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Differential γδ T Cell Response Controlled by Antigen Presenting Cells in *Toxoplasma gondii*-infected Mice

KUNIO SANO AND AKIHIKO YANO

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Abstract

We examined the potential role of γδ T lymphocytes in the defense against infection by *Toxoplasma gondii* (*T. gondii*). Intraperitoneal infection of BALB/c mice with *T. gondii* induced an increase in the number of γδ T cells both in the spleen and the peritoneal cavity, while the number of splenic αβ T cells remained unchanged. The increase of splenic γδ T cells was inhibited by injection with an antibody against I-A or mycobacterial heat shock protein65. In sharp contrast to BALB/c mice, B6 mice failed to mount a γδ T cell proliferation in response to *T. gondii* infection. The inability of γδ T cells from B6 mice to proliferate was ascribed to the impaired function of antigen presenting cells (APC) in these mice, since immunization of (BALB/c × B6) F_1 mice with *T. gondii*-infected lipopolysaccharide (LPS) blasts of BALB/c mice induced a strong increase in the γδ T cell subpopulation, while γδ T cells from the same F_1 strain did not proliferate in response to *T. gondii*-infected LPS blasts of B6 mice. These results suggest that Ia^+ APC play a pivotal role in the induction of γδ T cells upon infection with *T. gondii* by presenting a possible ligand recognized by γδ T cells. Since mice with H-2^d, but not H-2^b, were reported to be resistant to *T. gondii* infection (Williams *et al.*, 1978; Suzuki *et al.*, 1991; McLeod *et al.*, 1989; McLeod *et al.*, 1989; Brown *et al.*, 1990), the induction of γδ T cells in response to *T. gondii* in BALB/c mice, but not in B6 mice, implies a protective role of γδ T cells in *T. gondii* infection.

Key words: γδ T cells, *Toxoplasma gondii*, antigen presenting cells, heat shock protein

Introduction

Toxoplasma gondii (*T. gondii*) infection has recently achieved prominence as a severe opportunistic disease occurring under immune compromised conditions, such as in AIDS (Murray, 1991). In immunocompetent individuals, both humoral (Mineo *et al.*, 1993) and cell-mediated immunities (Suzuki and Remington, 1988; Gazzinelli *et al.*, 1991; Suzuki *et al.*, 1993) have been documented as constituting major protective mechanisms against this protozoa. The direct killing effector function of $CD8^+$ class I-restricted cytotoxic T lymphocytes against *T. gondii*-infected targets has been reported as playing an important role in defense against *T. gondii* infection by us and others (Yano *et al.*, 1989; Subauste *et al.*, 1991; Hakim *et al.*, 1991). $CD8^+$ T cells have also been shown to secrete interferon-γ and thereby potentiate resistance to *T. gondii* infection (Suzuki and Remington, 1990). These T cells manifest their functions following antigen recognition by surface T cell receptors (TCR) composed of α and β chains.

T cells bearing TCRγδ chains constitute a distinct T cell subpopulation, which comprises less than 5% of T cells in the thymus and peripheral lymphoid organs of mouse (Bank *et al.*, 1986; Brenner *et al.*, 1986). γδ T cells show a characteristic distribution as major T cell populations in epithelial tissues in the skin, lung, reproductive tract, and

Abbreviations used in this paper
APC, antigen presenting cells
FCM, flow cytometry
hsp65, myobacterial heat shock protein 65
LPS, lipopolysaccharide
mAb, monoclonal antibody
Mlg, mouse Immunoglobulin
MMC, mitomycin C
PEC, Peritoneal exudate cells
TCR, T cell receptors
T. gondii, *Toxoplasma gondii*

Department of Medical Zoology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan.
佐野公仁夫　矢野明彦（長崎大学医学部医動物学教室）

intestine (Tonegawa *et al.*, 1989), implying their role in host defense against certain bacterial and viral infections (Haas *et al.*, 1993). Although an increase in γδ T cell numbers was reported in *T. gondii*-infected patients (Scalise *et al.*, 1992; De-Paoli *et al.*, 1992), the significance of this γδ T cell proliferation in *T. gondii* infection remains unclear.

