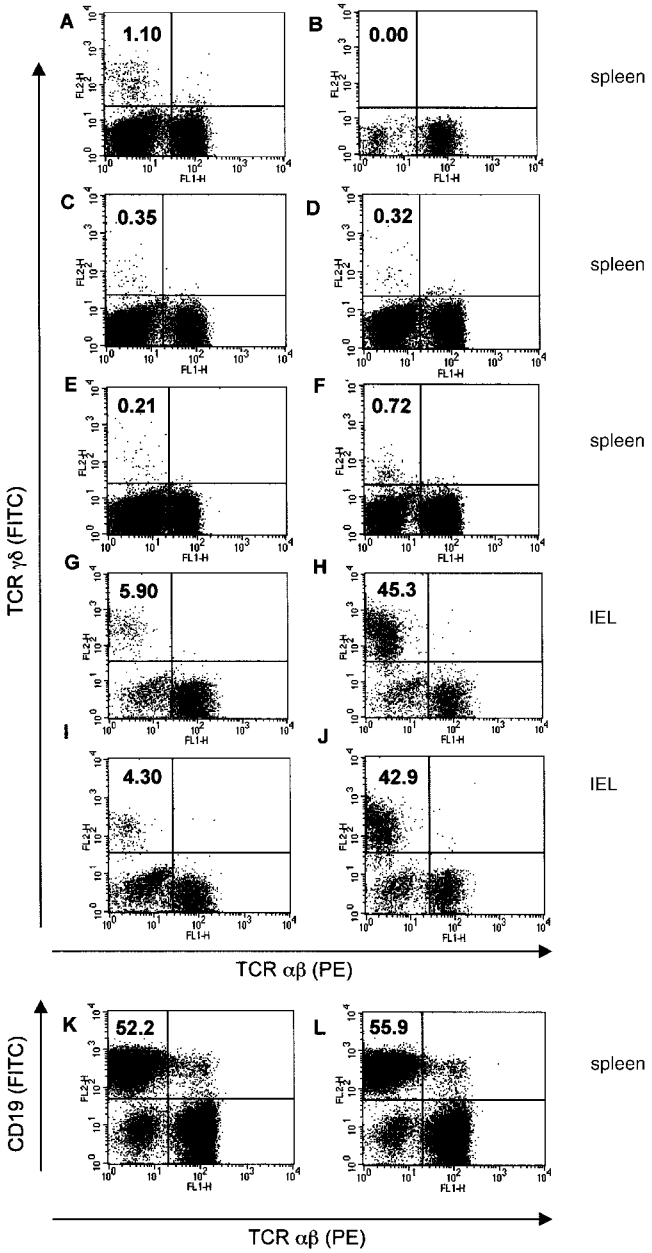

Figure 2. γδ cells are present in the spleen and gut of reconstituted animals. Splenocytes analyzed by flow cytometry for TCRγδ (A–J, y axis) and TCRαβ (x axis), and C19 (K and L, y axis) and TCRαβ (x axis). All plots shown were pregated on live lymphocytes. (A) Unmanipulated C57.BL/6 mice; (B) mock-reconstituted TCRδ$^{-/-}$ mice; (C–F) TCRδ$^{-/-}$ mice reconstituted with E14 fetal liver cells from wild-type donors (C and D); and TCR β $^{-/-}$ donors (E and F). (G–J) IELs were isolated 12 wk after neonatal reconstitution of TCRδ$^{-/-}$ mice with E14 fetal liver cells from wild-type (G and H) and TCRβ$^{-/-}$ (I and J) donors; this harvest date was 8 wk after infection. (A–J) Percentage of total splenic/gut lymphocytes comprising γδ cells is indicated in the top left quadrant. (K and L) Analysis of B cell composition of splenocytes from TCRδ$^{-/-}$ mice and TCRδ$^{-/-}$ mice reconstituted with E14 fetal liver cells from wild-type donors. Percent of total splenic lymphocytes comprising CD19^{+} B cells is indicated in the top left quadrant.

