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J Immunol 2000; 165:931-940; ;
doi: 10.4049/jimmunol.165.2.931
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Expression of Toll-Like Receptor 2 on $\gamma\delta$ T Cells Bearing Invariant V γ 6/V δ 1 Induced by *Escherichia coli* Infection in Mice¹

Yasuji Mokuno,^{*†} Tetsuya Matsuguchi,^{2*} Manabu Takano,^{*†} Hitoshi Nishimura,^{*} Junji Washizu,^{*†} Tomohiko Ogawa,[‡] Osamu Takeuchi,[§] Shizuo Akira,[§] Yuji Nimura,[†] and Yasunobu Yoshikai^{*}

We recently reported that the number of $\gamma\delta$ T cells was increased after infection with *Escherichia coli* in C3H/HeN mice. We here showed that an i.p. injection with native lipid A derived from *E. coli* induced an increase of $\gamma\delta$ T cells in the peritoneal cavity of LPS-responsive C3H/HeN mice and, albeit to a lesser degree, also in LPS-hyporesponsive C3H/HeJ mice. The purified $\gamma\delta$ T cells from C3H/HeN and C3H/HeJ mice expressed a canonical TCR repertoire encoded by V γ 6-J γ 1/V δ 1-D δ 2-J δ 2 gene segments and proliferated in response to the native lipid A derived from *E. coli* in a TCR-independent manner. The lipid A-reactive $\gamma\delta$ T cells bearing canonical V γ 6/V δ 1 expressed Toll-like receptor (TLR) 2 mRNA, while TLR4 mRNA was undetectable. Treatment with a TLR2 anti-sense oligonucleotide resulted in hyporesponsiveness of the $\gamma\delta$ T cells to the native lipid A. TLR2-deficient mice showed an impaired increase of the $\gamma\delta$ T cells following injection of native lipid A. These results suggest that TLR2 is involved in the activation of canonical V γ 6/V δ 1 T cells by native *E. coli* lipid A. *The Journal of Immunology*, 2000, 165: 931–940.

Most microorganisms are detected and destroyed within hours by innate immunity that preexists and is not Ag specific. Cells of innate immunity such as macrophages discriminate between self and nonself by receptors that identify molecules synthesized exclusively by microbes. LPS, a characteristic component found on the outer membrane of Gram-negative bacteria (1), is one of the most ideal targets for innate immunity. LPS typically consists of the polysaccharide region covalently bound to the lipid region, termed lipid A. Lipid A is generally regarded as a target for the LPS receptor and consequently as the bioactive center of LPS (2–6). CD14 Ag has been widely recognized as an LPS signaling receptor for immune cells (3, 7), and, until now, several classes of molecules on leukocytes have been recognized as receptors for LPS, such as CD11/CD18 integrins (3, 8–11), P-selectin (12), and L-selectin (13). However, CD14 lacks a transmembrane domain (3, 7), and cytoplasmic domains of CD11/CD18 integrins do not appear to be necessary for signaling translocation of NF- κ B in response to LPS binding (9). Thus, these receptors may function to transfer LPS to a second receptor that transduces the signal.

Several lines of evidence suggest that the Toll-like receptor (TLR)³ family is the cell-surface receptor for LPS, the prototypical activator of NF- κ B and other proinflammatory responses (14–17). Toll was first identified as a protein controlling dorsoventral pattern formation in the early development of *Drosophila* and was shown to participate in anti-microbial immune responses (18, 19). Recently, several mammalian Toll homologues have been identified (18–22). One of the human Toll homologues, TLR2, has been shown to be involved in LPS signaling (14–16). In mice, there is evidence for a missense mutation in the cytoplasmic domain of TLR4 in C3H/HeJ mice exhibiting impaired ability to respond to LPS (23, 24), strongly suggesting that TLR4 is the dominant receptor for at least some types of LPS. This was confirmed by experiments using TLR4 gene-knockout mice (25). More recently, it has been suggested that TLR2 functions not only as an LPS signal transducer (14, 16, 26) but also as a receptor for bacterial lipoproteins from *Mycobacteria* or Gram-positive bacteria (27–29).

Based on the type of TCR they express, T lymphocytes can be divided into two major groups, $\alpha\beta$ and $\gamma\delta$ T cells. $\gamma\delta$ T cells are further divided into subsets, based on their expression of certain γ - and δ -chains and their prevalence in certain tissues. Most of these subsets bear, as do $\alpha\beta$ T cells, junctionally diverse TCRs, but two $\gamma\delta$ T cell subsets in the mouse bear invariant TCRs. These include the V γ 5/V δ 1 subset in skin and the V γ 6/V δ 1 subset that comprises most of the $\gamma\delta$ T cells in the female reproductive tract (30–32). Under normal circumstances, these two subsets bear truly invariant TCRs, even at the nucleotide level in the TCR gene junction. These canonical sequences are very simple, with no apparent N-region contribution. Such characteristics have led to the hypothesis that $\gamma\delta$ T cells represent a more primitive, early line of cellular defense, preprogrammed to recognize a limited set of Ags.

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Received for publication December 27, 1999. Accepted for publication May 3, 2000.

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¹ This work was supported in part by a grant from Ministry of Education, Science, and Culture of Japan (JSPS-RFTF97L00703), as well as the Uehara Foundation, Ono Foundation, Aichi Cancer Foundation, Rinsyo Yakuri Foundation, and Yakult-Research Foundation.

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³ Abbreviations used in this paper: TLR, Toll-like receptor; PEC, peritoneal exudate cells; A-ODN, anti-sense oligonucleotide; S-ODN, sense oligonucleotide; *lps*^d, mutations of the gene *lps*.

We and others (33–35) previously reported that i.p. infection of mice with *E. coli* induced a marked increase in $\gamma\delta$ T cells in the peritoneal cavity (33, 34, 36) and that the $\gamma\delta$ T cells had a protective role against the infection (37). In the present study, we focused on the responsiveness of $\gamma\delta$ T cells to native lipid A. Our results demonstrated that the purified $\gamma\delta$ T cells in both C3H/HeN and C3H/HeJ mice responded to native lipid A from not only *E. coli* but also *Porphyromonas gingivalis* in a TCR-independent manner. The LPS/lipid A-reactive $\gamma\delta$ T cells, which used a canonical TCR repertoire encoded by V γ 6-J γ 1/V δ 1-D δ 2-J δ 2 gene segments, strongly expressed TLR2 mRNA. A TLR2 antisense oligonucleotide significantly inhibited the proliferation of $\gamma\delta$ T cells in response to the native lipid A. TLR2-deficient mice showed an impaired increase of the $\gamma\delta$ T cells following the injection of native lipid A. These results suggest that the canonical V γ 6/V δ 1 $\gamma\delta$ T cells respond to natural products from *E. coli* via TLR2. The implications of these findings for the mechanisms whereby a significant fraction of $\gamma\delta$ T cells are activated during *E. coli* infection are discussed.

Materials and Methods

Animals and microorganisms

C3H/HeN and C3H/HeJ mice were purchased from Japan SLC (Shizuoka, Japan). These mice were bred in our institute under specific pathogen-free conditions. Eight- to 10-wk-old female mice were used for the experiments. The mutant mouse (F₂ interbred from 129/Ola \times C57BL/6) strain deficient in TLR2 was generated by gene targeting, as described previously (29). Age- and sex-matched groups of TLR2-deficient (TLR2^{-/-}) mice and their littermate (TLR2^{+/+}) mice were used for the experiments. *E. coli* (no. 26; American Type Culture Collection, Manassas, VA) grown in a brain-heart infusion broth (Difco Laboratories, Detroit, MI) was washed repeatedly, resuspended in PBS, and stored at -70°C in small aliquots until use. The concentration of bacteria was quantitated by plate counts.

