Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen

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SEVERAL **bacterial pathogens of medical importance are able to persist and replicate inside host mononuclear phagocytes. Protective immunity depends on specific T lymphocytes that induce granulomatous lesions at the sites of bacterial multiplication[1,2]. *Listeria monocytogenes* is an intracellular pathogen that replicates inside mononuclear phagocytes and hepatocytes of mice[1–4]. Invasion from the phagosomal compartment into the cytoplasmic compartment is the principal mechanism of intracellular survival[5]. Early in infection, resistance against *L. monocytogenes* is mediated by polymorphonuclear phagocytes which destroy infected liver cells, followed by natural killer cells which activate macrophages by means of interferon-γ (refs 6, 7). A specific immune response by T cells then develops which leads to sterile eradication of the microbes[1,2,8]. T cells are also responsible for the highly effective protection in vaccinated mice against secondary infections[1,2]. Although the role of $\alpha\beta$ T cells has been demonstrated in these immune responses, that of $\gamma\delta$ T cells is unclear[2,9,10]. Here we use mice that selectively lack either $\alpha\beta$ or $\gamma\delta$ T cells as a result of targeted germ-line mutations in their T-cell receptor genes[11,12] to investigate the relative roles of these T-cell populations during experimental infection with *L. monocytogenes*. We find that in primary listeriosis, either $\alpha\beta$ or $\gamma\delta$ T cells are sufficient for early protection. Resistance to secondary infection is mediated mainly by $\alpha\beta$ T cells but also involves $\gamma\delta$ T cells. Thus $\alpha\beta$ T-cell-deficient mice can be rendered partially resistant by vaccination, and $\gamma\delta$ T cells are shown to be responsible for this protective effect. In infected $\gamma\delta$ T-cell-deficient mice we noticed the appearance of unusual liver lesions, indicating that $\gamma\delta$ T cells have a unique regulatory role in this bacterial infection.**

We previously reported the construction and initial characterization of T-cell antigen receptor (TCR)-α or TCR-β mutant mice (lacking $\alpha\beta$ T cells) and TCR-δ mutants (lacking $\gamma\delta$ T cells)[11,12]. These studies suggested the presence of a normal $\alpha\beta$ T-cell repertoire in the periphery of the $\gamma\delta$ T-cell-deficient mice[12] and of apparently normal $\gamma\delta$ T cells in the periphery of the $\alpha\beta$ T-cell-deficient mice[11]. Thus, we should be able to deduce whether the roles of $\alpha\beta$ or $\gamma\delta$ T cells in protection against the intracellular pathogen *L. monocytogenes* are essential and/or sufficient by analysing the mutant mice infected by this pathogen.

TCR-α, TCR-β and TCR-δ mutants as well as heterozygous littermates were intravenously infected with *L. monocytogenes* and the dose giving 50% lethality (LD_{50}) was determined as $\leqslant 1 \times 10^4$ (data not shown). Subsequently, TCR mutants were infected with sublethal doses of *L. monocytogenes* and colony-forming units (CFU) in spleens were determined after several

TABLE 1 Primary listeriosis in TCR-β, TCR-α, TCR-δ and TCR-$\beta \times \delta$ mutant mice*