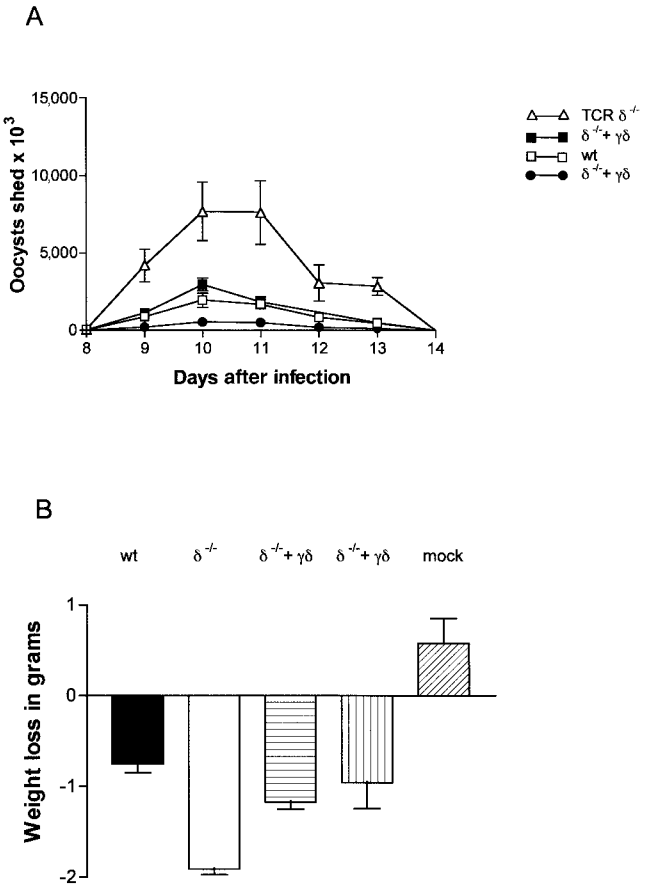

Figure 3. Replacement of γδ cells restores resistance to infection among 30-d-old TCRδ$^{-/-}$ mice. Reconstituted and control mice were gavage infected with 1,000 *E. vermiformis* oocysts. (A) Oocysts shed by TCRδ$^{-/-}$ mice (open triangles, $n = 3$; same group as in Fig. 1 D), wild-type mice (open squares, $n = 7$; same group as in Fig. 1 D), TCRδ$^{-/-}$ mice reconstituted with fetal liver from wild-type donors (closed squares, $n = 11$), and TCRδ$^{-/-}$ mice reconstituted with fetal liver from αβ T cell–deficient donors (closed circles, $n = 7$). (B) 30-d-old wild-type mice (shaded bars, $n = 7$), TCRδ$^{-/-}$ mice mock reconstituted (white bar, $n = 3$), and TCRδ$^{-/-}$ mice reconstituted with E14 fetal liver cells from either wild-type mice (horizontally striped bar, $n = 11$) or αβ cell–deficient mice (vertically striped bar, $n = 7$) were weighed daily at the peak of infection. Bars represent average weight loss in grams ± SD among members of a group during days 7–10 after infection. Age-matched control mice were mock infected with sterile water (diagonally striped bar, $n = 7$).

of oocyst shedding (7–10 DAI). Hence, daily weight measurements provide a robust and independent assessment of susceptibility among infected animals. C57.BL/6 mice infected at 30 d of age rarely lost >3–5% of total body mass. By contrast, TCRδ$^{-/-}$ mice lost up to 12% of total body mass in a 24–48-h period, a clinically significant dehydration (Fig. 1 E). Reflecting this, infected TCRδ$^{-/-}$ mice appeared sicker than infected C57.BL/6 mice, commonly displaying ruffled fur, hunched posture, and listlessness. Despite severe dehydration, most TCRδ$^{-/-}$ mice returned to normal body weight by 14 DAI.

Reconstitution of γδ Cells Restores Resistance to Young γδ Cell–deficient Mice. As an independent means to determine whether γδ cells provide immunoprotection in the period between weaning and sexual maturity, the potential of γδ cell reconstitution to restore immunocompetence in young TCRδ$^{-/-}$ mice was assessed. Because *E. vermiformis*

is a gut-tropic pathogen, it was important to reconstitute gut TCR$\gamma\delta^+$ IELs as well as systemic $\gamma\delta$ cells. Both compartments can be reconstituted by adoptive transfer of embryonic day 14 (E14) fetal liver cells to newborn recipients, up until 4 d of age (50). The use of neonatal TCR$\delta^{-/-}$ recipients accommodates the early development of many $\gamma\delta$ cell compartments (21) and obviates the need for radiation to ensure graft survival. Although all three lymphocyte cell types, $\alpha\beta$ T, $\gamma\delta$, and B cells will develop from inocula of unfractionated C57.BL/6 fetal liver, the lymphocyte progenitor potential of fetal liver from TCR$\beta^{-/-}$ mice is limited to $\gamma\delta$ and B cells.