Abs and reagents

Biotin-conjugated anti-CD3 ϵ mAb, FITC-conjugated anti-TCR $\alpha\beta$ mAb, PE-conjugated anti-TCR $\gamma\delta$ mAb, purified rat anti-mouse CD11a mAb, PE-conjugated anti-mouse CD11b mAb, biotin-conjugated anti-mouse CD11c mAb, purified rat anti-mouse CD14 mAb, and PE-conjugated anti-rat IgG mAb were purchased from PharMingen (San Diego, CA). Red-613-conjugated streptavidin was purchased from Life Technologies (Gaithersburg, MD). Murine anti-TCR $\gamma\delta$ (UC7-13D) mAb was obtained by growing hybridoma cells in serum-free medium (medium 101; Nissui Pharmaceutical, Tokyo, Japan) and collecting the supernatant. The Ab was then concentrated and purified by 50% ammonium sulfate precipitation. The purity of the preparation was confirmed by SDS-PAGE, and the concentration of Ab was determined by the Lowry method. The mAbs, diluted to 1 mg/ml in PBS, were stored at -70°C until use. LPS (*E. coli*, O26/B6) and lipid A from *E. coli* (F585 Rd mutant) were obtained from Sigma (St. Louis, MO). Lipid A from *Salmonella minnesota* (R595 Rd mutant) was obtained from List Biological Laboratories (Campbell, CA). Lipid A from *P. gingivalis* was prepared as described (6). An *E. coli*-type synthetic lipid A analogue with low toxicity (ONO-4007) was kindly provided by Ono Chemical (Osaka, Japan) (38, 39). LPS was dissolved in pyrogen-free water at the concentration of 1 mg/ml. Lipid A was dissolved at a concentration of 2 mg/ml in 0.1% (v/v) triethylamine aqueous solution. The solution was appropriately diluted with pyrogen-free PBS or culture medium before use for assay.

Cell line

Cell lines were grown as adherent monolayers in tissue culture dishes at 37°C in 5% CO₂ with 95% air and passaged twice a week to maintain logarithmic growth. The J774A.1 cell line was obtained from the American Type Culture Collection. The cells growing as monolayers in tissue culture dishes were detached from the surface and washed twice with HBSS before experiments.

Preparation of PEC $\gamma\delta$ T cells

Mice were i.p. inoculated with *E. coli* at a dose of 1×10^8 CFU/mouse (one-fifth the 50% lethal dose), LPS, or lipid A in 1.0 ml PBS on day 0. Peritoneal exudate cells (PEC) were harvested on day 3 after inoculation by

centrifugation at $110 \times g$ for 5 min, washed twice, and resuspended at optimal concentrations in RPMI 1640 medium (Life Technologies) supplemented with 10% serum. Smear specimens for differential counts were stained with Giemsa solution. PEC were spread on plastic plates and incubated for 1 h in a CO₂ incubator at 37°C to obtain nonadherent cells.

Sorting of $\gamma\delta$ T cells

The $\gamma\delta$ T cells were purified by cell sorting using a FACSVantage (Becton Dickinson, San Jose, CA) electric cell sorter from the plastic nonadherent cells on day 3 after *E. coli* or lipid A injection. The purity of sorted cells was >99% (data not shown).

Flow cytometry analysis

For three-color analysis, plastic-nonadherent cells of PEC were incubated with saturating amounts of biotin-conjugated and purified Abs for 30 min at 4°C. Cells were washed twice and incubated with FITC-, PE-, and Red-613-conjugated secondary Abs for 30 min. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). The cells were carefully gated by forward and side light scattering for live lymphocytes. The data were analyzed with FACSCalibur research software (Becton Dickinson).

V gene segment usage analysis

Total RNA was extracted by the acid-guanidium-phenol-chloroform method from $\gamma\delta$ T cells purified by cell sorting. cDNA synthesis and PCR were performed using a cDNA cycle kit (Invitrogen, San Diego, CA). RNA was primed either with 20 pmol of γ -chain C region (C γ) primers (5'-CTTATGGAGGATTGTTCACG-3') or 6.7 pmol of δ -chain J region (J δ) primers (5'-TTGGTCCACAGTCACTTGG-3') in 20- μ l reaction mixtures for reverse transcription. The PCR was performed on a PCR thermal cycler (Takara, Tokyo, Japan). PCR cycles were run for 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C. Before the first cycle, a denaturation step for 7 min at 94°C was included, and after 35 cycles the extension was prolonged for 4 min at 72°C. The 5' V primers are as follows: V γ 1/2, 5'-ACACAGCTATACATTGGTAC-3'; V γ 2, 5'-CGGCAAAAAACAATCAACAG-3'; V γ 4, 5'-TGTCTTGCACCCCTACCC-3'; V γ 5, 5'-TGTGCACTGGTACCAACTGA-3'; V γ 6, 5'-GGAATTCAAAAGAAAACATGTCT-3'; V γ 7, 5'-AAGCTAGAGGGGTCCTCTGC-3'; V δ 1, 5'-ATTCAGAAGGCAACAATGAAAG-3'; V δ 2, 5'-AGTTCCTGCAGATCAAGC-3'; V δ 3, 5'-TTCCTGGCTATTGCCTCTGAC-3'; V δ 4, 5'-CCGCTTCTCTGTGAACCTCC-3'; V δ 5, 5'-CAGATCCTTCCAGTTCATCC-3'; V δ 6, 5'-TCAAGTCCATCAGCCTTGTG-3'; V δ 7, 5'-CGCAGAGCTGCAGTGTAAC-3'; V δ 8, 5'-AAGGAAGATGGACGATTAC-3'.

PCR products (4 μ l) were subjected to electrophoresis on a 1.5% agarose gel (Life Technologies) and transferred to a Gene Screen Plus filter (New England Nuclear, Boston, MA). The Southern blots of γ and δ PCR products were hybridized with MNG6 cDNA containing the C γ 2 gene, J δ 1 probe (oligonucleotide; 5'-TTGGTCCACAGTCACTTGG-3'), or J δ 2 probe (oligonucleotide; 5'-CTCCACAAAGAGCTCTATGCCCA-3'). The C γ 2 probe was labeled with [α -³²P]dCTP using a Megaprime DNA labeling system (Amersham International, Amersham, U.K.) according to the manufacturer's instructions. The J δ 1 and J δ 2 probes were labeled with [γ -³²P]ATP using a Megalabel 5' labeling kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. Before hybridization, the filters were incubated in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 50 μ g/ml heat-denatured salmon sperm DNA for 18 h at 60°C, and then the filters were washed in 2 \times SSC, 1% SDS for 15 min at 60°C. The radioactivity of each band of PCR product was analyzed with a Fujix BAS2000 Bio-image analyzer (Fuji, Tokyo, Japan). For nucleotide sequencing, RT-PCR products were resolved in low-melting-point agarose gels, isolated, and cloned into the TA vector PCR II (Invitrogen). Purified dsDNAs were sequenced by using a Taq Dye primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT) and an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Expression of TLR genes

C3H/HeN mice were killed 3 days after i.p. inoculation with lipid A. Extraction of total RNA from sorted $\gamma\delta$ T cells or $\alpha\beta$ T cells in PEC, $\gamma\delta$ T cells in liver, or J774A.1 (as a positive control) and cDNA synthesis were performed as described above. Serial dilutions of total RNA were primed with 20 pmol of random primer (Life Technologies) in 20- μ l reaction mixture for reverse transcription. Synthesized cDNAs were amplified by PCR with primers derived from the murine cDNA. The specific primers were as follows: TLR2 sense, 5'-GGAGCGGCGGCTGCAGGACTC-3'; TLR2 antisense, 5'-CCAAAGAGCTCGTAGCATCC-3'; TLR4 sense, 5'-AGTGGGTCAAGGAACAGAAGCA-3'; TLR4 antisense, 5'-CTTTACCA GCTCATTCTCACC-3' (26).