In this study, we demonstrated a potential role of γδ T cells in host-defense against *T. gondii* in mice. We found that *T. gondii* infection induced an increase in the number of γδ T cells in both the spleen and in the peritoneal cavity. The ability to mount a γδ T cell response correlated well with the ability to resist to *T. gondii* infection, implying an important role of γδ T cells at an early stage of *T. gondii* infection.

Materials and Methods

Animals

BALB/c, C57BL/6 (B6), and (BALB/c × B6) F_1 mice were bred in our animal facility, and were used at 7 to 12 wk of age. Mice were injected with 5×10^5 live *T. gondii* tachyzoites (RH strain) or 2×10^6 *T. gondii*-infected LPS-activated B cell blasts prepared as described below. They were sacrificed 4 days after the injection unless otherwise indicated. Each experiment consisted of three to seven mice.

Preparation of T. gondii *tachyzoites*

T. gondii tachyzoites amplified in B cell lymphoma (ARH) cells were recovered in a supernatant fraction following centrifugation at 30g for 5 min to eliminate infected cells and cell debris.

Immunization of mice with Toxoplasma-*infected LPS-activated B cell blasts*

BALB/c or B6 splenic B cells adherent to dishes (Corning, Corning, NY) coated with goat anti-mouse Immunoglobulin (Mlg) (Caltag, South San Francisco, CA) were activated by 10 μg/ml LPS (Difco, Detroit, MI) for 2 days, and then cultured with *T. gondii* (RH strain) *in vitro* for 1 day. 2×10^6 *T. gondii*-infected LPS-activated B cell blasts were injected i.p. into (BALB/c × B6) F_1 mice following treatment with 100 μg/ml mitomycin C (MMC) (Kyowa Hakko Kogyo Co., Tokyo, Japan) for 2h at 37°C.

Preparation of peritoneal cells

Peritoneal exudate cells (PEC) were recovered by lavage of the peritoneal cavity with phosphate-buffered saline containing 2% fetal calf serum, and were plated in wells of 6-well tissue culture plates (Sumitomo Bakelite Co., Tokyo, Japan). After incubation for 2h at 37°C, nonadherent cells were collected and were used as mononuclear cells for flow cytometry (FCM) analysis.

Treatment of Toxoplasma-*infected mice with mAbs*

BALB/c mice infected with 5×10^5 live *T. gondii* tachyzoites i.p. were subsequently injected i.p. with 100 μl of ascites containing monoclonal antibody (mAb) against either I-A^d (MKD-6) (Kappler *et al.*, 1981), mycobacterial heat shock protein 65 (hsp65) (ML30) (Ivanyi *et al.*, 1983), or anti-TCRαβ (H57-597) mAb (Kubo *et al.*, 1989). After 4 days, PEC were analyzed using FCM.

FCM analysis

Anti-CD3ε (145-2C11) (Leo *et al.*, 1987) and anti-TCRγδ (3A10) (Itohara *et al.*, 1989) mAbs were conjugated to fluorescein isothiocyanate (FITC) (Sigma, St Louis, MO) and biotin (Sigma), respectively, as described previously (Sano *et al.*, 1990). Spleen cells or PEC were incubated with FITC-anti-CD3 and biotin-anti-TCRγδ mAbs, followed by R-phyco-erythrin-conjugated streptavidin (Biomeda, Foster City, AC). The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA). 50,000 viable cells were collected with logarithmic intensity scale, and cells positive for both CD3 and TCRγδ were defined as γδ T cells. Propidium iodide (PI) (Sigma) was used to exclude dead cells from analysis.

