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Evidence that γδ T cells play a limited role in resistance to murine listeriosis

A. L. RAKHMILEVICH *Trudeau Institute, Saranac Lake, New York, USA*

SUMMARY

A role for αβ and γδ T cells in protection against primary and secondary infection with *Listeria monocytogenes* was studied. The results show that mice depleted of either γδ T cells with 3A10 monoclonal antibody (mAb), or αβ T cells with anti-CD4 plus anti-CD8 mAb, or both types of T cells, remained capable of controlling *Listeria* multiplication during the first 4 days of primary sublethal infection. Moreover, mice depleted of either or both types of T cells also remained capable of resolving primary infection, although the absence of αβ T cells, but not γδ T cells, caused resolution to be slower. Likewise, *Listeria*-immune mice depleted of either αβ or γδ T cells remained capable of resolving secondary infection with a large inoculum of *L. monocytogenes*, although depletion of αβ T cells, and to a much lesser extent γδ T cells, resulted in early exacerbation of infection. However, immune mice depleted of both types of T cells lost their ability to resist a lethal *Listeria* challenge. Taken together, the results show that whereas neither type of T cell is needed for resistance to sublethal primary listeriosis, αβ T cells may act in concert with γδ T cells in protecting mice against lethal secondary infection. In addition, the results indicate that the role of γδ T cells in anti-*Listeria* resistance is much less important than the role of αβ T cells, and can be demonstrated mainly in the absence of αβ T cells.

INTRODUCTION

Immunity to infection with *Listeria monocytogenes* is regarded as an example of T-cell-mediated immunity[1] in which $CD8^+$ T cells[2] and $CD4^+$ T cells[3] make the contribution to protection. However, this view is based on the results of adoptive immunization studies, and has been challenged by the demonstration[4] that resolution of primary and secondary listeriosis, although delayed, is not prevented by depleting mice of $CD4^+$ and $CD8^+$ T cells. Similar results were recently obtained with mice that selectively lack αβ T cells as a result of targeted germ-line mutations in their T-cell receptor (TCR)-α or TCR-β genes. These mice were also capable of resolving primary and secondary infection, although at a slower tempo than control mice.[5] Similarly, TCR-δ mutant mice (lacking γδ T cells), or mice depleted of γδ T cells with an anti-γδ T-cell monoclonal antibody (mAb),[6] remained even more capable of resolving *Listeria* infection. Although these results suggest that neither αβ T cells nor γδ T cells are needed for resolution of infection, it is also possible that either T-cell subset alone may exert the levels of protection that are sufficient for the resolution of infection. In this respect, it has been demonstrated[4] that anti-*Listeria* resistance is abrogated by treating mice with anti-Thy-1 mAb, suggesting, among other possibilities, that γδ T cells may contribute in concert with αβ T cells to provide anti-*Listeria* protection. Indeed, the involvement of both αβ and γδ T cells in resistance is indicated by the finding[5] that mutant knock-out mice lacking both αβ and γδ T cells had more *Listeria* in their organs at a given time of infection than mutant mice lacking either type of T cells. However, whether the absence of both types of T cells prevents mice from eventually resolving infection is not known.

The purpose of this study is to show that although αβ T cells may act in concert with γδ T cells in protecting immune mice against a lethal secondary challenge of *L. monocytogenes*, neither type of T cell is needed for early defence against and resolution of sublethal primary infection. In addition, it is shown that the contribution of γδ T cells to anti-*Listeria* resistance is much less than that of αβ T cells.

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Correspondence: Dr A. Rakhmilevich, Trudeau Institute, PO Box 59, Saranac Lake, NY 12983, USA.

MATERIALS AND METHODS

Mice

AB6F1 (A/Tru × C57Bl/6Tru) male and female mice between 8 and 12 weeks of age were obtained from the Trudeau Institute Animal Breeding Facility. They were free from the common murine viral pathogens according to screening tests routinely performed by the Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO).

Bacteria

Listeria monocytogenes, strain EGD, was grown in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), harvested in log phase, and aliquoted and frozen at −70°. An inoculum of bacteria was prepared for i.v. injection by thawing an aliquot and diluting it in 0·9% NaCl solution. The 50% lethal dose of mice was about 10^4 colony-forming units (CFU).