Experiment (group)	Type of mice	No. of mice	Inoculum (in $\log_{10}$)	Day of testing	CFU per spleen (in $\log_{10}$)†	Significant difference in CFU‡
1(1)	TCR-$\beta^{-/-}$	5	3.4	8	3.3 (2.7–4.6)	vs 1(2)
1(2)	TCR-$\beta^{-/-}$ +anti-TCR-δ	5	3.4	8	5.1 (4.3–6.0)	vs 1(1), vs 1(3)
1(3)	Control	5	3.4	8	3.3 (<2–4.0)	vs 1(2)
2(1)	TCR-$\alpha^{-/-}$	5	3.4	7	4.0 (3.5–4.4)	vs 2(2)
2(2)	TCR-$\alpha^{-/-}$ + anti-TCR-δ	5	3.4	7	5.4 (4.9–5.8)	vs 2(1), vs 2(3)
2(3)	Control	5	3.4	7	3.7 (3.3–4.3)	vs 2(2)
3(1)	TCR-$\delta^{-/-}$	4	3.7	2	5.0 (4.8–5.2)	NS§
3(2)	Control	4	3.7	2	5.2 (5.1–5.3)	NS
4(1)	TCR-$\delta^{-/-}$	4	3.7	9	2.8 (2.0–3.7)	NS
4(2)	Control	4	3.7	9	3.0 (2.3–3.7)	NS
5(1)	TCR-$\delta^{-/-}$	4	5.0	2	7.2 (5.4–7.5)	NS
5(2)	Control	4	5.0	2	6.6 (6.2–7.6)	NS
6(1)	TCR-$\beta^{-/-}$	5	3.4	7	2.3 (<2–3.7)	vs 6(2)
6(2)	RAG-1 $^{-/-}$	4	3.4	7	5.9 (<2–6.4)	vs 6(1), vs 6(3)
6(3)	Control	5	3.4	7	2.3 (<2–3.7)	vs 6(2)
7(1)	TCR-$\beta^{-/-}$	5	3.3	8	3.7 (3.4–3.9)	vs 7(2)
7(2)	TCR-$\beta \times \delta^{-/-}$	5	3.3	8	4.8 (3.6–5.1)	vs 7(1), vs 7(3)
7(3)	Control	5	3.3	8	3.2 (<2–3.5)	vs 7(2)
8(1)	TCR-$\beta^{-/-}$	4	3.0	20	<2 (<2–<2)	NS
8(2)	TCR-$\delta^{-/-}$	4	3.0	20	<2 (<2–<2)	NS
8(3)	Control	4	3.0	20	<2 (<2–<2)	NS
9(1)	TCR-$\beta^{-/-}$	5	3.7	21	2.5 (<2–3.2)	vs 9(3), vs 9(4)
9(2)	TCR-$\delta^{-/-}$	5	3.7	21	<2 (<2–<2)	vs 9(3), vs 9(4)
9(3)	TCR-$\beta \times \delta^{-/-}$	4	3.7	21	5.5 (5.1–6.2)	vs 9(1), vs 9(2), vs 9(5)
9(4)	RAG-1 $^{-/-}$	4	3.7	21	5.4 (5.0–6.3)	vs 9(1), vs 9(2), vs 9(5)
9(5)	Control	5	3.7	21	<2 (<2–<2)	vs 9(3), vs 9(4)

* Mice were infected with a sublethal dose of *L. monocytogenes* and on days 2–21 thereafter, colony-forming units (CFU) in spleens were determined by plating serial dilutions of organ homogenate on trypticase soy plates[3]. Some groups of mice received 0.5 mg GL3 antibody intraperitoneally 2 days before infection. All experiments were done with >10-week-old mice of (129 × C57BL/6) or (129 × BALB/c) background. In a given experiment the background, age and sex were matched. Mice were kept in isolators and fed autoclaved food and water *ad libitum*.
† Median values of a range.
‡ Mann–Whitney *U* test; $P < 0.05$ was considered to be significant.
§ NS, not significant.