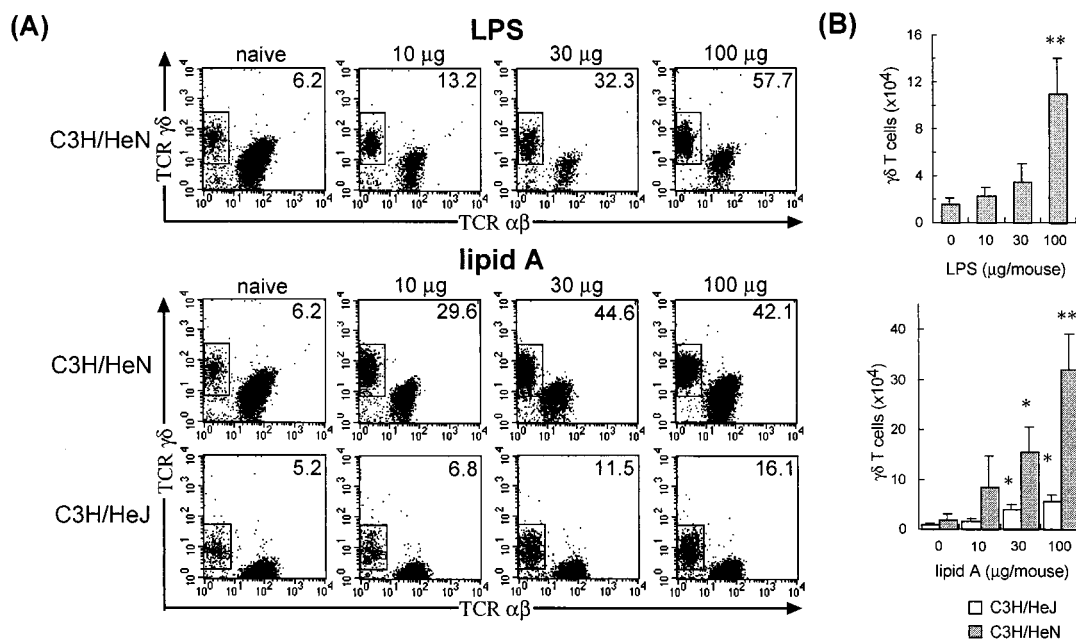


FIGURE 1. Increase in peritoneal $\gamma\delta$ T cells after injection of the LPS or native lipid A derived from *E. coli*. C3H/HeN and C3H/HeJ mice were inoculated i.p. with the LPS or lipid A on day 0. **A**, Nonplastic-adherent PEC from injected mice on day 3 were stained with anti-TCR $\gamma\delta$, anti-TCR $\alpha\beta$ mAb, and anti-CD3 ϵ mAb and analyzed with FACSCalibur. Analysis gate was set on lymphocytes using forward and side scatter gating and on CD3 $^+$. The number in each panel indicates the percentage of $\gamma\delta$ T cells in the whole lymphocyte population. **B**, Absolute numbers of $\gamma\delta$ T cells in the peritoneal cavity after LPS or lipid A injection. The numbers were calculated by multiplying the percentage of $\gamma\delta$ T cells by the total number of peritoneal nonadherent cells. The results are representative of those from three independent experiments. Values are the means \pm SD of five mice for each group. *, $p < 0.05$ vs the control group. **, $p < 0.01$ vs the control group.

Proliferation assay

Tissue culture 96-well plates were incubated overnight at 4°C with 100 μ g/ml anti-TCR $\gamma\delta$ mAb. The plates were then washed thoroughly and incubated for 1 h at 37°C with RPMI 1640 medium containing 10% FCS. The sorted $\gamma\delta$ T cells (1×10^5 /well) were incubated in the 96-well plates for 48 h with or without immobilized anti-TCR $\gamma\delta$ mAb in the presence or absence of LPS or lipid A. During the last 8 h of incubation, 1.0 μ Ci of [3 H]TdR/well was added. The cells were then harvested, and the amount of [3 H]TdR incorporated was determined by scintillation counting. In some experiments, the sorted $\gamma\delta$ T cells (1×10^5 /well) were cultured with phosphorothioate-modified anti-sense oligonucleotide (A-ODN), 5'-GACCGC CTGCCCGGAGCCTAGG -3', or sense oligonucleotide (S-ODN), 5'-CCTAGGCTCCGGCAGGCGGTC-3', specific for mouse TLR2 gene (5 μ mol/L) in the presence of LPS for 48 h at 37°C.

IFN- γ assay

The sorted $\gamma\delta$ T cells (1×10^5 /well) were incubated in the anti-TCR $\gamma\delta$ mAb-coated plates for 48 h in the presence of LPS or lipid A. IFN- γ levels in the culture supernatants were determined by ELISA (Genzyme, Cambridge, MA). ELISA for IFN- γ was performed in triplicate using Genzyme mAb according to the manufacturer's instructions.

Statistical analysis

Data were analyzed by Student's *t* test, and a Bonferroni correction was applied for multiple comparison. The value of $p < 0.05$ was considered statistically significant.

Results

In vivo response of $\gamma\delta$ T cells after an i.p. administration of lipid A

We have previously reported that $\gamma\delta$ T cells significantly increased in the peritoneal cavity of C3H/HeN mice on day 3 after *E. coli* infection (37). To determine whether native lipid A derived from *E. coli* can induce an increase in $\gamma\delta$ T cells, flow cytometry analysis for the expression of CD3, TCR $\alpha\beta$, and TCR $\gamma\delta$ was conducted with nonadherent PEC of LPS-responsive C3H/HeN mice

or LPS-hyporesponsive C3H/HeJ mice on day 3 after the inoculation with LPS or lipid A. A representative result from three independent experiments is shown in Fig. 1A. The absolute numbers of peritoneal $\gamma\delta$ T cells were calculated by multiplying the absolute number of the nonadherent PEC by the percentage of the $\gamma\delta$ T cells, and they are shown in Fig. 1B. The relative number of $\gamma\delta$ T cells in the PEC of C3H/HeN mice were increased, constituting >30% of the total CD3-positive cell population after inoculation of 100 μ g/mouse of LPS or 30 μ g/mouse of lipid A (Fig. 1A). The $\gamma\delta$ T cells were also significantly increased in the peritoneal cavity of C3H/HeJ mice, albeit to a lesser degree compared with those in C3H/HeN mice (Fig. 1, A and B). There is a possibility that the native LPS and lipid A include the contaminated materials such as lipoproteins. Therefore, flow cytometry analyses for the expression of CD3, TCR $\alpha\beta$, and TCR $\gamma\delta$ were conducted with nonadherent PEC of C3H/HeN mice or C3H/HeJ mice on day 3 after the inoculation with a synthetic lipid A analogue, ONO-4007. A representative result from three independent experiments is shown in Fig. 2A. The absolute numbers of peritoneal $\gamma\delta$ T cells were calculated by multiplying the absolute number of the total nonadherent PEC by the percentage of the $\gamma\delta$ T cells and are shown in Fig. 2B. Both relative and absolute numbers of the PEC $\gamma\delta$ T cells in C3H/HeN and C3H/HeJ mice were significantly increased after the inoculation of ONO-4007 (1000 μ g/mouse), although a >10 times dose of native lipid A was required.