The standard i.v. inoculum for sublethal primary infection was $2-3 \times 10^3$ CFU in 0·2 ml, whereas the lethal challenge inoculum was 10^5 or 10^6 CFU. Infection was followed in the spleens and livers at progressive times by plating 10-fold serial dilutions of whole organ homogenates on trypticase soy agar and counting colonies after 24 hr incubation at 37°.

In vivo *cell depletion*

Mice were depleted of $CD4^+$ T cells and $CD8^+$ T cells, or γδ T cells by treatment with mAb directed against surface markers of these cells. Rat anti-mouse hybridomas secreting mAb against the CD4 (clone GK1.5, ATCC no. TIB 207) and CD8 (clone 2.43, ATCC no. TIB 210) T-cell determinants were obtained from the American Type Culture Collection [(ATCC), Rockville, MD]. A hamster anti-mouse 3A10 hybridoma secreting mAb against pan γδ T cells[7] was obtained from Dr S. Tonegawa, Massachusetts Institute of Technology (Cambridge, MA). Hybridoma ascites fluids were enriched for IgG by ammonium sulphate precipitation, and mAb were further purified on a DEAE column. For *in vivo* depletion, mice were injected i.p. with 250 μg each of anti-CD4 mAb and anti-CD8 mAb, or with 200 μg of anti-γδ mAb. Control mice were given 500 μg of rat IgG (Sigma Chemical Co., St Louis, MO) or 200 μg of hamster IgG (Rockland, Gilbertsville, PA). Monoclonal antibody or control IgG were injected 3 days before *Listeria* challenge, and, if stated, every 4–5 days thereafter. The extent of cell depletion was determined by flow cytometric analysis.

Flow cytometric analysis

$F(ab')_2$ fragments of mAb directed against surface antigens Thy-1, CD3 and αβ TCR (hybridomas 30.H.12, 145–2C11 and H57–597; ATCC) as well as CD4 and CD8 were prepared and conjugated to either fluorescein isothiocyanate (FITC) or biotin as described previously.[8] A hamster anti-mouse hybridoma UC7–13D5 producing mAb against γδ TCR[9] was obtained from Dr J. Bluestone, The University of Chicago (Chicago, IL). Monoclonal antibodies were purified from ascites fluid by ammonium sulphate precipitation followed by DEAE chromatography. FITC-conjugated $F(ab')_2$ goat anti-hamster IgG was purchased from Jackson ImmunoResearch Lab. (West Grove, PA). Peritoneal and spleen cells ($0·5-1 \times 10^6$) were first incubated with biotinylated $F(ab')_2$ anti-Thy-1.2 mAb followed by streptavidin–phycoerythrin (PE) (Becton Dickinson, Mountain View, CA). After this, the cells were either directly stained with FITC-conjugated $F(ab')_2$ fragments of anti-CD4 plus anti-CD8 mAb, anti-CD3 mAb, anti-αβ TCR mAb, or incubated with 10 μg/ml of whole anti-γδ mAb (UC7–13D5), followed by staining with FITC-conjugated $F(ab')_2$ goat anti-hamster IgG. It was confirmed in a preliminary experiment that 3A10 mAb or UC7–13D5 mAb, when used for indirect staining, were not cross-reactive with αβ T cells. All treatments were carried out at 4° for 40 min. Stained cells were submitted to two-colour analysis on a FACScan cytofluorometer (Becton Dickinson). Data were acquired for 7500 or 10 000 events per sample and analysed using LYSIS II software. Lymphocytes were gated according to side and forward light scatter characteristics. The results are expressed as the percentage of positively stained cells.

RESULTS

Evidence inconsistent with an essential role for αβ and γδ T cells in early defence against primary infection with *L. monocytogenes*

To study the role of αβ and γδ T cells in *Listeria* infection, it was necessary to determine first the efficacy of the anti-CD4/CD8 mAb and anti-γδ mAb (3A10) in depleting mice of αβ and γδ T cells, respectively. As the number of γδ T cells in the lymphoid organs and blood of normal mice is very low (1–2%), it was decided to take advantage of the knowledge[6] that i.p. listeriosis is characterized by a significant accumulation of γδ T cells in the peritoneal cavity. Therefore, AB6F1 mice were inoculated i.p. with 10^4 *L. monocytogenes* and were given an injection of neutral casein solution 3 days later. To deplete αβ T cells or γδ T cells, mice were injected i.p. with either anti-CD4/CD8 mAb or 3A10 mAb, respectively, 2 days after inoculation of *Listeria*. On day 6 of infection, peritoneal exudate cells were harvested and stained with mAb for flow cytometric analysis. The results in Fig. 1 show that a single injection of anti-CD4/CD8 mAb caused depletion of more than 90% of αβ T cells in the peritoneal cavity, and an injection of 3A10 mAb caused depletion of more than 95% of γδ T cells. It will be shown later that these T-cell subsets were depleted for at least 6 days after a single i.p. administration of mAb.