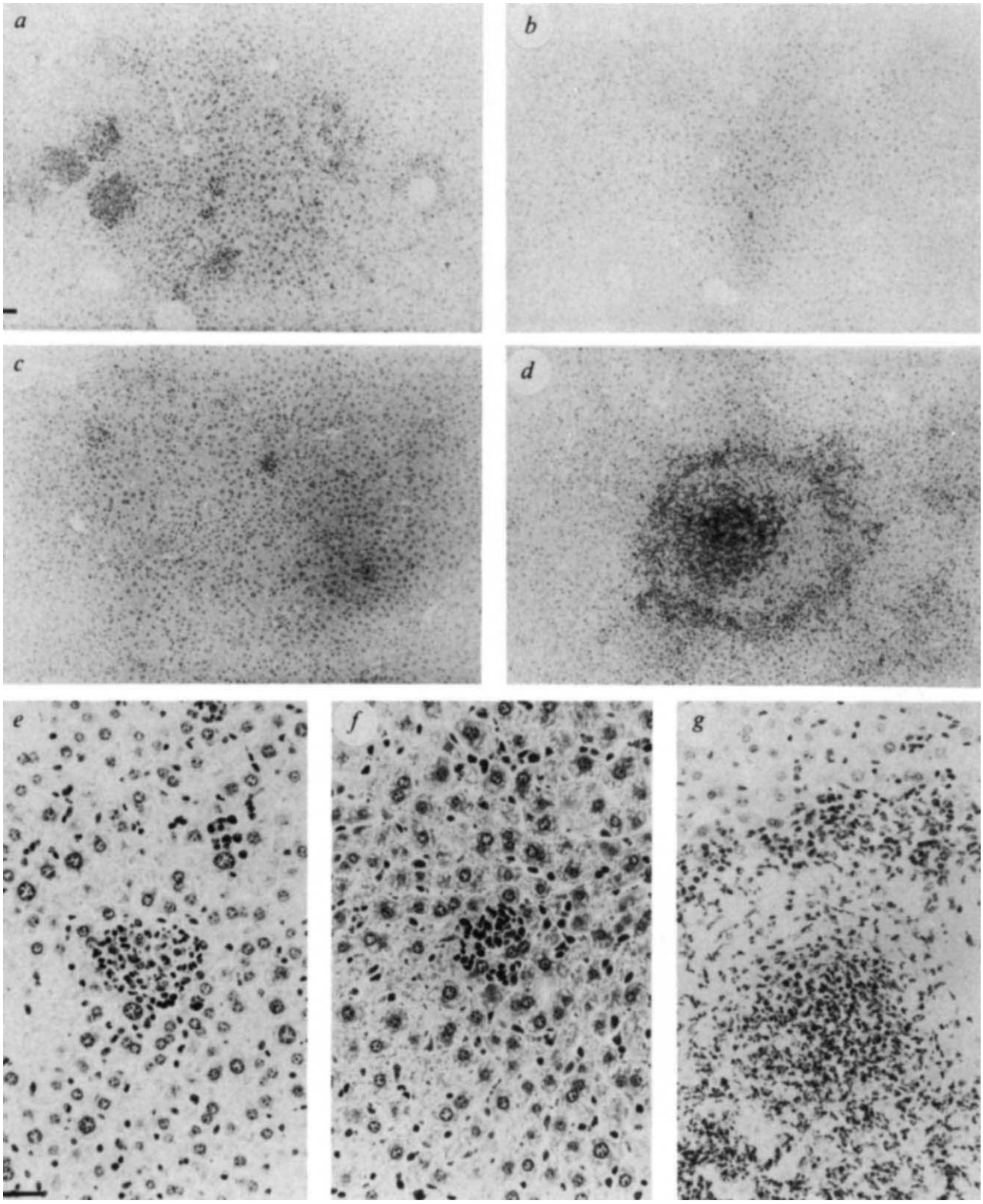

FIG. 1 Liver lesions in *L. monocytogenes*-infected TCR-β, TCR-δ, TCR-$\beta \times \delta$ mutants and control littermates. Mice were infected with *L. monocytogenes* ($\sim 5 \times 10^3$ CFU) and 7 d later their livers were removed. Sections representative of 10 to 15 animals per group are shown. *a–d*, Low magnification (100×; bar, 50 μm); *e–g*, detailed view (400×; bar, 30 μm). *a* and *e*, Liver section of control mice showing early granuloma formation. Granulomatous lesions (5 to 7 lesions per 100× fields, 30–35 lesions per section) contained macrophages associated with a few granulocytes at the centre which were surrounded by lymphocytes. In peripheral regions, double-nucleated as well as macro-nucleated hepatocytes were detectable. *b*, Lack of lesions in TCR-$\beta \times \delta$ mutants. Although there was some increase in macrophages, indicating general activation of the reticulo-endothelial system, distinct lesions were not identified. *c* and *f*, Lack of well-structured lesions in TCR-β mutants. A few small infiltrative lesions (2–3 lesions per 100× fields, that is, about 15–20 lesions per section) containing granulocytes and macrophages but lacking lymphocytes were present. *d* and *g*, Development of abscess-like lesions in TCR-δ mutants (1–2 lesions per section). Acute inflammatory processes that manifested as abscess formation and were characterized by a centre of pycnotic granulocytes surrounded by a necrotic circle dominated in lesions of all 10 mice. Demarcation to the liver parenchyma was formed by an outer circle of macrophages, granulocytes and lymphocytes. Liver tissue was fixed in formalin, embedded in paraffin, cut to 4-μm-thick sections, and stained with haematoxylin and eosin. Liver sections were evaluated for lesions per five random 100× fields.

TABLE 2 DTH responses to listerial antigens and IFN-γ production by spleen cells in TCR-β and TCR-δ mutant and control littermates

Mutant	DTH (mm^{-1})*	IFN-γ secretion (U)†		
		In vitro restimulation	Cell number per well 5×10^5	1×10^5
TCR-$\beta^{-/-}$	0	HKL	2.8	0.1
		Con A	21.5	0.2
		Nil	0.1	0.1
TCR-$\delta^{-/-}$	15	HKL	>100	>100
		Con A	>100	>100
		Nil	0.9	0.1
Control	13	HKL	>100	>100
		Con A	>100	>100
		Nil	3.3	1.0

* Mice were infected with *L. monocytogenes* and on day 8, DTH reactions were elicited by injection of 50 µl soluble listerial antigen into the right-hand footpad. After 24 h, footpad swelling was measured with a dial gauge caliper. DTH reactions represent differences between the right (injected) and left (non-injected) hind footpad. In uninfected control mice, footpad swelling was <2 mm^{-1}, excluding any influence of non-specific inflammatory response on DTH reactions. Medians of 5 mice per group.