18 neonatal recipients of E14 liver cells were assessed for their resistance to *E. vermiformis* infection after reaching 30 d of age, and, after recovery, were killed and assessed for cellular reconstitution. Fig. 2 depicts the analysis of splenocytes isolated from wild-type unmanipulated mice (Fig. 2 A), mock-reconstituted TCR$\delta^{-/-}$ mice (Fig. 2 B), or TCR$\delta^{-/-}$ mice reconstituted with E14 liver from C57.BL/6 (Fig. 2, C and D) or TCR$\beta^{-/-}$ donor mice (Fig. 2, E and F). In unmanipulated wild-type mice, $\gamma\delta$ cells commonly comprise 0.5–2.0% of the total lymphocytes in the spleen; in mice reconstituted with E14 liver from either C57.BL/6 or TCR$\beta^{-/-}$ donor mice, splenic $\gamma\delta$ cells accounted for 0.2–0.8% of total lymphocytes. Intestinal TCR$\gamma\delta^+$ IELs were similarly reconstituted to varying degrees, composing 4–45% of CD3$^+$ IELs, compared with 25–56% in unmanipulated wild-type mice (Fig. 2, G–J).

The heightened levels of oocyst shedding shown by young TCR$\delta^{-/-}$ mice across the patent period reverted to wild-type levels in mice receiving E14 liver cells from either C57.BL/6 or TCR$\beta^{-/-}$ donors (Fig. 3 A). The rescue of the TCR$\delta^{-/-}$ susceptibility phenotype was also evident in measures of body weight from 7 to 10 DAI (Fig. 3 B). Because functional reconstitution from TCR$\beta^{-/-}$ donors was as effective as that from C57.BL/6 mice (Fig. 3), the result cannot be attributed to a bolstered $\alpha\beta$ T cell compartment. Likewise, it is unlikely that functional reconstitution can be attributed to an altered B cell compartment, because although E14 liver can give rise to B cells, recipients of neither type of inoculum showed an altered representation of B cells relative to TCR$\delta^{-/-}$ mice (Fig. 2, K and L).