To determine whether αβ and γδ T cells are important in early host defence against primary *Listeria* infection, mice were injected with anti-CD4/CD8 mAb and 3A10 mAb 3 days before inoculating them i.v. with a sublethal dose of *L. monocytogenes*. Bacterial colonies in the spleens and livers were enumerated 30 min, 1, 2 and 4 days after the *Listeria* challenge. As can be seen in Fig. 2, the ability of normal mice to control *Listeria* growth in these organs was acquired between days 2 and 4 of infection. Depletion of αβ T cells, γδ T cells, or both T-cell subsets with appropriate mAb did not interfere with the ability of mice to control infection during the first 4 days.

αβ T cells and γδ T cells contribute to, but are not essential for, the ultimate resolution of primary infection with *L. monocytogenes*

As has been previously shown in this laboratory,[1] mice infected sublethally with *L. monocytogenes* generate T cells with maximal protective activity between days 4 and 6 of infection. From this time on, a significant though not critical contribution of αβ T cells to resolution of infection is seen in mice depleted of $CD4^+$ and $CD8^+$ T cells with appropriate mAb.[4,10] To determine whether γδ T cells are also involved in resolution of primary infection, mice were depleted of this T-cell subpopulation alone, αβ T cells alone, or both types of T cells throughout 10 days of infection. This was achieved by injecting mice with the appropriate mAb on days −3, 1 and 6 of infection. The results in Table 1 show that depletion of αβ T cells alone resulted in a delay before *Listeria* was completely cleared from the livers and spleens. However, depletion of γδ T cells did not affect the rate at which *Listeria* was inactivated in these organs. On the other hand, depleting both types of T cells resulted in a lower tempo of *Listeria* inactivation in the same organs compared with depleting αβ T cells alone, revealing that γδ T cells make some contribution to resolution of infection. However, *Listeria* numbers in the organs of mice depleted of both αβ and γδ T cells had decreased significantly by day 10 of