† At day 9 after infection, spleen cells from *L. monocytogenes*-infected mice were cultured together with antigen (10^7 heat-killed listeriae (HKL) per well) or with mitogen (1 µg concanavalin A (Con A) per well) in supplemented Iscove's medium in round-bottomed microtitre plates[23]. After 48 h, supernatants were collected and IFN-γ and interleukin-4 production measured by enzyme-linked immunosorbent assay (ELISA) as described[24]. Interleukin-4 was virtually undetectable. Means are shown of at least three samples per group; s.d. <15%.

days (Table 1). All three types of mutant mice (TCR-α, TCR-β and TCR-δ mutants) were able to control early listeriosis (Table 1, experiments 1 to 7) as efficiently as heterozygous littermates used as controls. (Hereafter the latter mice are termed 'normal' littermates.) Treatment with anti-TCR-δ monoclonal antibody GL3 (ref. 13) exacerbated primary listeriosis in TCR-α and TCR-β mutants (Table 1, experiments 1 and 2), suggesting that $\gamma\delta$ T cells are involved in clearance of infection in the $\alpha\beta$ T-cell-deficient mice. Mice carrying the mutations for both TCR-β and TCR-δ lacking all T lymphocytes[11,12], or mice homozygous for a mutation in the recombination activating gene-1 (RAG-1) and lacking T and B lymphocytes altogether[14], were more sensitive to listeriosis than TCR-β mutants (Table 1, experiments 6 and 7). TCR-$\beta\times\delta$ and RAG-1 mutants were incapable of clearing infection by three weeks. In contrast, at least some TCR-β mutants could achieve sterile clearance of listeriosis (Table 1, experiments 8 and 9). In some other TCR-β mutants, the bacterial counts at day 21 were ~1,000-fold lower than those in the T-cell-deficient animals (Table 1, experiment 9). These findings confirm the protective role of $\gamma\delta$ T lymphocytes in the absence of $\alpha\beta$ T cells.

Transient exacerbation of listeriosis by anti-TCR-δ or anti-CD4 plus anti-CD8 monoclonal antibody treatment has been reported[4,15–17]. Earlier studies with mice carrying the genes for severe combined immunodeficiency (*SCID*) and *nude* provided evidence for T-cell-independent macrophage activation by natural killer cells as a major effector mechanism of early antilisterial immunity[7,18]. But both of these types of T-cell-deficient mice become chronic carriers of *L. monocytogenes* and succumb to the disease after several weeks.

Delayed-type hypersensitivity (DTH) is considered to reflect the part of protective immunity conferred by $CD4^+$ T cells against intracellular bacteria[1,2,8,19]. DTH responses against listerial antigens were equally strong in TCR-δ mutant and normal littermates, but were absent in TCR-β mutants, suggesting that DTH is exclusively dependent on $\alpha\beta$ T cells and independent of $\gamma\delta$ T cells (Table 2). Macrophage activation by interferon (IFN)-γ is thought to be central to protection against intracellular bacteria[2,20,21]. We observed increased IFN-γ production by spleen cells from TCR-δ mutants and controls after antigen and mitogen stimulation (Table 2). We assume that concanavalin A (con A) causes IFN-γ secretion in both natural killer cells and T cells, reflecting maximal IFN-γ production, whereas IFN-γ production by antigen (heat-killed listeriae) is restricted to T cells. In TCR-β mutants only marginal amounts of IFN-γ were observed after antigen stimulation. These findings show that $\alpha\beta$ T cells are the principal source of antigen-induced IFN-γ pro-

TABLE 3 Secondary listeriosis in TCR-β, TCR-α, TCR-δ and TCR-$\beta\times\delta$ mutant mice*