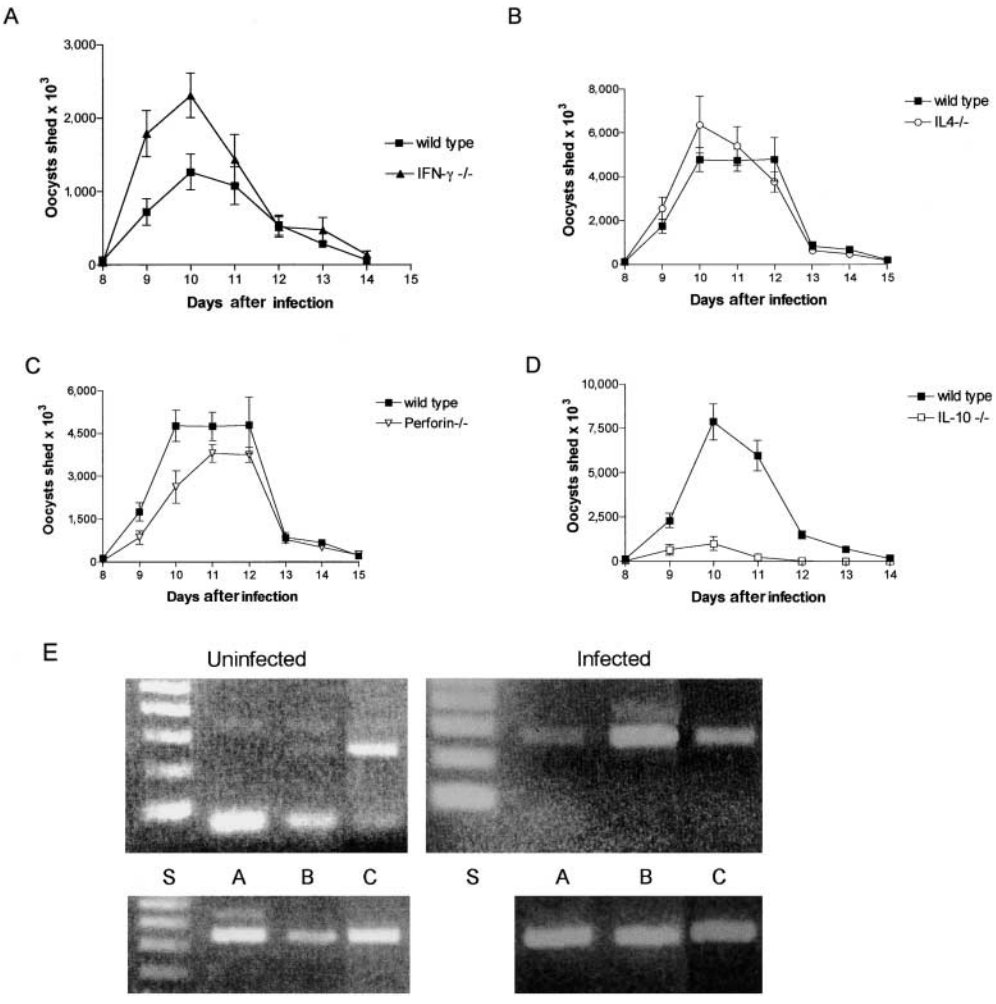

Figure 4. IFN$\gamma^{-/-}$ mice partially phenocopy susceptibility of TCR$\delta^{-/-}$mice. The susceptibility of 30-d-old wild-type and various cytokine deficient mice was compared as follows: (A) 30-d-old IFN$\gamma^{-/-}$ mice (closed triangles, $n = 4$) and wild type (closed squares, $n = 4$); (B) 30-d-old IL-4$^{-/-}$ mice (open circles, $n = 4$) and wild type (closed squares, $n = 3$); (C) perforin$^{-/-}$ mice (inverted open triangles, $n = 4$) and wild type (closed squares, $n = 3$); and (D) IL-10$^{-/-}$ mice (open squares, $n = 4$) and wild type (closed squares, $n = 6$). (E) RT-PCR analysis of IFNγ (top) and HPRT (bottom) RNA expression in splenic and intestinal $\alpha\beta$ T (lane A) and $\gamma\delta$ cells (lane B) harvested from mice that 6 d previously, at the age of 30 d, were either uninfected (left) or *E. vermiformis* infected (right). S, 100 bp size marker. IFNγ, bottom marker = 100 bp, product = 267 bp. HPRT, bottom marker = 200 bp, product = 351 bp. C, positive control (activated adult mouse spleen cells). The bona fide band is in each case the lower of a doublet; the fast running band in the top panel for the uninfected samples corresponds to primer dimers. The $\gamma\delta$ cell sample from infected mice shows an IFNγ signal comparable to the HPRT signal, whereas the signal in uninfected mice was much weaker than the HPRT signal.

Variable Susceptibilities to Infection of Young Cytokine-deficient Mice. IFNγ inhibits the growth of *E. vermiformis* within infected cells (38), and IFNγ-deficient mice infected as adults are severely susceptible to primary *E. vermiformis* infection, showing higher parasite yields and longer patent period, and generally becoming more ill than wild-type control mice (38, 39). Furthermore, αβ T cells but particularly γδ cells harvested from the splenic and intestinal IEL compartments of infected 30-d-old mice show increased levels of RNA for a spectrum of immunological mediators, including IFNγ and perforin (Fig. 4 E and not depicted). To test whether the immunoprotective effects of γδ cells in young mice are attributable to IFNγ, the susceptibilities of wild-type and IFNγ-deficient mice infected at 30 d of age were compared. Although the IFNγ$^{-/-}$ mice shed more oocysts, their increased susceptibility was largely confined to 9–10 DAI (Fig. 4 A), whereas that shown by young TCRδ$^{-/-}$ mice was more extensive (Fig. 1, C and D; and Fig. 3 A). To investigate this further, infections of age-matched 30-d-old wild-type, IFNγ$^{-/-}$, and TCRδ$^{-/-}$ mice were directly compared (Table I, Experiment 5). Although IFNγ$^{-/-}$ mice again shed many more oocysts on 9 DAI (7,800 compared with 2,300 by wild-type mice), the aggregate yield over the full course of infection was not significantly greater than that of wild-type mice, unlike the TCRδ$^{-/-}$ mice (Table I). Therefore, although IFNγ production is a parameter of host resistance, it alone cannot account for the protection provided by γδ cells in the young mouse.