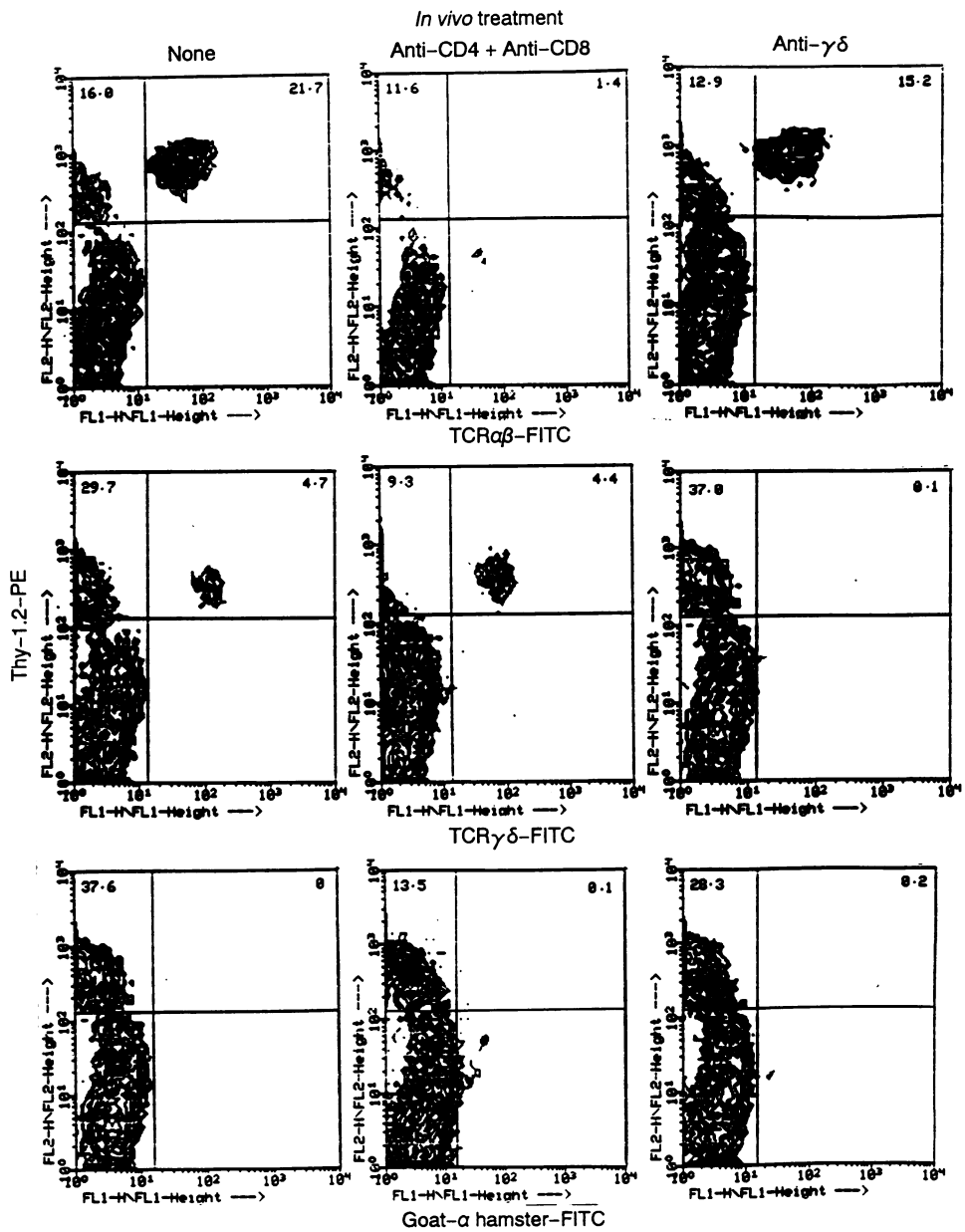

Figure 1. Two-colour flow cytometric analysis of peritoneal cells from mice with i.p. listeriosis after administration of anti-CD4/CD8 mAb and anti-$\gamma\delta$ mAb. Mice were given 1×10^4 *Listeria* i.p. and 2 days later injected i.p. with 200 μg of anti-$\gamma\delta$ mAb (3A10), 250 μg each of anti-CD4 mAb and anti-CD8 mAb, or left untreated. Mice were given 1 ml of casein i.p. 1 day later. Peritoneal cells were collected 4 days after mAb treatment, depleted of plastic-adherent cells and stained with the mAb as described in the Materials and Methods. The relative numbers of stained cells are shown. Note that anti-CD4/CD8 mAb treatment caused more than 90% depletion of $\alpha\beta$ T cells, and anti-$\gamma\delta$ mAb treatment led to depletion of more than 95% $\gamma\delta$ T cells.

infection, indicating that both types of T cells were not needed for resolution of primary sublethal infection.

$\alpha\beta$ and $\gamma\delta$ T cells are needed for resolution of lethal secondary *Listeria* infection

To determine whether $\alpha\beta$ and $\gamma\delta$ T cells have a larger role to play in host defence against secondary *Listeria* infection, mice were immunized with a sublethal dose of *L. monocytogenes*, depleted of $\alpha\beta$ T cells, $\gamma\delta$ T cells, or both types of T cells with appropriate mAb 30 days later, and challenged with a lethal dose of *Listeria* 3 days later. Bacterial colonies in the spleens and livers were enumerated 30 min, 1, 2 and 3 days after challenge. On day 3 of secondary infection, half of each spleen was used for obtaining cells for flow cytometry, and the other half for enumerating *Listeria*. The results in Fig. 3 show that more than 90% of $\alpha\beta$ T cells and 90% of $\gamma\delta$ T cells were depleted from the spleens of immune mice on day 6 after