Results

Kinetics of responses of αβ- and γδ-bearing T cells to T. gondii *infection*

We examined the effects of *T. gondii* infection on T cell subpopulations in the spleen and the peritoneal cavity at different time points after i.p. infection with *T. gondii* tachyzoites. Spleen cells and peritoneal exudate cells nonadherent to plastic plates were stained with anti-CD3 and anti-TCRγδ mAbs, and were analyzed by FCM. Two representative stain-

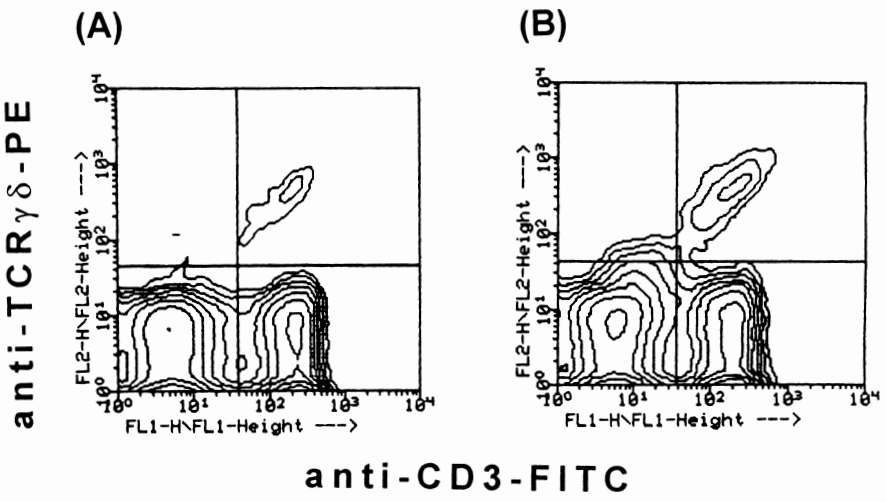

Fig. 1 Representative staining profiles of γδ T cells with anti-CD3ε and anti-TCR γδ mAbs. Spleen cells of mice either 2 days (A) or 6 days (B) after i.p. infection with 5×10^5 live *T. gondii* tachyzoites (RH strain) were stained with anti-CD3ε and anti-TCRγδ mAbs, and were analyzed by FCM. Note that γδ T cells which comprised less than 1% of spleen cells (0.37% in A, 0.77% in B) could be defined as a clearly distinct subpopulation on the diagonal.

ing profiles are shown in Fig. 1. After removal of PI-stained dead cells, viable TCRγδ$^+$CD3$^+$ lymphocytes could be detected as a clearly defined subpopulation on the diagonal. γδ T cells comprised as few as 0.37% of total cells in the spleen of mice 2 days after infection with *T. gondii*, and could easily be detected as a discernible subpopulation on a contour plot (Fig. 1A). γδ T cells accounted for 0.77% of total cells in the spleen of mice 6 days after infection and were observed as a proportionally bigger contour (Fig. 1B). Thus, staining of lymphocytes with a combination of anti-CD3 and anti-TCR γδ mAbs allowed a reliable quantitative analysis of γδ T cells.

Kinetics showed that γδ T cells comprised 0.6% of splenocytes of uninfected normal BALB/c mice (Fig. 2A). Following the initial small dip on day 2 after i.p. injection with *T. gondii*, the proportion of specific γδ T cells increased on day 4 and day 6 up to 0.92%. The number of γδ T cells in the spleen of *T. gondii*-infected mice did not increase by day 2, but reached a maximum on day 6, when the number of γδ T cells was more than twice that of uninfected normal mice (Fig. 2B). In sharp contrast to the responsive induction of γδ T cells, the proportion of αβ T cells declined from 41.5% in normal mice to 27.7% in mice on day 4 after infection (Fig. 2C). The absolute number of αβ T cells remained unchanged since there was an increase in the number of total spleen cells at all time points examined (Fig. 2D), arguing against any contributory effect of αβ T cells to host resistance in the early phase of infection.

Among nonadherent PEC, the proportion of γδ T cells did not significantly increase for the first 4 days of infection. However, on day 6, the γδ T cells comprised 4.8% of PEC, which constituted a greater than three-fold increase over normal BALB/c mice (Fig. 3A). The absolute number of γδ T cells started increasing on day 2, and reached a maximum on day 4, when the number of γδ T cells was more than 25-fold higher than that of normal BALB/c mice (Fig. 3B). Thus, infection of mice with *T. gondii* caused a strong induction of γδ T cells at an early stage of infection prior to any discernible αβ T cell response.