Consistent with this, recent gene profiling analyses showed that gut-associated γδ cells express a wide variety of conventional and unconventional effector and regulatory genes (45), (51), any or each of which may contribute to γδ cell function in young mice. To begin to assess the potential contributions of some of these molecules, separate infections were undertaken in 30-d-old mice lacking IL-4, perforin, and IL-10, respectively. Neither 30-d-old IL-4$^{-/-}$ mice nor 30-d-old perforin$^{-/-}$ mice showed enhanced susceptibility to *E. vermiformis* infection (Fig. 4, B and C). Conversely, 30-d-old IL-10$^{-/-}$ mice showed enhanced resistance to primary infection with *E. vermiformis* (Fig. 4 D). This implies that under normal circumstances, infection of young mice is followed by secretion of IL-10 by cells that effectively dampen the primary immune response. Were these cells to be in the αβ T cell compartment (e.g., T-reg cells), young mice lacking αβ cells might actually be more resistant to infection (see Fig. 6).

αβ T Cells Are Not Critical to the Primary Response of Young Mice to E. vermiformis. αβ T cells are critical to the efficacious protection of adult mice against *E. vermiformis* infection. To assess their role in young mice, infections were undertaken in TCRβ$^{-/-}$ mice. First, as with TCRδ$^{-/-}$ mice (Fig. 1 A), freshly weaned, 21–24-d-old TCRβ$^{-/-}$ mice showed no increased susceptibility to infection (Fig. 5 A). Together, these data demonstrate that neither T cell subset contributes significantly to primary protection in very young mice. However, in contrast to

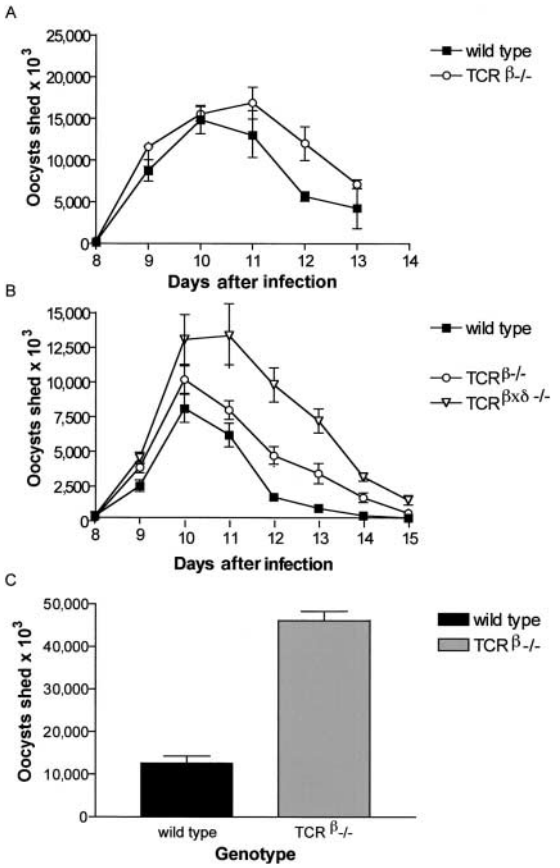

Figure 5. Young αβ T cell–deficient mice display variable susceptibility to infection. (A) Wild-type (closed squares, $n = 7$) and TCRβ$^{-/-}$ mice (open circles, $n = 7$) were infected (1,000 oocytes) at 21–24 d of age. (B) Wild-type mice (closed squares, $n = 6$), TCRβ$^{-/-}$ mice (open circles, $n = 7$), and TCRβxδ$^{-/-}$ mice (inverted triangles, $n = 6$) were infected (1,000 oocysts) at 30 d of age. (C) Wild-type (shaded bar) and TCRβ$^{-/-}$ mice (gray bar, $n = 7$) were infected (2,000 oocysts) as adults. Bars reflect total oocyst production ±SEM over the patent period.

the susceptibility of 28–30-d-old mice lacking γδ cells (Fig. 1 A), TCRβ$^{-/-}$ mice were commonly no more susceptible to infection than were wild-type mice (Fig. 5 B), and in some cases, they actually showed enhanced resistance to the early stages of infection (see Fig. 6). In contrast, adult TCRβ$^{-/-}$ mice were highly susceptible to infection (Fig. 5 C), consistent with all previous analyses (37, 46, 47). These works provide further evidence for the qualitative differences between the cellular immune responses of young and adult mice.