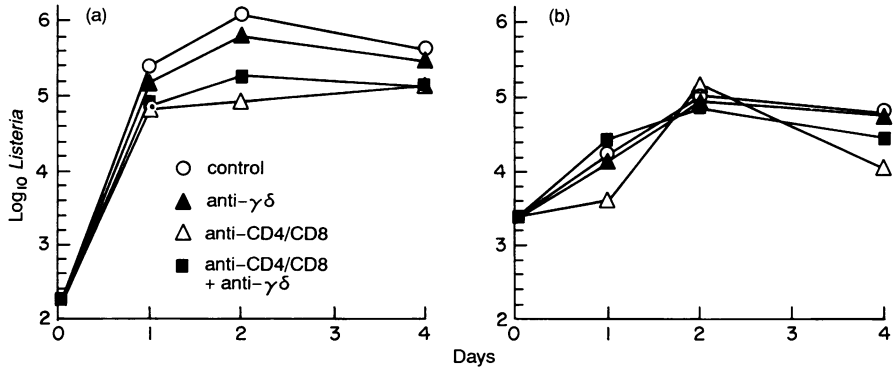

Figure 2. Evidence that αβ and γδ T cells play no role in early host defence against primary infection with *L. monocytogenes*. Mice were injected i.p. with 200 μg of anti-γδ mAb, 250 μg each of anti-CD4 mAb and anti-CD8 mAb, or left untreated. Three days later, mice were infused i.v. with 3×10^3 *Listeria*, and the numbers of colonies in the spleens (a) and livers (b) were determined 30 min, 1, 2 and 4 days later. Means of five mice per group. Standard deviations in this and the experiment in Fig. 4 did not exceed 15% and are not shown for the sake of clarity.

treatment with anti-CD4/CD8 mAb and 3A10 mAb, respectively. Administration of mAb against both types of T cells resulted in depletion of more than 95% of each. It can be seen in Fig. 4 that depleting immune mice of αβ T cells alone resulted in the significant exacerbation of infection in the spleen and liver before resolution began. In contrast, depleting immune mice of γδ T cells only marginally affected *Listeria* growth, resulting in a small but temporary increase of exacerbation of infection. On the other hand, immune mice depleted of both types of T cells were less capable than immune mice depleted of either type of T cells at controlling infection. That is, these mice had significantly more bacteria in the organs than mice depleted of either type of T cells. The results presented in the Table 2 support the finding that both αβ and γδ T cells are needed for combating lethal secondary infection by showing that 40% of mice depleted of both types of T cells died by day 7 of secondary infection, and that the mice that survived had more than 4 logs of *Listeria* in their spleens and 7 logs in their livers on day 9. At the same time, mice depleted of either type of T-cell subset cleared the infection by day 9. Thus, the results indicate that γδ T cells aid αβ T cells in protecting mice against lethal secondary infection.

DISCUSSION

The results of this study show that depleting αβ T cells or γδ T cells alone, or depleting both types of T cells, failed to prevent AB6 mice from resolving a sublethal primary infection with *L. monocytogenes*. The results also show that neither type of T cell is essential for immunized mice to control a lethal secondary infection. However, depleting mice of both types of T cells removed their capacity to combat a lethal secondary infection,

Table 1. Non-essential role of αβ and γδ T cells in resolution of primary sublethal infection with *Listeria monocytogenes**

Organ	Treatment of mice†	Log_{10} *Listeria*/organ‡ Day 3	Day 6	Day 10
Spleen	None	5·76 ± 0·39	4·02 ± 0·43	< 2
	Anti-γδ	6·20 ± 0·42	4·55 ± 0·32	2·53 ± 0·50
	Anti-CD4/CD8	5·13 ± 0·31	5·49 ± 0·28§	4·99 ± 0·30§
	Anti-γδ + anti-CD4/CD8	5·21 ± 0·34	5·77 ± 0·13§	5·37 ± 0·14§¶
Liver	None	5·34 ± 0·25	4·47 ± 0·77	< 2
	Anti-γδ	5·76 ± 0·62	5·31 ± 1·42	3·38 ± 2·42
	Anti-CD4/CD8	4·69 ± 0·43	3·99 ± 0·72	2·35 ± 0·50
	Anti-γδ + anti-CD4/CD8	5·05 ± 0·30	5·65 ± 0·75	4·77 ± 1·62§

* AB6F1 mice were given i.v. 3×10^3 *Listeria monocytogenes*, and the numbers of bacterial colonies were estimated in the spleens and livers 3, 6 and 10 days later.

† Antibodies were injected i.p. on days −3, 1 and 6 relative to the injection of *Listeria*.

‡ Mean ± SD of five mice per group.

§ Significant exacerbation of infection to compare with the non-treated control.

¶ Significant difference to compare with the groups of mice treated with either anti-γδ mAb, or anti-CD4/CD8 mAb.