Experiment (group)	Type of mice	No. of mice	Vaccination: Day of inoculation	Vaccination: Dose (in $\log_{10}$)	Secondary infection (day 0): Dose (in $\log_{10}$)	Secondary infection (day 0): Day of testing	Secondary infection (day 0): CFU per spleen (in $\log_{10}$)†	Significant difference in CFU‡
1(1)	TCR-$\beta^{-/-}$	5	−14	3.3	4.5	3	3.7 (2.9–3.9)	vs 1(2)
1(2)	Control	5	−14	3.3	4.5	3	<2 (<2–<2)	vs 1(1)
1(3)	TCR-$\beta^{-/-}$	5	−14	3.3	4.5	6	2 (<2–3.5)	NS§
1(4)	Control	5	−14	3.3	4.5	6	<2 (<2–<2)	NS
2(1)	TCR-$\alpha^{-/-}$	5	−14	3.2	4.4	3	3.8 (3.6–6.1)	vs 2(2)
2(2)	Control	5	−14	3.2	4.4	3	2.8 (<2–3.7)	vs 2(1)
2(3)	TCR-$\alpha^{-/-}$	5	−14	3.2	4.4	6	3.0 (<2–5.7)	NS
2(4)	Control	5	−14	3.2	4.4	6	<2 (<2–3.9)	NS
3(1)	TCR-$\alpha^{-/-}$	5	−17	3.8	5.1	2	4.6 (3.9–5.0)	vs 3(2), vs 3(3),
3(2)	TCR-$\alpha^{-/-}$ + anti-TCR-δ	6	−17	3.8	5.1	2	5.5 (4.7–5.8)	vs 3(1), vs 3(3)
3(3)	Control	5	−17	3.8	5.1	2	<2 (<2–4.3)	vs 3(1), vs 3(2)
4(1)	TCR-$\delta^{-/-}$	5	−15	3.3	5.3	3	2.0 (<2–2.2)	NS
4(2)	Control	5	−15	3.3	5.3	3	2.0 (<2–2.0)	NS
4(3)	TCR-$\delta^{-/-}$	5	−15	3.3	5.3	8	<2 (<2–<2)	NS
4(4)	Control	5	−15	3.3	5.3	8	<2 (<2–<2)	NS
5(1)	TCR-$\beta^{-/-}$	5	−15	3.0	4.8	3	3.7 (3.6–3.9)	vs 5(2), vs 5(3), vs 5(4)
5(2)	TCR-$\beta\times\delta^{-/-}$	5	−15	3.0	4.8	3	5.1 (4.2–5.4)	vs 5(1), vs 5(4)
5(3)	RAG-$1^{-/-}$	4	−15	3.0	4.8	3	5.4 (4.3–5.5)	vs 5(1), vs 5(4)
5(4)	Control	5	−15	3.3	4.8	3	<2 (<2–<2)	vs 5(1), vs 5(2), vs 5(3)

* Mice were vaccinated with the indicated sublethal dose of *L. monocytogenes* 14 to 17 d before secondary infection (day 0). Between 5–7 d before the secondary infection, mice were treated with ampicillin (twice, subcutaneously, with 10 mg each time; ref. 23). On the indicated day after secondary infection, CFU in spleens were determined. All mice used were of (129 × C57BL/6) or (129 × BALB/c) background and were more than 10 weeks old. In a given experiment, the genetic background, age, and sex were matched. Mice were kept in isolators and fed autoclaved food and water *ad libitum*.

† Median values of a range.

‡ Mann–Whitney *U* test; $P<0.05$ was considered to be significant.

§ NS, not significant.

duction in listeriosis, with $\gamma\delta$ T cells making only a minor contribution. This may mean that antilisterial resistance mediated by $\gamma\delta$ T cells involves mechanisms other than IFN-γ secretion[22].