αβ T Cells Are Critical to the Development of Immunological Memory in Young Mice. Some of the T cells that effect a primary immune response acquire immunological memory and protect the host against subsequent infection with the same organism. Because γδ cells and not αβ cells are the primary immunoprotective T cells in 30-d-old mice, it was important to determine whether γδ cells could also protect against rechallenge. Fig. 6 A shows the result of an experiment in which wild-type, TCRδ$^{-/-}$, or TCRβ$^{-/-}$ mice were initially infected at 30 d of age (1° infection) and re-

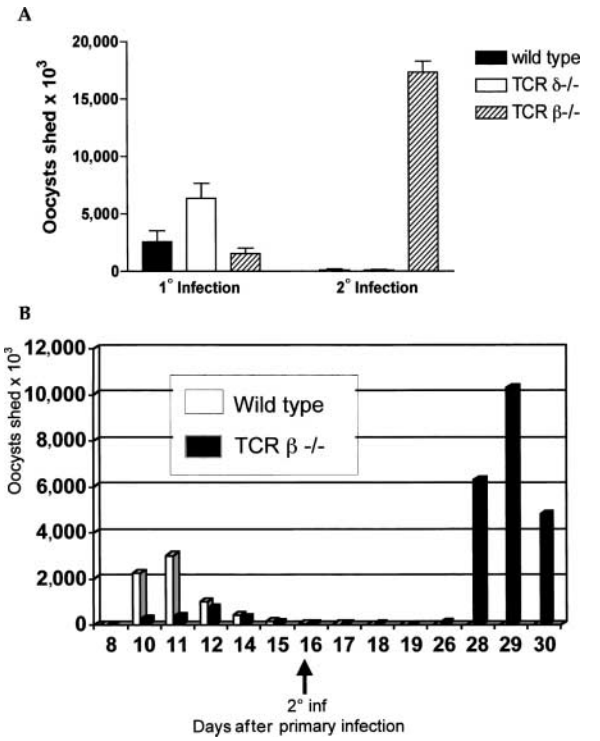

Figure 6. αβ cells are required for the acquisition of memory. (A) 30-d-old wild-type mice (shaded bars, $n = 5$), TCR$\delta^{-/-}$ mice (white bars, $n = 3$), and TCR$\beta^{-/-}$ mice (striped bars, $n = 2$) mice were infected with 1,000 *E. vermiformis* oocysts (1° infection), and rechallenged at 8 wk of age with 10,000 oocysts (2° infection). (B) 11-d-old wild-type (white bars, $n = 6$) and TCR$\beta^{-/-}$ mice (shaded bars, $n = 4$) were infected with 100 oocysts and rechallenged at 28 d of age with 1,000 oocysts. In both cases, only αβ T cell–intact mice were immune to reinfection.

challenged at 8 wk of age (2° infection). Consistent with previous results, TCR$\delta^{-/-}$ mice but not TCR$\beta^{-/-}$ mice showed enhanced susceptibility to primary infection. However, upon rechallenge, all αβ cell–sufficient mice (wild-type and TCR$\delta^{-/-}$ mice) were completely immune. In contrast, TCR$\beta^{-/-}$ mice, which were comparatively resistant to primary challenge, developed no protective immunity and were highly susceptible to rechallenge. Thus, although γδ cells are integral to the primary anti–*E. vermiformis* response in 30-d-old mice, they do not provide pathogen-specific protective immunity.