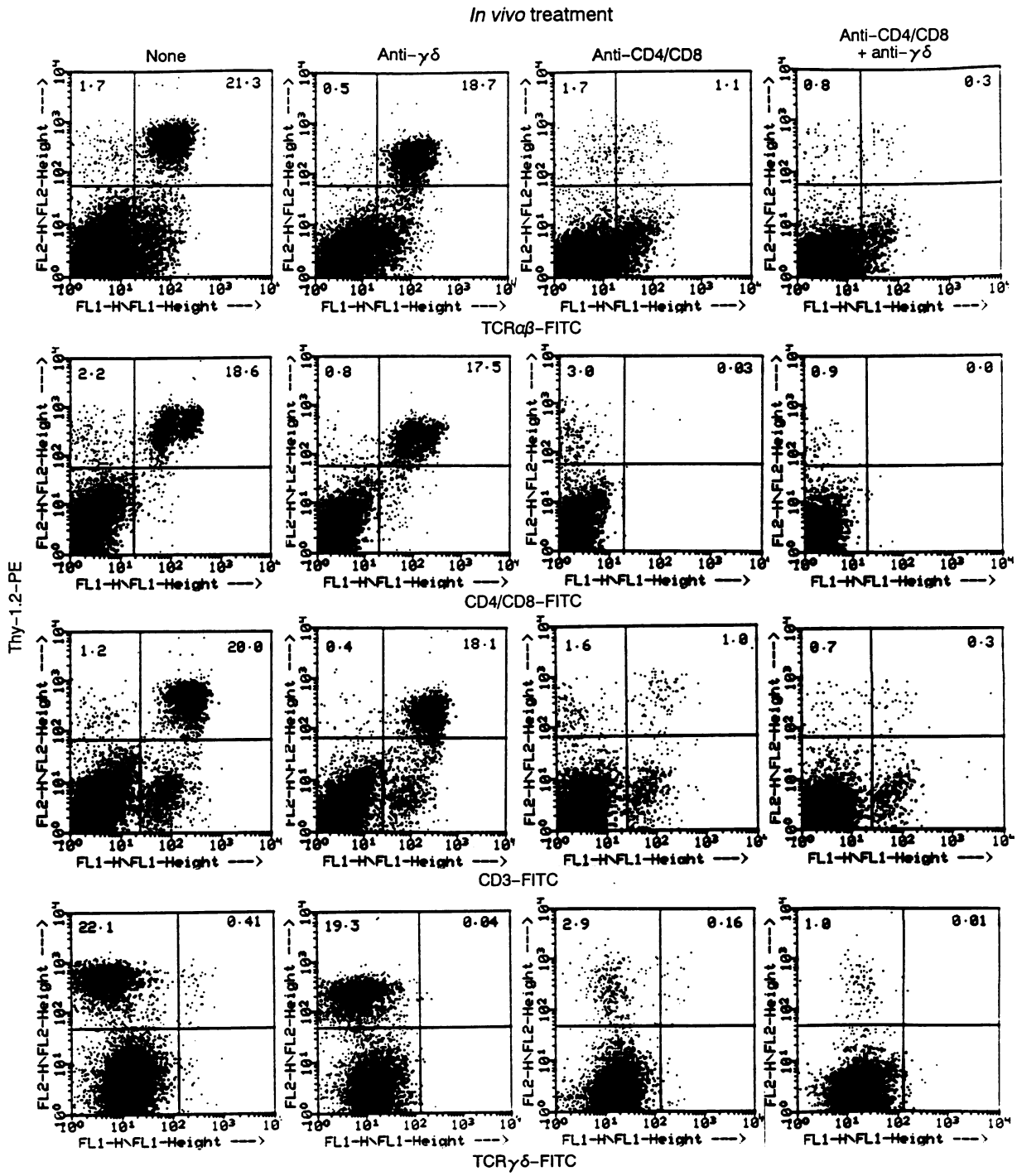

Figure 3. Flow cytometric analysis of spleen cells from secondary-infected *Listeria*-immune mice 6 days after mAb treatment. Mice were immunized with a sublethal dose of *L. monocytogenes* and 30 days later were injected i.p. with 200 μg of anti-γδ mAb or 250 μg each of anti-CD4 mAb and anti-CD8 mAb. Mice were given a lethal challenge of *Listeria* 3 days later. Spleen cells were harvested on day 3 of secondary infection, pooled from three mice per group, stained with the mAb and analysed using flow cytometry. The relative numbers of stained cells are shown.

although it is almost certain that a smaller secondary challenge would have been resisted. Therefore, the results indicate that γδ T cells play a minor role in anti-*Listeria* resistance, to the extent that it is necessary to give mice a large inoculum of the organism and to deplete their αβ T cells, to show that γδ T cells contribute to resistance.