Inhibition of γδ T cell induction by mAbs

In an attempt to explore the mechanisms by which *T. gondii* infection induced the amplification of the γδ T subset, we treated mice with three distinct mAbs at the time of i.p. infection of *T. gondii* to assess their effects on the γδ T cell induction. In the

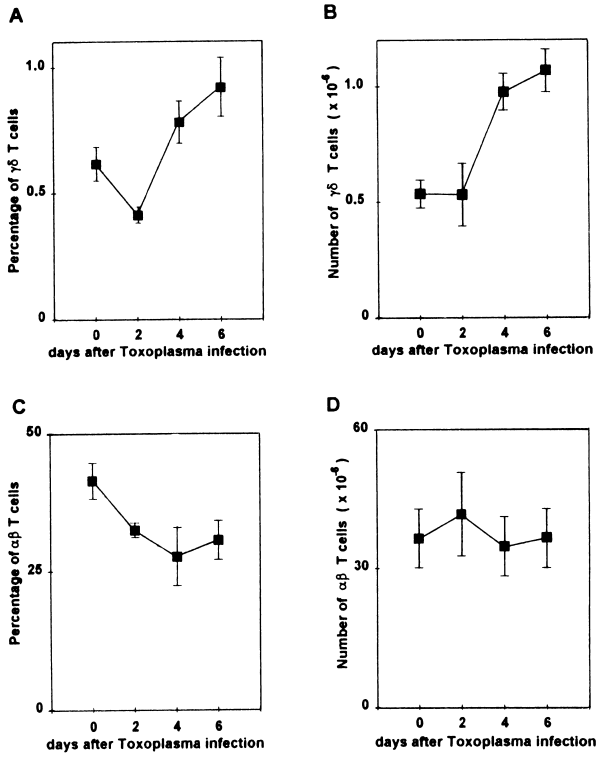

Fig. 2 Kinetics of responses of γδ and aβ T cells in the spleen to *T. gondii* infection.
BALB/c mice were infected i.p. with 5×10^5 live *T. gondii* tachyzoites. On day 2, 4, or 6 after the infection, spleen cells were stained with anti-CD3e and anti-TCRγδ mAbs, and were analyzed with FCM. Cells positive for both CD3 and TCRγδ and $CD3^+TCR\gamma\delta^-$ cells were scored as γδ T cells and αβ T cells, respectively. The proportion of γδ T cells (A) and aβ T cells (C), the total number of γδ T cells (B) and aβ T cells (D) in the spleen at the indicated time points are shown.

T. gondii-infected mice which received no mAbs, the number of γδ T cells in PEC was 140 thousand (Fig. 4). The injection of a mAb against mycobacterial hsp65 reduced γδ T cell numbers to 88 thousand. A more profound decrease in the γδ T cell subset (47 thousand) was observed upon the injection of an mAb against $I\text{-}A^d$. These results suggest possible involvement of presentation of hsp65 by $I\text{-}A^+$ antigen presenting cells (APC) in the stimulatory process of γδ T cells. Treatment with an anti-TCRαβ mAb did not inhibit the induction of γδ T cells, indicating that participation of αβ T cells is not required for the induction of γδ T cells.

Correlation of the ability to induce γδ T cells with the resistance to T. gondii *infection*

Host response against *T. gondii* infection has been found to be genetically controlled (Williams *et al.*, 1989). The resistance against infection with *T. gondii* was mapped to H-2D and/or L subregion(s), since mice with the $H\text{-}2^d$ (e.g. BALB/c) or $H\text{-}2^a$ haplotype were resistant to cyst numbers, encephalitis, and mortality following the infection of *T. gondii*, while mice with $H\text{-}2^b$ (e.g. B6) were susceptible (Suzuki *et al.*, 1991; McLeod *et al.*, 1989; McLeod *et al.*, 1989; Brown *et al.*, 1990). We examined whether there is a correlation between the