One concern regarding this result was that αβ T cells might not be acquiring memory in very young mice, but rather through exposure to antigen retained in adult mice in which αβ T cells have gained full immunocompetence. To test this, both primary and secondary infections were performed before 30 d of age (Fig. 6 B). C57.BL/6 mice and TCR$\beta^{-/-}$ mice were initially infected with 100 oocysts at 11 d of age. As in some infections of 30-d-old mice, the TCR$\beta^{-/-}$ mice were more resistant to infection than their T cell–intact counterparts. However, upon reinfection at 30 d of age, only the C57.BL/6 mice and not the TCR$\beta^{-/-}$ mice were immune to reinfection (Fig. 6 B). This result confirms that even when αβ T cells are not critical for the resolution of primary infection,

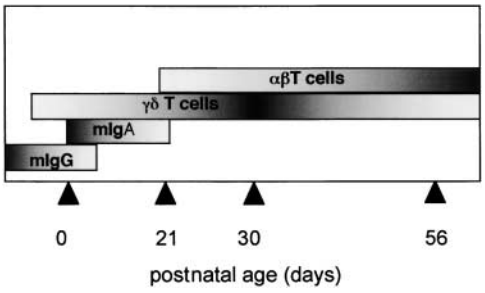

Figure 7. Development of murine immune competence proceeds in overlapping stages. The model depicts the proposed relative contributions of maternal antibody (mIgG and mIgA), γδ T cells, αβ T cells, and innate factors to immune protection among young mice as age increases. Darker shading indicates higher dependence on a particular factor. Age increases from left to right with 0, 21, 30, and 56 d of age (arrowheads). Weaning typically occurs at 21 d, and animals are considered adults by 56 d of age.

they are essential to the development of pathogen-specific immunity.

Discussion

The vertebrate immune system is a dynamic entity, changing to respond to the unique environment and sets of challenges that confront animals at each stage of life. For mammals, the period between weaning and the onset of sexual maturity is characterized by the need to discriminate between newly encountered pathogens, ongoing colonization by nonpathogenic, symbiotic organisms, and the expression de novo of self antigens specific to particular developmental stages. Some of these challenges will be particularly relevant at specific anatomical sites (e.g., the gut).

The results presented here demonstrate that when young mice were infected with a natural gut pathogen, their capacity to resolve infection increased with age, ranging from relative immunodeficiency at weaning (21 d) to full immune competence at adulthood (10 wk). This schedule of developing immunologic autonomy was perturbed in mice lacking γδ cells, which remained highly susceptible to infection at around 1 mo of age. In most experiments, mice lacking αβ T cells were not similarly compromised. Instead, it was only after ~6 wk that αβ T cell–deficient mice showed an invariable and severe immunodeficiency, whereas mice lacking γδ cells were less affected by then. Collectively, these results permit us to propose a revised "time line" for the development of primary responses to *E. vermiformis* infection (Fig. 7).

The time line depicted raises the question of whether, as mice reach adulthood, there is a developmental loss of function of γδ cells or a gain of function of αβ cells. Several results are germane to this issue. First, in *E. vermiformis*–infected adult mice, αβ and γδ cell double-deficient mice are more susceptible than TCR$\beta^{-/-}$ mice alone (39, 40). This suggests that γδ cells retain function in the adult, but that their immunoprotective role is largely redundant in the presence of a fully mature and expanded αβ T cell compartment. This conclusion is consistent with several other pa-

pers that confirm a continued role for γδ cells in adult animals, including humans. Thus, γδ cells protect adult mice against epithelial tumors (52, 53), appear effective against mycobacteria in adult humans (54), and may protect against various viruses in mice and humans alike (55, 56).

Second, many works have demonstrated the immunodeficiency de facto of neonatal αβ T cell responses in both humans and mice (10–12, 57). Our own studies have confirmed reports that cord blood αβ T cells are distinct from their adult counterparts being highly compromised in IFNγ production, even after activation (unpublished data). The results presented here would indicate that vis-a-vis a natural gut infection of the mouse, this immunodeficency persists into the postweaning period, rendering the animal more dependent on γδ cells for immune protection than is the case in the adult.

Third, the level of protection provided by γδ cell–dependent mechanisms in 1-mo-old mice is substantially lower than that provided by αβ T cell–dependent mechanisms in adults. In sum, it is as if γδ cells provide a contingency protection until αβ T cell–dependent mechanisms have fully developed. That the protection mediated by γδ cells differs qualitatively from that mediated by αβ T cells is supported by the observation that it fails to confer long-term, pathogen-specific immunity toward *E. vermiformis*.