TCR mutants were next vaccinated with viable bacteria, treated with ampicillin to ensure complete eradication of any remaining bacteria[23], and subsequently challenged with a lethal dose of bacteria (Table 3). Control experiments had shown that all groups of mice died by day 2 at inocula of $\geqslant 5 \times 10^4$ (log 4.6) *L. monocytogenes* organisms unless they had been vaccinated (data not shown). As expected, normal littermates were fully protected by vaccination (Table 3, experiments 1 to 5). Vaccinated TCR-δ mutants were equally resistant (Table 3, experiment 4). In contrast, secondary listeriosis was markedly exacerbated in vaccinated TCR-α and TCR-β mutants (Table 3, experiments 1, 2, 3 and 5) compared with the vaccinated normal littermates or vaccinated TCR-δ mutants. Secondary listeriosis was even more pronounced, however, in TCR-$\beta \times \delta$ double mutants, RAG-1 mutants (Table 3, experiment 5) or in TCR-α mutants treated with the anti-TCR-δ antibody (Table 3, experiment 3), indicating that $\gamma\delta$ T cells do have a role in vaccine-induced immunity in the $\alpha\beta$ T-cell-deficient mice. The failure of $\gamma\delta$ T cells to compensate fully for $\alpha\beta$ T cells in this immunity could be quantitative or qualitative in nature. In the $\alpha\beta$ T-cell-deficient mice and normal littermates, few $\gamma\delta$ T cells are present (1–10%) compared with the number of $\alpha\beta$ T cells in normal littermates. The absolute numbers of T cells must influence the kinetics of infectious diseases, or perhaps the $\gamma\delta$ T cells did not develop their full protective potential after *L. monocytogenes* vaccination. The $\gamma\delta$ T cells isolated from mice after secondary infection were potent IFN-γ producers after antigen stimulation *in vitro* (C. Ladel and S.H.E.K., unpublished), indicating that further characterization of this cytokine will be necessary to determine its *in vivo* role in TCR-β mutants using either *in vivo* reconstitution with recombinant IFN-γ or *in vivo* neutralization with anti-IFN-γ antibody[20,21].

Lesions in infected normal littermates were granulomatous, although epitheloid cells did not develop (Fig. 1*a* and *e*). In contrast, in infected anti-TCR-δ-antibody-treated, TCR-β mutants (data not shown) and in infected TCR-$\beta \times \delta$ double mutants (Fig. 1*b*), liver lesions were virtually absent. Livers of infected TCR-β mutant mice not only contained two- to threefold fewer lesions, but these were also smaller than and unlike those found in infected normal littermates: they showed phagocyte infiltration only and lacked any organoid structure (Fig. 1*c* and *f*). We also noted the appearance of large atypical lesions in livers of $\gamma\delta$ T-cell-deficient mice that had been infected with *L. monocytogenes* (Fig. 1*d* and *g*). The pronounced inflammation was characterized by pronounced abscess formation, not reported previously for infections of normal mice with intracellular bacteria. These histological findings indicate that in primary listeriosis: (1) $\alpha\beta$ T cells are crucial for the development of granulomatous lesions; (2) $\gamma\delta$ T cells are not sufficient for the generation of such organoid lesions, but that (3) $\gamma\delta$ T cells regulate $\alpha\beta$ T-cell functions so that in their absence $\alpha\beta$ T cells induce numerous abscess-like lesions rather than granulomatous lesions.

In summary, our studies of primary *L. monocytogenes* infections of mice selectively deficient in $\alpha\beta$ or $\gamma\delta$, or both types of T cells, have demonstrated that either T-cell subset can confer protective immunity against early listeriosis in the absence of the other subset. In the secondary infection, the protective role of $\gamma\delta$ T cells is limited but still significant. In addition, our results indicate that $\gamma\delta$ T cells may regulate (suppress) the formation of liver lesions in listeriosis. We aim to investigate the mechanisms of this regulatory function and to determine whether this finding is generalizable to other types of immune response. □

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Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to-bone transition

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THE vertebrate skeleton is formed primarily by endochondral ossification, starting during embryogenesis when cartilage anlagens develop central regions of hypertrophic cartilage which are replaced by bony trabeculae and bone marrow[1,2]. During this process chondrocytes express a unique matrix molecule, type X collagen[3]. We report here that mice carrying a mutated collagen X transgene develop skeletal deformities including compression of hypertrophic growth plate cartilage and a decrease in newly formed bone, as well as leukocyte deficiency in bone marrow, reduction in size of thymus and spleen, and lymphopenia. The defects indicate that collagen X is required for normal skeletal morphogenesis and suggest that mutations in COL10A1 are responsible for certain human chondrodysplasias, such as spondylometaphyseal dysplasias and metaphyseal chondrodysplasias[4].

The programmed tissue substitution of endochondral ossification (EO) leads to the emergence of growth plates that provide bones with longitudinal growth potential. Within growth plates a chondrocytic differentiation gradient culminates in a zone of hypertrophy where collagen X is a unique and major product[3,5,6]. To define its regulation and function in EO, we generated transgenic mice with a dominant negative mutation in collagen X. Transgene constructs were designed assuming that homotrimeric collagen X molecules, composed of $\alpha 1$(X) subunits, assemble through interactions of carboxy-terminal domains followed by folding of the triple-helix from the carboxy to the amino end[7] (Fig. 1*c*). The constructs contained either