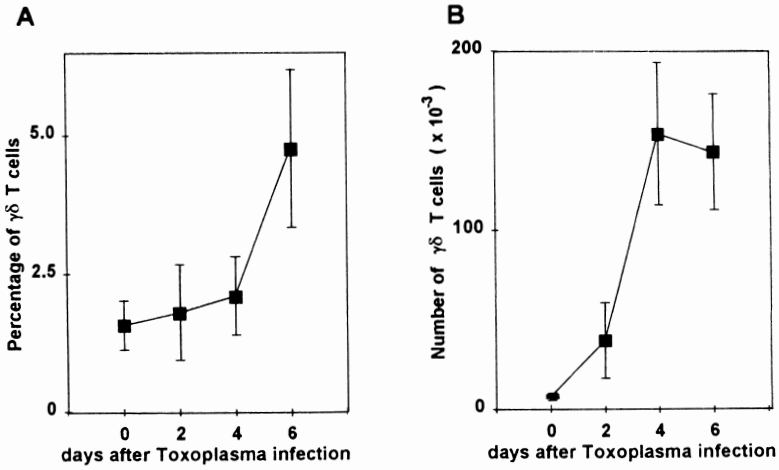

Fig. 3 Kinetics of responses of γδ T cells in the peritoneal cavity to *T. gondii* infection. BALB/c mice were infected i.p. with 5×10^5 live *T. gondii* tachyzoites. On day 2, 4, or 6 after the infection, PEC nonadherent to a plastic plate were stained with anti-CD3ε and anti-TCRγδ mAbs, and were analyzed with FCM. Cells positive for both CD3 and TCRγδ were counted as γδ T cells. The proportion (A) and the total number (B) of γδ T cells in the PEC at the indicated time points are shown.

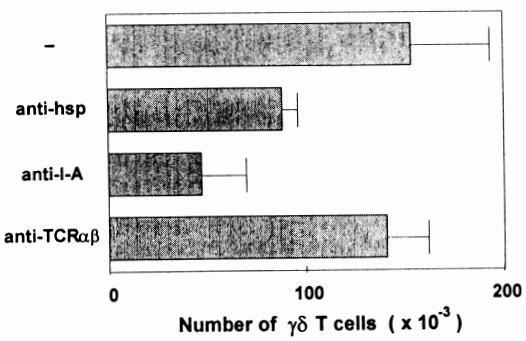

Fig. 4 Inhibition of *T. gondii*-induced γδ T cell stimulation by anti-I-A or anti-hsp mAb.
BALB/c mice were treated i.p. with 100 μl of ascites containing an mAb against either I-A, mycobacterial hsp, or TCRγδ following i.p. infection with 5×10^5 live *T. gondii* tachyzoites. 4 days after the infection, PEC nonadherent to a plastic plate were stained with anti-CD3 and anti-TCRγδ, and were analyzed with FCM. The total numbers of γδ T cells were calculated and are shown in the figure.

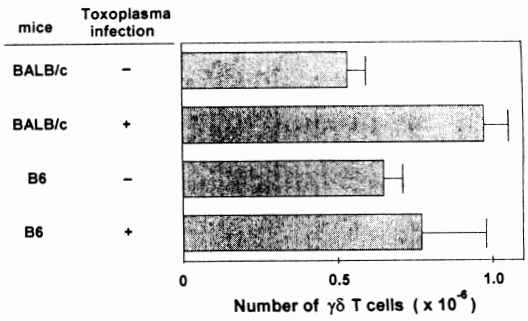

Fig. 5 Induction of a γδ T cell response in resistant (BALB/c), but not susceptible (B6), mice by *T. gondii* infection. BALB/c and B6 mice were infected i.p. with 5×10^5 live *T. gondii* tachyzoites. On day 4, spleen cells were stained with anti-CD3 and anti-TCRγδ mAbs, and were analyzed with FCM. The total numbers of γδ T cells in the spleen are shown. Uninfected mice were used as negative controls.

inducibility of γδ T cells by *T. gondii* infection and the resistance to this pathogen. BALB/c and B6 mice were injected i.p. with 5×10^5 *T. gondii* tachyzoites, and the induction of splenic γδ T cells was assessed on day 4 by FCM. Uninfected normal BALB/c mice, which are a resistant strain, were found to have 530 thousand γδ T cells in spleen (Fig. 5). The spleen cells from BALB/c mice infected with *T. gondii* for 4 days contained 980 thousand,