V γ and V δ gene expression by the PEC $\gamma\delta$ T cells in the peritoneal cavity induced by injection with native lipid A

To compare the V gene expressions of the $\gamma\delta$ T cells induced by injection with *E. coli*, LPS, or lipid A in C3H/HeN and C3H/HeJ mice, total RNA was extracted from $\gamma\delta$ T cells sorted from nonadherent PEC of mice inoculated with *E. coli*, LPS, or lipid A 3 days previously, and the V gene expressions analyzed by RT-PCR

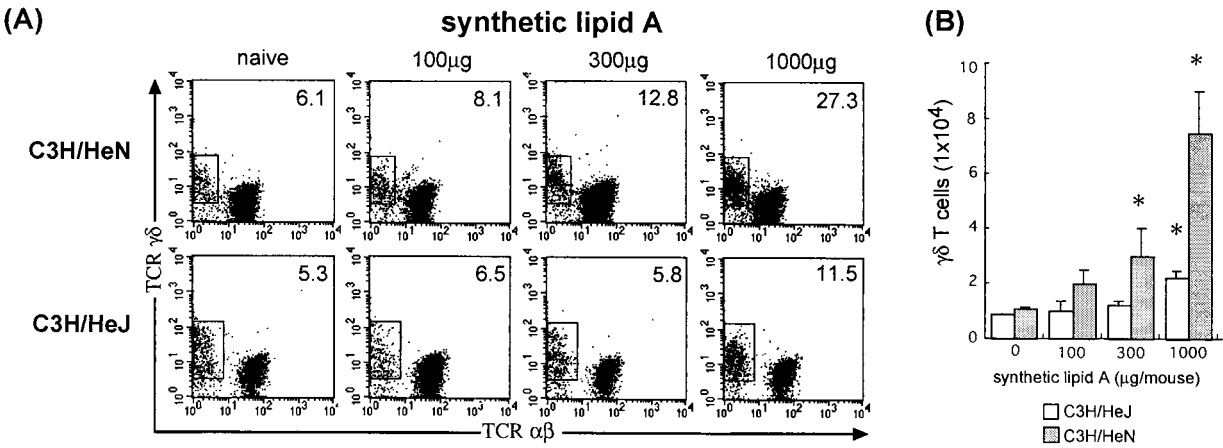


FIGURE 2. Increases of peritoneal $\gamma\delta$ T cells after the injection of a synthetic lipid A analogue ONO-4007. C3H/HeN and C3H/HeJ mice were inoculated i.p. with ONO-4007 on day 0. **A**, Nonplastic-adherent PEC from injected mice on day 3 were stained with anti-TCR $\gamma\delta$, anti-TCR $\alpha\beta$ mAb, and anti-CD3 ϵ mAb and analyzed with FACSCalibur. Analysis gate was set as described in Fig. 1. The number in each panel indicates the percentage of $\gamma\delta$ T cells in the whole lymphocyte population. **B**, Absolute numbers of $\gamma\delta$ T cells in the peritoneal cavity after synthetic lipid A injections. The numbers were calculated as described in Fig. 1. The results are representative of those from three independent experiments. Values are the means \pm SD of five mice for each group. *, $p < 0.05$ vs the control group.

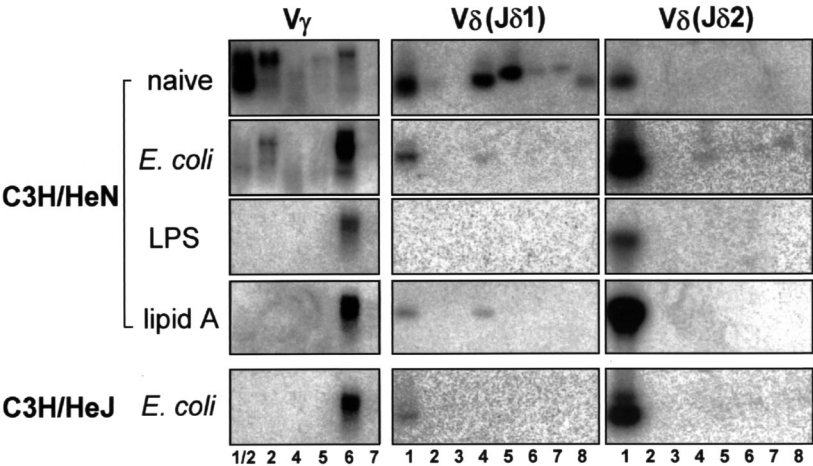
are shown in Fig. 3. The PEC $\gamma\delta$ T cells from naive mice expressed V γ 1/2, 2, 4, and a diversity of V δ genes, whereas the PEC $\gamma\delta$ T cells of C3H/HeN mice inoculated with *E. coli* preferentially expressed V γ 6 and V δ 1 genes, findings that are consistent with those obtained in our previous study (37). Similarly, V γ 6 and V δ 1 genes were exclusively used by the PEC $\gamma\delta$ T cells in both C3H/HeN and HeJ mice injected with LPS or lipid A. These results suggest that the $\gamma\delta$ T cells expressing V γ 6/V δ 1 genes were selectively induced by the native lipid A, a natural product of *E. coli*. To determine the junctional diversity of the V γ 6-J γ 1 and V δ 1-J δ 2 gene rearrangements in the $\gamma\delta$ T cells induced by the native lipid A, we examined the nucleotide sequences of the V γ 6 and V δ 1 transcripts of the peritoneal $\gamma\delta$ T cells in the lipid A-injected mice. As shown in Fig. 4, all 20 V γ 6-J γ 1 transcripts and 18 of 20 V δ 1-J δ 2 transcripts of the $\gamma\delta$ T cells from the lipid A-injected mice showed no junctional diversity, resulting in in-frame invariant canonical sequences, which are preferentially expressed in fetal thymocytes at the late stage (approximately day 17) of gestation (31) and in the intraepithelial lymphocytes of reproductive organs such as the uterus (30). Taken together, these results suggest that the lipid A-induced $\gamma\delta$ T cells in the peritoneal cavity expressed a

canonical V γ 6/V δ 1 TCR, which was the same as that of *E. coli*-induced $\gamma\delta$ T cells.

Proliferation and cytokine production of lipid A-induced $\gamma\delta$ T cells in response to LPS in vitro

We previously reported that the $\gamma\delta$ T cells induced by *E. coli* infection in C3H/HeN mice exhibited a strong proliferative response to LPS even in the absence of APC under TCR engagement (37). Therefore, we examined the proliferative response and cytokine production of the lipid A-induced $\gamma\delta$ T cells from C3H/HeN and C3H/HeJ mice in response to LPS. The $\gamma\delta$ T cells were purified by cell sorting from the nonadherent peritoneal cells on day 3 after lipid A injection, and they were incubated for 48 h with immobilized anti-TCR $\gamma\delta$ mAb in the presence or absence of an optimum dose (10 μ g/ml) of LPS. Fig. 5A shows that the lipid A-induced $\gamma\delta$ T cells exhibited a strong proliferative response in the presence of LPS. Notably, the $\gamma\delta$ T cells in LPS-hyporesponsive C3H/HeJ mice proliferated more vigorously in response to LPS than did those in LPS-responsive C3H/HeN mice. Fig. 5B shows that $\gamma\delta$ T cells stimulated with LPS produced a large amount of IFN- γ , whereas neither IL-2 nor IL-4 was detected in

FIGURE 3. V γ or V δ usage of $\gamma\delta$ T cells in PEC on day 3 after *E. coli*, LPS, or native lipid A injection. Total RNA extracted from $\gamma\delta$ T cells sorted from five C3H/HeN and C3H/HeJ mice injected with *E. coli*, LPS, or a native lipid A 3 days previously was reverse transcribed into cDNA and amplified by PCR with primers for C γ or C δ and various V γ or V δ segments. The Southern blot of the γ PCR products was hybridized with MNG6. The Southern blot of δ PCR products was hybridized with an oligonucleotide probe for J δ 1 or J δ 2. The results are representative of those from three independent experiments.