The results of published studies of others who analysed the contribution of αβ T cells to host defence against listeriosis

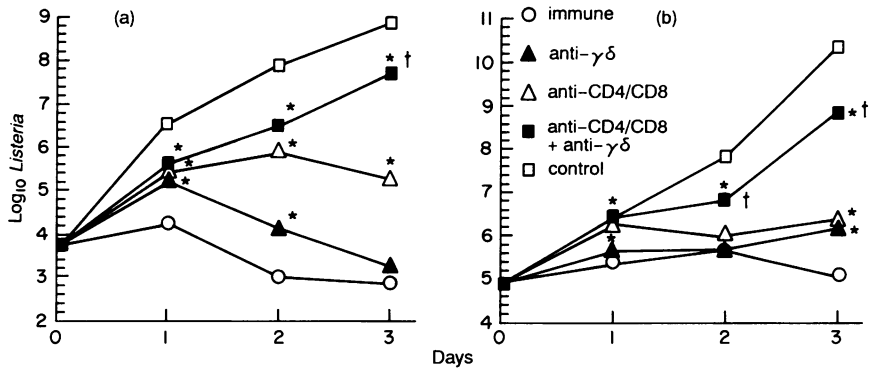

Figure 4. αβ T cells together with γδ T cells are needed for host defence against secondary *Listeria* infection. Mice were immunized, treated with mAb and challenged with a lethal dose of *Listeria* as described in the legend to Fig. 3. Numbers of bacterial colonies in the spleens (a) and livers (b) were determined 30 min, 1, 2 and 3 days after the challenge. * Significant difference between the groups of control immune mice and mice treated with mAb. † Significant difference between the groups of immune mice treated with mAb against either type of T cells and mice treated with anti-CD4/CD8 mAb plus anti-γδ mAb. Means of five mice per group.

essentially agree with the findings presented here, in that they show that although depleting αβ T cells causes exacerbation of infection,[4,6,10,11] it does not prevent mice from ultimately controlling and resolving infection.[4,10] According to the study presented here, γδ T cells have an even smaller role to play than αβ T cells in anti-*Listeria* defence. This interpretation, however, is not in keeping with published findings of others[5,6,11] showing that γδ T cells contribute as much as αβ T cells to resistance to primary infection. On the other hand, the results of this study are in agreement with those of Mombaerts *et al.*,[5] showing that

Table 2. Both αβ and γδ T cells are important for resolution of lethal secondary infection with *Listeria monocytogenes**

Organ	Treatment of immune mice†	Log_{10} *Listeria*/organ‡		
		Day 1	Day 3	Day 9
Spleen	None	4·48 ± 0·17	5·05 ± 0·26	< 2
	Anti-γδ	4·65 ± 0·35	3·14 ± 0·19	< 2
	Anti-CD4/CD8	5·42 ± 0·19	5·70 ± 0·67	< 2
	Anti-CD4/CD8 + anti-γδ	6·14 ± 0·25§	6·77 ± 0·46§	4·15 ± 0·62§¶
	Anti-CD4/CD8 + ham IgG	ND††	5·53 ± 0·47	2·39 ± 0·66
	Anti-γδ + rat IgG	ND	2·55 ± 0·38	< 2
	Ham IgG + rat IgG	4·70 ± 0·31	ND	ND
	Non-immune control	6·68 ± 0·50	8·39 ± 0·26	Death**
Liver	None	5·16 ± 0·25	5·45 ± 1·03	< 2
	Anti-γδ	5·37 ± 0·34	4·79 ± 0·98	< 2
	Anti-CD4/CD8	6·15 ± 0·17	5·69 ± 0·37	2·84 ± 0·47
	Anti-CD4/CD8 + anti-γδ	6·42 ± 0·15§	7·05 ± 0·66§	7·40 ± 0·76§¶
	Anti-CD4/CD8 + ham IgG	ND	5·53 ± 0·57	3·44 ± 0·96
	Anti-γδ + rat IgG	ND	5·26 ± 0·31	< 2
	Ham IgG + rat IgG	4·87 ± 0·18	ND	ND
	Non-immune control	6·30 ± 0·16	10·1 ± 0·19	Death**

* AB6F1 mice were immunized with 2×10^3 *Listeria monocytogenes* and challenged 30 days later with the lethal dose of *Listeria*. The numbers of bacterial colonies were estimated in the spleens and livers on days 1, 3 and 9 after the challenge.