The different schedules by which γδ cells and αβ T cells may reach immunocompetence in the gut may be affected by several factors, including microbial colonization. Thus, colonization with segmented filamentous bacteria (SFB) is rapidly followed by a marked expansion of intraepithelial T cells (58), production of IgA by lamina propria B cells (59), and formation of Peyer's patch germinal centers (60). In mice, SFB are first detectable in the small intestine at 16 d of age, peaking at 32 d of age, and rapidly declining thereafter (61). This schedule clearly overlaps the development of functional γδ cell immunocompetence as measured by the increasing resistance of normal mice to primary infection between 21 and 30 d of age (Fig. 1). Interestingly, withdrawal of maternal IgA is associated with increased colonization by SFB, and likewise, SFB colonization and expansion occurs earlier (13 d of age), peaks sooner (28 d of age), and persists longer in animals in which either the host or the mother is immunodeficient (e.g. scid/scid; references 61, 62). These observations raise the possibility that some cellular components of the gut immune system, including γδ cells, may develop precociously in TCRβ$^{-/-}$ colonies, in which there would be less maternal Ig, and thereby confer enhanced resistance to primary infections experienced by young αβ T cell–deficient mice (e.g., Fig. 6 and not depicted).

The relative resistance of young TCRβ$^{-/-}$ mice to infection demonstrates that the actions of γδ cells are independent of αβ T cells. Second, the susceptibility of IFNγ$^{-/-}$ mice during the early stages of infection is consistent with the general Th1 bias of γδ cells, and with the vulnerability of *E. vermiformis*–infected cells to IFNγ. Nonetheless, the failure of IFNγ$^{-/-}$ mice to fully phenocopy TCRδ$^{-/-}$ mice suggests that γδ cells are pleiotropic, making multiple contributions to immunoprotection, as do B and T cells in adult mice. For example, TCRγδ^{+} IELs express high levels of several other immunological effectors, including granzymes (45, 51). Although we show that perforin-deficient mice are no more susceptible to infection than are wild-type mice, perforin-independent effects of granzymes have been described previously (63). TCRγδ^{+} IELs also highly express RNA for chemokines that may have a strong influence over innate immunoprotective mechanisms. Clearly, further experiments will determine the full mechanistic basis for the immunoprotective role of γδ cells.

It will be important to determine the generality of the results presented here, and the time line that has been developed (Fig. 7). To this end, rather similar data were obtained in a single experiment by Waters and Harp for young mice infected by *C. parvum* (41), although because no adoptive transfers were used, an immunoprotective function could not be assigned unequivocally to γδ cells. It might be argued that the generality of the results will be limited to specific tissues in animals that show a reasonably high representation of γδ cells. Thus, beyond mice, the data may be relevant to intestinal infections of chickens, where coccidiosis is a major economic problem.

Alternatively, γδ cells have been proposed to be prototypes of a broader range of cells that include γδ cells and nonconventional αβ T cells (64). Were this coclassification to prove appropriate, the generality of the results presented here may extend to species in which γδ cells are less abundant, but that harbor sets of unconventional TCRαβ^{+} T cells (e.g., CD8αα TCRαβ^{+} IELs). In that case, the proposal would be that young animals progress through a developmental window during which primary responses to gut infections are dependent largely on unconventional T cells, whereas memory to rechallenge would remain dependent on conventional T cells.

The reasons why γδ cells fail to develop pathogen-specific immunity toward *E. vermiformis* remain to be elucidated. Possibly, the nature of the signaling events that follow TCRγδ engagement do not provoke memory cell differentiation. This cannot be resolved until the general nature of the ligands directly recognized by TCRγδ is elucidated. Currently, the murine MHC class IB molecule, T10/T22, is the only ligand shown biochemically to bind TCRγδ (65), and analyses are underway to determine whether such stress-induced self antigens are induced on epithelial cells by *E. vermiformis* infection (66). Likewise, any of several mechanisms may explain why αβ T cells in young mice contribute strongly to memory, but not primary effector responses. Among those mechanisms is the possibility that αβ T cells can adopt memory status without prior transit through an effector stage (67). The improved understanding of these various aspects of cell signaling can hopefully enhance the design of prophylactic and therapeutic strategies for the protection of young animals and children. At the same time, the data presented in this paper may provide some clue as to the selective advantage that

may have been conferred by the presence of γδ cells over >400 million yr of vertebrate evolution.

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