Vγ-Jγ

germline	TGT GCA TGC TGG GAT A (Vγ6)	N / P	AT AGC TCA GGT TTT (Jγ1)	
<i>E. coli</i>	TGT GCA TGC TGG GAT		AGC TCA GGT TTT	in frame 20/20
Lipid A	TGT GCA TGC TGG GAT		AGC TCA GGT TTT	in frame 20/20

Vδ-Dδ-Jδ

germline	TGT GGG TCA GAT A (Vδ1)	ATCGGAGGGATACGA (Dδ2)	N / P	CT ACC GAC AAA CTC (Jδ1)	
				C TCC TGG GAC ACC C (Jδ2)	
<i>E. coli</i>	TGT GGG TCA GAT	ATCGGAGGGGA		G C TCC TGG GAC ACC C	in frame 17/20
	TGT GGG TCA GAT	ATCGGAGGGATAC		C GAC AAA CTC	out of frame 3/20
Lipid A	TGT GGG TCA GAT	ATCGGAGGGGA		G C TCC TGG GAC ACC C	in frame 18/20
	TGT GGG TCA GAT	ATCGGAGGGATAC		C GAC AAA CTC	out of frame 2/20

FIGURE 4. Junctional sequences of Vγ6 and Vδ1 from γδ T cells in PEC on day 3 after *E. coli* or a native lipid A injection. RT-PCR products were sequenced and analyzed as described in *Materials and Methods*.

the supernatant (data not shown). Moreover, the γδ T cells in LPS-hyporesponsive C3H/HeJ mice produced more IFN-γ in response to LPS than those did in LPS-responsive C3H/HeN mice.

To determine whether the responsiveness of the γδ T cells to LPS is mediated by TCR signal, sorted γδ T cells induced by lipid A were incubated for 48 h with or without LPS (10 μg/ml) in the presence or absence of immobilized anti-TCR-γδ mAb. As shown

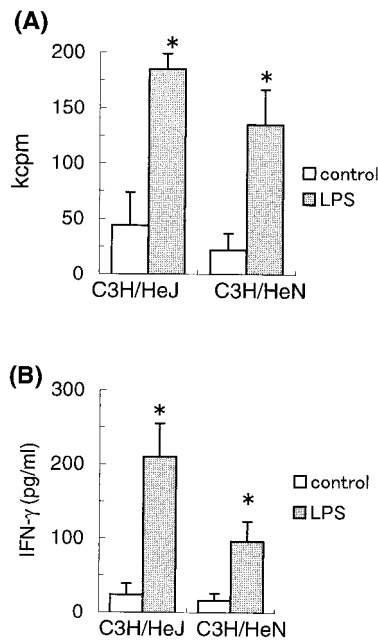


FIGURE 5. The proliferative response and cytokine production of the lipid A-induced γδ T cells from the peritoneal cavities of C3H/HeN or C3H/HeJ mice in the presence of LPS. *A*, Purified γδ T cells (1×10^5 /well) were incubated on anti-TCR γδ mAb-coated 96-well plates for 48 h in the presence or absence of LPS (10 μg/ml). During the last 8 h of incubation, 1.0 μCi of [3 H]thymidine was added, and the thymidine incorporation was determined by scintillation counting. *B*, Purified γδ T cells (1×10^5 /well) were cultured similarly in the presence or absence of LPS (10 μg/ml) for 48 h, and the culture supernatant was collected. The IFN-γ concentration in the culture supernatant was determined by ELISA. The data are representative of three separate experiments and are expressed as the means of triplicates \pm SD. *, $p < 0.05$ vs the control group.

in Fig. 6A, the γδ T cells exhibited a significant proliferative response even without TCR stimulation, and the proliferative response was augmented by TCR stimulation. In contrast, Fig. 6B shows that γδ T cells stimulated with LPS produced a small amount of IFN-γ in the absence of TCR stimulation compared with those in the presence of TCR stimulation. These results suggested that stimulation with LPS induced γδ T cell proliferation but that TCR stimulation was required for the IFN-γ production. Dose responses of LPS for proliferation of γδ T cells induced by *E. coli* are shown in Fig. 6C. In the presence of >0.1 μg/ml of the LPS, the γδ T cells exhibited a significant proliferative response.

We next examined the effect of soluble anti-TCR γδ mAb on the proliferative response of γδ T cells to LPS. Sorted γδ T cells induced by native lipid A were incubated for 48 h with the LPS (10 μg/ml) in the presence or absence of an optimum dose (10 μg/ml) of neutralizing anti-TCR γδ mAb or the same dose of control IgG. We confirmed that this concentration of anti-TCR γδ mAb could inhibit the proliferative response of the heat-killed *Salmonella*-specific γδ T cells (40). Fig. 7A shows that the proliferative response to the LPS was not inhibited by the anti-TCR-γδ neutralizing mAb, confirming that the lipid A-induced γδ T cells can proliferate in response to the LPS in a TCR-independent manner.

Although macrophages from C3H/HeJ mice are hyporesponsive to many types of lipid A, they are nearly as responsive as their normal counterparts when stimulated with *P. gingivalis* native lipid A (2). Therefore, we next examined the proliferative response of the lipid A-induced γδ T cells to the lipid A from *P. gingivalis* besides the lipid A derived from *E. coli* or *S. minnesota*. When the sorted γδ T cells were incubated for 48 h with each kind of the lipid A (10 μg/ml), they exhibited a strong proliferative response with each of them (Fig. 7B). Taken together with the findings for the γδ T cells from C3H/HeJ mice, these results suggest that the γδ T cells respond to the native lipid A using an LPS receptor other than TLR4, which is mutated in C3H/HeJ mice (23, 24).

Expression of the LPS receptors in the γδ T cells induced by injection with the native lipid A

CD14 and β₂ integrins (CD11/CD18) have been widely recognized as LPS receptors for immune cells (3, 7–11). Recently, two TLR family proteins, TLR2 and 4, have been identified as LPS signaling receptors (14–17, 25, 26, 29). We attempted to determine which LPS receptors are used by the lipid A-induced γδ T