† Monoclonal antibodies were injected i.p. on days −3, 1 and 6 relative to the secondary *Listeria* challenge.

‡ Mean ± SD of five mice per group.

§ Significant exacerbation of infection to compare with all other groups of immune mice.

¶ Two mice from five died by day 7.

** All five mice died by day 5.

†† ND, not determined.

in order to reveal a role for γδ T cells in resistance to murine listeriosis it was necessary to remove the contribution of αβ T cells. Similar findings were made by those who studied the role of γδ T cells in resistance to murine malaria.[12] The results of the studies with αβ T-cell-deficient mice,[5,12] or β_2 microglobulin-deficient mice,[13] suggest that γδ T cells mediate compensatory antibacterial immunity in such immunodeficient mice. In contrast, the results presented here indicate that γδ T cells function in immunocompetent mice rather than compensate for the loss of αβ T cells. Thus, the contribution of γδ T cells to host defence against *Listeria* could be noted either in the presence of αβ T cells, or within a few days of mAb-induced depletion of αβ T cells.

The findings of this study that T-cell depletion did not alter the ability of mice to control the early stages of infection confirm the data of others[14] who described the T-cell-independent mechanism of early resistance to listeriosis. In contrast to this evidence, the original belief that γδ T cells are important in defence against *L. monocytogenes* was based on results showing that these T cells appear earlier than αβ T cells in the peritoneal cavity of mice infected i.p. with *Listeria*,[15] and that treatment of i.p.-infected mice with anti-γδ mAb results in an early enhancement of *Listeria* growth.[6] However, attempts by others to reproduce these results were unsuccessful.[11] It is worth mentioning, moreover, that the appearance of increased numbers of γδ T cells at sites of infection, for example with influenza[16] and leishmania,[17] do not represent cause-and-effect evidence that these T cells are involved in immunity. The present study also failed to show a significant role for γδ T cells in early defence against i.v.-administered *L. monocytogenes* (Fig. 2), as well as against intestinal primary listeriosis (data not shown). Instead, the findings presented indicate that if γδ T cells are involved, they contribute at a later stage of the host response when infection is in the process of being resolved. Their role, however, appears to be less important than that of αβ T cells which themselves are not essential for the control of infection.

The limited effect of depleting mice of αβ and γδ T cells on resistance to listeriosis contrasts with the devastating effect of depleting them of Thy-1$^+$ CD4$^-$ CD8$^-$ cells.[4] It is apparent, therefore, that Thy-1$^+$ cells that are not T cells are needed for resistance to primary and secondary listeriosis. Even though 40% of immune mice depleted of αβ T cells plus γδ T cells died on day 7 of a lethal *Listeria* infection (Table 2), the results of a previous study showed that treatment of immune mice with anti-Thy-1.2 mAb resulted in death of all mice by day 4.[4] It is almost certain that Thy-1$^+$ NK cells are important for resistance to primary and, to a lesser extent, secondary *Listeria* infection. Indeed, it has been shown[18] that treatment of mice with anti-NK1.1 mAb or anti-asialoGM1 antibody causes a marked exacerbation of infection. In addition to NK cells, it has been demonstrated[19–21] that neutrophils play an essential role in anti-*Listeria* resistance. When it is considered that depleting NK cells or neutrophils can have a lethal exacerbating effect on primary *Listeria* infection, whereas depleting mice of αβ or γδ T cells does not prevent them from eventually resolving infection, it is suggested that anti-*Listeria* immunity perhaps should not be considered a clear-cut model of T-cell-mediated immunity. The latter view originally was based on the results of adoptive immunization experiments.[1] However, it was recently shown[22] that results obtained with adoptive immunization should be treated with caution, in that they may not be reflective of events causing control of infection in donor mice at the time that T cells are harvested from these mice for passive transfer.

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