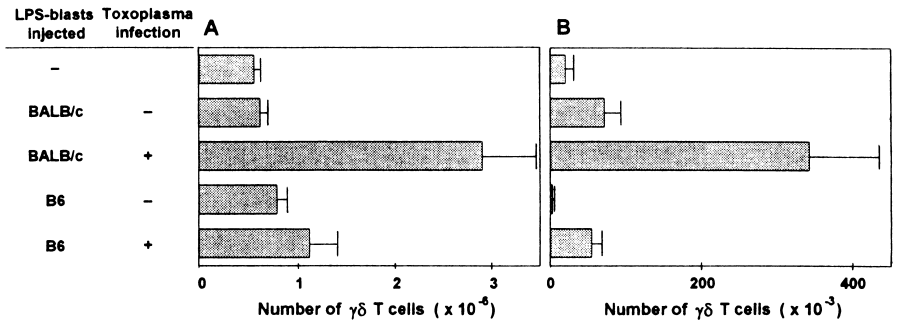

Fig. 6 APC determine the strain difference in the γδ T cell inducibility. BALB/c or B6 splenic B cells adherent to anti-Mlg-coated dishes were activated by LPS for 2 days, and then infected with *T. gondii in vitro* for one day. 2×10^6 *T. gondii*-infected LPS-activated B cell blasts were injected i.p. into (BALB/c × B6) F_1 mice following the treatment with MMC. Untreated F_1 mice and F_1 mice which were immunized with uninfected LPS blasts of either parental strain were used as controls. 4 days after the immunization, spleen cells or PEC were stained with anti-CD3 and anti-TCRγδ mAbs, and were analyzed with FCM. The calculated total number of γδ T cells in the spleen (A) or in the peritoneal cavity (B) is shown.

almost double that of normal mice. Uninfected B6, which are susceptible to *T. gondii* infection, possessed 650 thousand γδ T cells in the spleen, while infection of B6 did not result in a significant increase in the number of splenic γδ T cells. Thus, the resistant strain, BALB/c, responded to *T. gondii* infection by inducing γδ T cells, whereas the susceptible strain, B6, failed to mount a significant early response among the γδ T cell subpopulation.

Pivotal role by APC in determining the inducibility of γδ T cells

Since Ia^+ APC appear to play an important role in the induction of γδ T cells, we used LPS-activated B cell blasts as a source of APC to further address the issue of the strain difference in the γδ T cells response. (BALB/c × B6) F_1 mice were immunized with MMC-treated LPS-activated B cell blasts of either parental strain which were infected with *T. gondii. in vitro*, and the responsive induction of γδ T cells in the spleen and the peritoneal cavity was examined. The average number of γδ T cells in the spleen of normal F_1 animals was 560 thousand (Fig. 6A), and this number was not increased by immunization with MMC-treated LPS-activated B cell blasts of BALB/c. Strong induction of γδ T cells was observed when F_1 mice were immunized with *in vitro T. gondii*-infected BALB/c B cells blasts, with the number of γδ T cells reaching levels of 4 times that of the control. In sharp contrast, the same F_1 mice showed little γδ T cell induction in response to immunization with *T. gondii*-infected LPS-activated B cell blasts of the other parent, B6. The degree of γδ T cell response to *T. gondii*-presenting B6 LPS blasts was almost equal to that induced by control uninfected B6 LPS blasts. Similar results were obtained for PEC. The most striking induction of γδ T cells was observed by the immunization of F_1 mice with *T. gondii*-infected blasts of BALB/c (344 thousand), while B6 *T. gondii*-infected blasts failed to enhance the number of γδ T cells (56 thousand) (Fig. 6B). These results verified the previous conclusion that the resistance of mice to *T. gondii* infection correlates well with the ability of mice to induce γδ T cells in response to *T. gondii* infection. In addition, these results indicate that the ability of γδ T cells to respond against infection by *T. gondii* is primarily determined by APC which present the possible ligand recognized by γδ T cells.