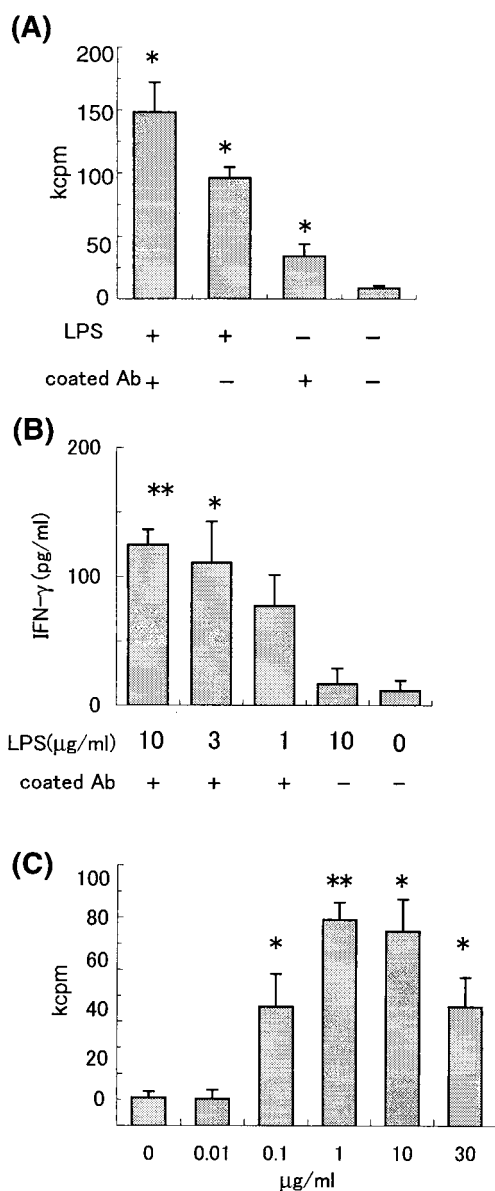


FIGURE 6. Proliferative responses and cytokine production of the lipid A-induced $\gamma\delta$ T cells from the peritoneal cavities of C3H/HeN mice in the presence of LPS. **A**, Purified $\gamma\delta$ T cells (1×10^5 /well) were incubated for 48 h in the presence or absence of LPS (10 μ g/ml) with or without immobilized anti-TCR $\gamma\delta$ mAb. During the last 8 h of incubation, 1.0 μ Ci of [3 H]thymidine was added, and the thymidine incorporation was determined by scintillation counting. **B**, Purified $\gamma\delta$ T cells (1×10^5 /well) were cultured in the presence or absence of immobilized anti-TCR $\gamma\delta$ mAb with different doses of LPS for 48 h, and the culture supernatant was collected. The IFN- γ concentration in the culture supernatant was determined by ELISA. **C**, Purified $\gamma\delta$ T cells (1×10^5 /well) were incubated for 48 h with different doses of LPS. The [3 H]thymidine incorporation was measured as described above. The data are representative of three separate experiments and are expressed as the means of triplicates \pm SD. *, $p < 0.05$ vs the control group. **, $p < 0.01$ vs the control group.

cells. Flow cytometry analysis for the expression of β_2 integrins and CD14 was conducted with nonadherent PEC on day 3 after the inoculation with lipid A. A representative result from three independent experiments is shown in Fig. 8A. The $\gamma\delta$ T cells expressed CD11a but not CD11b, CD11c, or CD14. To examine whether CD11a is involved in mediating LPS response, sorted $\gamma\delta$ T cells

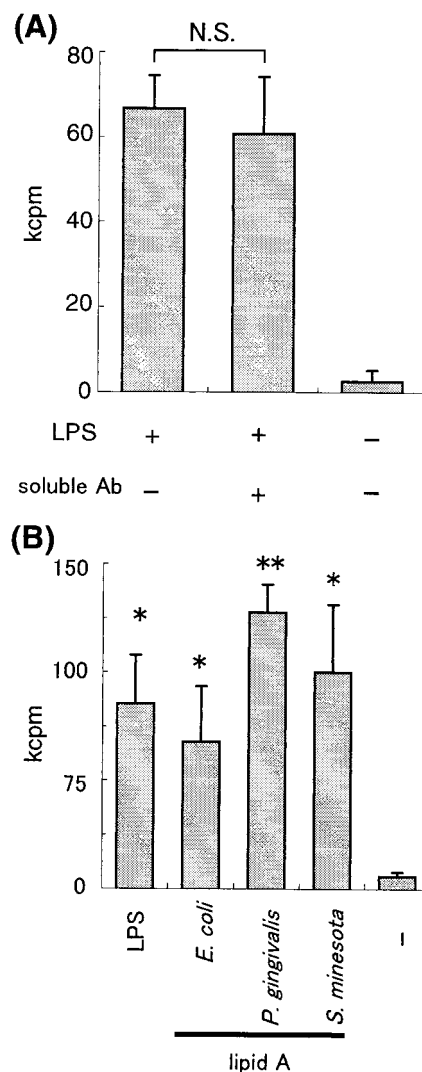


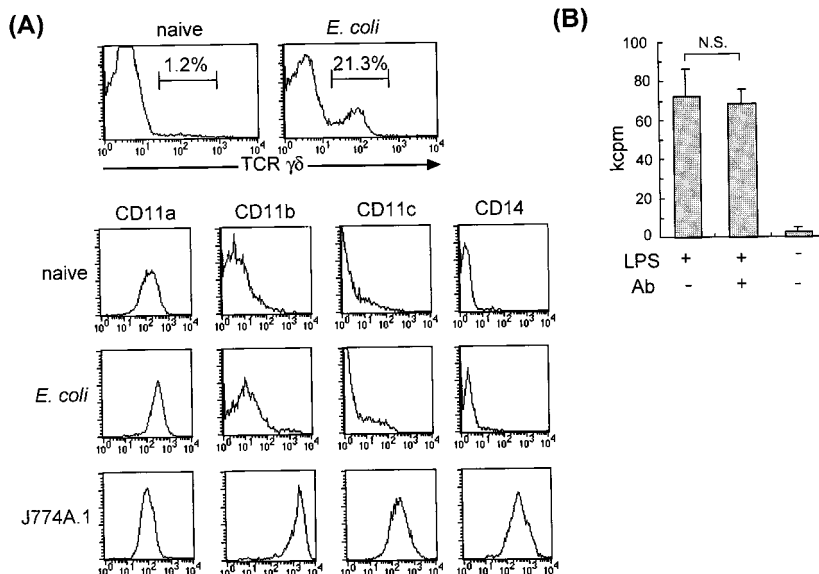
FIGURE 7. Proliferative responses of the lipid A-induced $\gamma\delta$ T cells from the peritoneal cavities of C3H/HeN mice in the presence of LPS or a native lipid A. **A**, Purified $\gamma\delta$ T cells (1×10^5 /well) were cultured in the presence of LPS (10 μ g/ml) for 48 h with or without the neutralizing anti-TCR $\gamma\delta$ mAb. **B**, Purified $\gamma\delta$ T cells (1×10^5 /well) were cultured in the presence of a native lipid A (10 μ g/ml) derived from *E. coli*, *S. minnesota*, or *P. gingivalis* for 48 h. During the last 8 h of incubation, [3 H]thymidine was added to the culture, and the thymidine incorporation was determined by scintillation counting. The data are representative of two separate experiments and are expressed as the means of triplicates \pm SD. *, $p < 0.05$ vs the control group. **, $p < 0.01$ vs the control group.

were incubated for 48 h with LPS derived from *E. coli* (10 μ g/ml) in the presence or absence of a neutralizing anti-CD11a mAb (10 μ g/ml) or the same dose of control IgG. As shown in Fig. 8B, the proliferative response of the $\gamma\delta$ T cells to LPS was not inhibited by the neutralizing anti-CD11a mAb.

An essential role of TLR2 in the LPS response by the lipid A-induced $\gamma\delta$ T cells

To examine the TLR2 and TLR4 expressions by $\gamma\delta$ T cells, total RNA was extracted from $\gamma\delta$ T cells sorted from nonadherent PEC of mice inoculated with lipid A 3 days previously, and TLR2 and TLR4 expressions were analyzed by RT-PCR. As shown in Fig. 9A, the $\gamma\delta$ T cells in the peritoneal cavity expressed a significant level of TLR2 mRNA but not TLR4 mRNA. In contrast, both

FIGURE 8. Expression of the LPS receptors in $\gamma\delta$ T cells in the peritoneal cavity induced by a native lipid A. **A**, Non-plastic-adherent PEC were stained with anti-TCR $\gamma\delta$, anti-CD3 ϵ mAb, and mAb against CD11a, CD11b, CD11c, and CD14 and analyzed with FACSCalibur. Analysis gate was set on lymphocytes using forward and side scatter gating and on the TCR C δ^+ cells. **B**, Purified $\gamma\delta$ T cells (1×10^5 /well) were cultured in the presence of LPS ($10 \mu\text{g/ml}$) for 48 h with or without the neutralizing anti-CD11a mAb. During the last 8 h of incubation, $1.0 \mu\text{Ci}$ of [^3H]thymidine was added, and the incorporation was determined by scintillation counting. The data are representative of two separate experiments and are expressed as the means of triplicates \pm SD.



TLR2 and TLR4 mRNA were only marginally expressed by the $\alpha\beta$ T cells in PEC and $\gamma\delta$ T cells in the liver, which did not respond to LPS. Thus, these results suggest that the $\gamma\delta$ T cells in the peritoneal cavity may respond to lipid A via TLR2. To test this issue, we examined the effect of a TLR2 A-ODN treatment on the proliferation response of the $\gamma\delta$ T cells to LPS. As shown in Fig. 9B, treatment with the A-ODN reduced the expression of TLR2 mRNA in the $\gamma\delta$ T cells, and the proliferation of the $\gamma\delta$ T cells in response to LPS was significantly impaired by this treatment compared with that of S-ODN treatment (Fig. 9C). To further confirm the involvement of TLR2 in the $\gamma\delta$ T cell-response to native lipid A, we examined the flow cytometry analysis for the expression of CD3, TCR $\alpha\beta$, and TCR $\gamma\delta$ with nonadherent PEC in TLR2 $^{-/-}$ or TLR2 $^{+/-}$ mice on day 3 after the inoculation of native lipid A. A representative result from three independent experiments is shown in Fig. 10A. The absolute numbers of the peritoneal $\gamma\delta$ T cells were calculated by multiplying the absolute number of the nonadherent PEC by the percentage of $\gamma\delta$ T cells and are shown in Fig. 10B. The increase of $\gamma\delta$ T cells in TLR2 $^{+/-}$ mice with C57BL/6/129 background was relatively less as compared with C3H/HeN

mice. However, the increase of $\gamma\delta$ T cells following an i.p. injection of lipid A were significantly impaired in TLR2 $^{-/-}$ mice compared with that of TLR2 $^{+/-}$ mice ($p < 0.05$, Fig. 10, A and B). Thus, these results indicate that TLR2 is at least partly involved in the proliferation of $\gamma\delta$ T cells in response to the native lipid A from *E. coli*.

Discussion

A significant fraction of $\gamma\delta$ T cells is reported to respond to the LPS fraction through an apparently TCR-independent mechanism (41–43). Nitta et al. reported that $\gamma\delta$ T cells in the peritoneal cavity of mice proliferate in response to TCR triggering in synergy with LPS (43). Leclercq and Plum reported that TCR V γ 5 cells, which are exclusively associated with canonical V δ 1 chain and are preferentially present in the early fetal thymus and the epidermis of mice, are activated to produce cytokines upon interaction with LPS via a TCR-independent pathway (41). Similarly, in the present study, we found that the $\gamma\delta$ T cells expressing V γ 6/V δ 1 genes

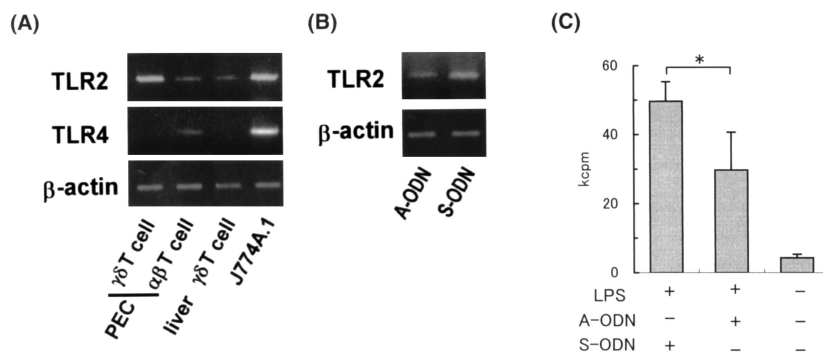


FIGURE 9. RT-PCR analysis of TLR2 and TLR4 of the $\gamma\delta$ T cells induced by a native lipid A. **A**, Total RNA extracted from $\gamma\delta$ T cells or $\alpha\beta$ T cells in peritoneal cavities or livers sorted from five C3H/HeN mice injected with lipid A 3 days previously was reverse transcribed into cDNA and amplified by PCR with primers for TLR2 or TLR4. **B**, Purified $\gamma\delta$ T cells (1×10^5 /well) were cultured in the presence of LPS ($10 \mu\text{g/ml}$) for 24 h with TLR2 A-ODN or S-ODN in anti-TCR $\gamma\delta$ mAb-coated 96-well plates, and total RNA was extracted, reverse transcribed into cDNA, and amplified by PCR with primers for TLR2. **C**, Purified $\gamma\delta$ T cells (1×10^5 /well) were incubated in anti-TCR $\gamma\delta$ mAb-coated 96-well plates for 48 h in the presence of LPS ($1 \mu\text{g/ml}$) with A-ODN or S-ODN. During the last 8 h of incubation, $1.0 \mu\text{Ci}$ of [^3H]thymidine was added to the culture, and the thymidine incorporation was determined by scintillation counting. The data are representative of two separate experiments and are expressed as the means of triplicates \pm SD. *, $p < 0.05$ vs the control group.

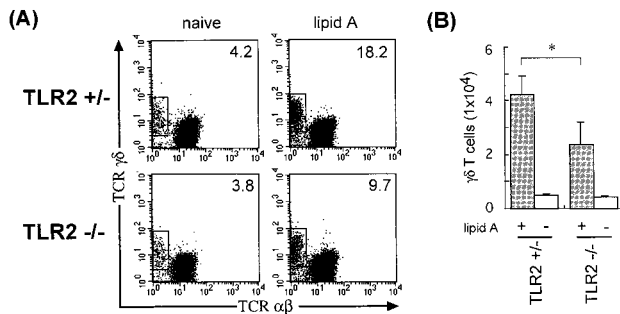


FIGURE 10. Impaired increase of peritoneal $\gamma\delta$ T cells after the injection of native lipid A in TLR2-deficient mice. TLR2^{-/-} and control TLR2^{+/+} mice were inoculated i.p. with native lipid A (100 μ g/mouse) on day 0. A, Nonplastic-adherent PEC from injected mice on day 3 were stained with anti-TCR $\gamma\delta$, anti-TCR $\alpha\beta$ mAb, and anti-CD3 ϵ mAb and analyzed with FACSCalibur. Analysis gate was set as described in Fig. 1. The number in each panel indicates the percentage of $\gamma\delta$ T cells in the whole lymphocyte population. B, Absolute numbers of $\gamma\delta$ T cells in the peritoneal cavity after lipid A injection. The numbers were calculated as described in Fig. 1. The results are representatives of those from three independent experiments. Values are the means \pm SD of five mice for each group. *, $p < 0.05$.

responded to native lipid A in vivo and in vitro in a TCR-independent manner. The $\gamma\delta$ T cells in the peritoneal cavity expressed V γ 6-J γ 1 and V δ 1-J δ 2 mRNA with no N diversity, as they do in the fetal thymus and uterus. Thus, it would appear that only primitive $\gamma\delta$ T cells with invariant TCR such as V γ 5/V δ 1 and V γ 6/V δ 1, which develop in the thymus at the early stage of gestation, respond directly to the bacterial products from Gram-negative bacteria. We have previously demonstrated that $\gamma\delta$ T cell-deficient mice with a truncated C δ gene are resistant to LPS-induced lethal shock with impaired TNF- α production (44). Furthermore, mice depleted of $\gamma\delta$ T cells by TCR- δ gene mutation showed impaired host defense against *E. coli* (37). Like phagocytes, the primitive $\gamma\delta$ T cells may play an important role in innate immunity against bacterial infection through rapid responses to the bacterial components via TLR2.

Bacterial LPS, a constituent of the outer membrane of the cell wall of Gram-negative bacteria, is one of the main causative agents of septic shock in humans. Recognition of LPS is a key event in host antimicrobial defense reactions. LPS is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A (45, 46). The conserved lipid A structure has been identified as the LPS component responsible for LPS-induced biological effects (45–47).