Discussion

In this paper, we addressed the issue of a possible protective role of γδ T cells in *T. gondii* infection. We observed that upon infection with *T. gondii*, γδ T cells are induced both in the spleen and in the

peritoneal cavity during the early stage of infection, when accumulation of αβ T cells is not yet observed (Fig. 2 and 3). The prompt response among the γδ T cell subset in the absence of an amplification of the αβ T cell subset implies that recruited γδ T cells, but not αβ T cells, can function as one of the vital defense members in the early phase of infection. Mechanisms of host defense by γδ T cells have been suggested similar to those by αβ T cells, including cytotoxic elimination (Davodeau *et al.*, 1993; Bukowski *et al.*, 1994) and production of a variety of cytokines, such as IFN-γ, IL-2, and TNF-α (Morita *et al.*, 1991; Hiromatsu *et al.*, 1992). An increase in the number of γδ T cells are known to significantly regulate an immune response (McMenamin *et al.*, 1994).

To identify potential molecules involved in the γδ T cell induction, we tested *in vivo* effects of mAbs on the γδ T cell response, and found that both I-A^+ APC and hsp65 are required for adequate induction of γδ T cells. γδ T cells which recognize hsp65 have been reported in other murine experimental systems (O'Brien *et al.*, 1989; Born *et al.*, 1990; Pajasekar *et al.*, 1990; O'Brien *et al.*, 1992; Hiromatsu *et al.*, 1992; Emoto *et al.*, 1992, Hisaeda *et al.*, 1993). Since the anti-I-A^d mAb also inhibited the γδ T cell response, it is conceivable that the induced γδ T cells recognize hsp65 as a ligand presenting on I-A^+ APC. In this process, direct recognition of I-A molecules on APC by γδ T cells does not seem to be an obligatory step (O'Brien *et al.*, 1989; Matis *et al.*, 1989; Janis *et al.*, 1989).

An indispensable function of I-A^+ APC in the γδ T cell activation was also verified in experiments in Figure 6. Based on the observed correlation between the resistance to *T. gondii* and γδ T cell inducibility (Fig. 5), we immunized (BALB/c × B6) F_1 mice with *T. gondii*-infected APC from either parental strain. We found that the immunization of F_1 mice with *T. gondii*-infected APC of the resistant strain, BALB/c, but not the susceptible strain, B6, elicited a strong γδ T cell response both in the spleen and in the peritoneal cavity in the F_1 mice, suggesting that the nature of APC determines the response of γδ T cells to infection.

The lack of an appropriate repertoire of γδ T cells could be another reason for failure of susceptible mice to mount a γδ T cell response. The repertoire of αβ T cells is adaptively determined through positive and negative selection during development in the thymus (Teh *et al.*, 1988; Kappler *et al.*, 1987). γδ T cells using the Vγ4 gene have been reported to undergo both negative and positive selection as in the case of αβ T cells (Bonneville *et al.*, 1990; Dent *et al.*, 1990; Wells *et al.*, 1991; Pereira *et al.*, 1992). There is, however, no evidence for selection of a restricted repertoire of γδ T cells during development as is the case for αβ T cells, the repertoire of which is determined under the influence of MHC molecules in the thymus (Bevan, 1977; Zinkernagel *et al.*, 1978). The involvement of class I or class II MHC molecules as restriction elements for γδ T cells seems unlikely because γδ T cell responses could not be inhibited by antibodies against the classical MHC proteins (Haas *et al.*, 1993). Therefore, it is most likely that the difference in the γδ T cell response between resistant and susceptible strains results from a difference in the capacity of APC to present ligands, including hsp. It should be noted that the immunization with *in vitro* infected LPS-activated B cell blasts as APC could trigger far stronger response in γδ T cells (Fig. 6) than a direct infection with *T. gondii* in which few pre-activated B cells were available as APC at the time of infection (Fig. 5). Thus, the way in which APC present the putative ligand to γδ T cells exerts a considerable influence on the degree of γδ T cell response, arguing for a critical role of the APC in γδ T cell induction and the expression of resistance.

It has been reported that resistance to *T. gondii* infection is regulated by a minimum of five genes, including the H-2 complex (Williams *et al.*, 1989). Resistance can be ascribed in part to the appropriate stimulation of cytotoxic T lymphocytes against *T. gondii*-infected targets (Yano *et al.*, 1989; Subauste *et al.*, 1991; Hakim *et al.*, 1991). Here we describe the possibility that induction of γδ T cells can also contribute to the reinforcement of resistance to this pathogen, particularly at an early stage of infection.

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