Recently, several members of the mammalian TLR family have been identified (18–22). Several lines of evidence suggest that one or more members of the TLR family are the cell-surface receptors for LPS, the prototypical activators of NF- κ B and other proinflammatory responses (14–17, 23, 24, 48). In C3H/HeJ and C57BL/10ScCr mice, mutations of the gene *lps* (*lps*^d) selectively impede LPS signal transduction, rendering them resistant to endotoxin yet highly susceptible to Gram-negative infection (23, 24). TLR4 from the C3H/HeJ mouse has a point mutation at amino acid 712 (Pro to His), and the C57BL10/ScCr mouse appears to be null for the TLR4 locus (23, 24). These observations suggested that TLR4 is a cell-surface component of the LPS signaling pathway. However, although *lps*^d mice are hyporesponsive to LPS, they are not unresponsive, and LPS-dependent gene transcription will occur if a very large dose of LPS is administered (15). Moreover, cells from C3H/HeJ mice are nearly as sensitive as their normal counterparts when stimulated with LPS components derived from certain bac-

teria such as *P. gingivalis* (2, 49). These observations suggested that proteins other than TLR4 may replace the function of TLR4 in signal transduction for LPS responsiveness. Transfection of cell lines with TLR2 confers them with the ability to respond to LPS with activation of NF- κ B, thus directly suggesting that TLR2 serves in place of TLR4 (14, 16, 26). Our results demonstrated that not only in C3H/HeN mice but also in C3H/HeJ, the lipid A-induced $\gamma\delta$ T cells exhibited a strong proliferative response in the presence of *E. coli* native lipid A as well as the lipid A fraction from *P. gingivalis*. Furthermore, the $\gamma\delta$ T cells in the peritoneal cavity strongly expressed TLR2 mRNA, whereas those from the liver that did not respond to the naive LPS in vitro (37) expressed only a marginal level of TLR2 mRNA. Treatment with TLR2 A-ODN significantly inhibited the proliferation response of the $\gamma\delta$ T cells to the lipid A. It has to be noted, though, that the inhibition of cell proliferation was partial. This was probably because the TLR2 mRNA inhibition by the A-ODN was moderate (Fig. 9B). In serial dilutions of the samples, the inhibition was \sim 75% compared with the S-ODN treatment. Moreover, the increase of $\gamma\delta$ T cells following the i.p. injection of native lipid A was significantly impaired in TLR2^{-/-} mice compared with that of TLR2^{+/+} mice. However, we cannot exclude the possibility that a minor expression of TLR4 on the $\gamma\delta$ T cells also acts as a LPS receptor for signal transduction in the $\gamma\delta$ T cells in vivo. We previously reported that after *E. coli* infection the $\gamma\delta$ T cells of the PEC included many V γ 6/V δ 1 $\gamma\delta$ T cells but those in the liver did not, and the former exhibited a strong proliferative response to LPS but the latter did not (37). Taken together, these results suggested that V γ 6/V δ 1 $\gamma\delta$ T cells responded to the lipid A and that TLR2 in $\gamma\delta$ T cells is responsible, at least partly, for the LPS signaling in the V γ 6/V δ 1 $\gamma\delta$ T cells.

It has recently reported that, TLR2 mediates monocyte activation by peptidoglycans, lipoteichoic acids, and microbial lipoproteins (27–29, 50). Roark et al. reported that V γ 6/V δ 1 T cells preferentially increase among $\gamma\delta$ T cells infiltrating inflamed tissues induced by infection with *Listeria monocytogenes*, a Gram-positive bacteria (51). Takeuchi et al. have most recently reported that TLR4 mainly recognize lipid A and lipoteichoic acid from Gram-negative or -positive bacteria, respectively, whereas TLR2 plays a major role in recognition of peptidoglycan and lipoprotein from both Gram-negative or -positive bacteria (29). The primitive $\gamma\delta$ T cells may play protective roles in infection with not only Gram-negative bacteria but also Gram-positive bacteria via TLR2 signal. The native LPS and lipid A we used here may contain some bacterial proteins, raising a possibility that bacterial proteins contaminated in LPS stimulate the V γ 6/V δ 1 $\gamma\delta$ T cells via TLR2. However, the experiments using a synthetic lipid A analogue, ONO-4007, which does not contain any other bacterial materials, demonstrated that it induced an increase of $\gamma\delta$ T cells in the peritoneal cavities in the C3H/HeJ mice that have the mutated TLR4 gene. These results indicate that TLR2 serve to function as one of the lipid A signaling receptors. The ability of ONO-4007 to induce $\gamma\delta$ T cells in the PEC was lower than that of the naive lipid A. Hence, it is possible that the $\gamma\delta$ T cells respond more vigorously to the naive lipid A than synthetic lipid A via TLR2-mediating signals for contaminated materials such as lipoproteins.

In the present study, the $\gamma\delta$ T cells from C3H/HeJ mice responded more vigorously to LPS in vitro compared with those from C3H/HeN mice. Nevertheless, the $\gamma\delta$ T cells were increased less in C3H/HeJ mice than in C3H/HeN mice after in vivo administration of lipid A, similar to *E. coli* infection as reported previously (37). Skeen and Ziegler reported that the peritoneal $\gamma\delta$ T cells proliferated in response to IL-1 and IL-7 (52). It has been reported that TNF- α and IL-12 synergistically stimulate human $\gamma\delta$

T cell proliferation (53). We have previously reported that $\gamma\delta$ T cells proliferate in response to IL-15 in vitro (37, 54). Therefore, these cytokines derived from infected macrophages may preferentially stimulate the invariant $\gamma\delta$ T cells to proliferate in the inflamed sites. We have previously demonstrated that the macrophages induced by *E. coli* infection in C3H/HeJ mice showed an impaired expression of monokine genes such as TNF- α , IL-6, IL-12, and IL-15 compared with those in C3H/HeN mice (37). Administration of anti-IL-15 mAb inhibited, albeit partially, the increase in $\gamma\delta$ T cells after *E. coli* infection in C3H/HeN mice (37). Therefore, IL-15 derived from LPS-stimulated macrophages may be partly responsible for local expansion of $\gamma\delta$ T cells in the peritoneal cavity in vivo. Furthermore, IL-15 is reported to have a strong chemotactic activity for T cells (55, 56). Thus, impaired accumulation and expansion of $\gamma\delta$ T cells in C3H/HeJ mice after in vivo administration of the native lipid A may be attributable in part to impaired cytokine production by macrophages that may preferentially use TLR4 for LPS signaling.

In conclusion, $\gamma\delta$ T cells expressing invariant V γ 6/V δ 1 TCR responded to the native lipid A not only from *E. coli* but also from *P. gingivalis* in a TCR-independent manner. The LPS/lipid A-reactive $\gamma\delta$ T cells expressed TLR2 mRNA but no detectable TLR4 mRNA. Treatment with TLR2 A-ODN significantly inhibited the proliferative response of $\gamma\delta$ T cells to the lipid A. Additionally, TLR2-deficient mice showed an impaired increase of the $\gamma\delta$ T cells following in vivo injection of the native lipid A. These results suggest that the invariant V γ 6/V δ 1 $\gamma\delta$ T cells respond to the lipid A fraction via TLR2. The primitive $\gamma\delta$ T cells bearing invariant TCR may play an important role in innate immunity against microbial infection through TLR2 activation.

Acknowledgments

We thank Ono Chemical and Dr. J. A. Bluestone for providing an *E. coli*-type synthetic lipid A analogue (ONO-4007) and the UC7-13D hybridoma, respectively.

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