Research article

Induction of hypersensitivity to endotoxin lethality in mice by treatment with trehalose 6,6′-dimycolate but not with 2,3,6,6′-tetraacyl trehalose 2′-sulfate

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The mechanism by which priming with trehalose 6,6′-dimycolate (TDM, cord factor) induced hypersensitivity to endotoxin lethality was investigated. C57BL/6 and BALB/c mice primed with TDM succumbed to endotoxin shock, but BALB/c IFN-γ knock-out (IFN-$\gamma^{-/-}$) mice showed resistance to LPS lethality. The levels of serum IFN-γ peaked on day 4 after priming with TDM and kept significant levels, indicating that IFN-γ plays a critical role for inducing hypersensitivity to LPS lethality. After challenge with LPS, TDM-primed mice produced higher amounts of serum TNFα and soluble CD14. A sulfolipid (SL, 2,3,6,6′-tetraacyl trehalose 2′-sulfate) did not induce the hypersensitivity and, conversely, suppressed the activity of TDM when administered together. Administration of TDM induced infiltration of mononuclear cells in liver, and apoptosis of cells present in the liver sinus was observed after LPS challenge. These results suggest that the hypersensitivity to LPS lethality is due to overproduction of cytokines and other molecules.

INTRODUCTION

LPS is known as an agent leading to sepsis, multiple organ failure and shock in the course of Gram-negative infections.[1] The pathogenesis of LPS is mediated by large amounts of several cytokines, particularly TNFα, produced by macrophages and other cells stimulated with LPS. Previous studies showed that mice became hypersensitive to endotoxin lethality when infected with *Mycobacterium bovis* BCG.[2,3] Induction of hypersensitive conditions to LPS lethality in BCG-infected mice seems to be due to the activation of macrophages with significant levels of IFN-γ produced by NK and Th1 cells during infections.

Injections of LPS and *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP), the minimal structure for inducing adjuvanticity, into guinea pigs and mice increased lethal toxicity to endotoxin.[4–7] Trehalose 6,6′-dimycolate (TDM, cord factor), a mycolic acid-containing glycolipid as one of characteristic components of the cell wall of *Mycobacterium*, plays a role as not only virulent factor but also immunostimulatory molecule possessing the following activities: adjuvanticity, granuloma-formation, apoptosis induction in the thymus, activation of macrophages and NK cells.[8–12] The first observation by Suter that the treatment of mice with TDM dissolved in mineral oil increased the LPS lethality was supported by the results of Parant *et al*. who injected TDM dissolved in metabolizable peanut oil or saline.[2,13] Parant *et al*. also showed that the hypersensitivity induced by TDM depended on the chain length of mycolic acid in TDM.[14,15] On the other hand, Yarkoni *et al*. could demonstrate that the enhancement by TDM was dependent on the conditions for making the emulsion,[16] *i.e*. increasing the oil concentration and decreasing the Tween 80 concentration were important for induction of hypersensitivity to the LPS lethality. A glycolipid without mycolic acid, 2,3,6,6′-tetraacyl trehalose 2′-sulfate (sulfolipid, SL), is

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also one of the constituents of the mycobacterial cell wall and relates comparably to the virulence of *Mycobacterium tuberculosis*.[17] Both of these glycolipids are similar in possessing trehalose as the hydrophilic moiety. However, it is not clear whether injections with not only TDM but also SL in a form of water-in-oil-in-water (w/o/w) emulsion into mice induce hypersensitive conditions to endotoxin toxicity. In addition, the molecular mechanisms of hypersensitivity induced by mycobacterial glycolipids still remain unclear. In this study, we have investigated whether or not mycobacterial glycolipids, TDM and SL prepared from *M. tuberculosis* Aoyama B, are able to induce hypersensitive conditions to LPS lethality when administered intraperitoneally (i.p.) in the form of w/o/w emulsion. We describe here that TDM but not SL is able to induce hypersensitivity to LPS lethality, and that this induction is due to the production of IFN-γ by priming with TDM and higher amounts of TNFα production after LPS challenge.

Materials and Methods

Animals

Female C57BL/6, C3H/HeN and BALB/c mice, purchased from Japan SLC Co., Shizuoka, Japan, were housed for at least one week before being used for priming. BALB/c IFN-$\gamma^{-/-}$ mice were obtained as breeder pairs from Jackson Laboratory (Bar Harbor, ME, USA) and raised in the Animal Facility, School of Science, Kitasato University. All mice were used at 8-weeks-old for experiments carried out under specific pathogen-free conditions.

Mycobacterial preparations

M. bovis BCG strain Pasteur, a kind gift from Dr Mitsuyama (School of Medicine, Kyoto University, Kyoto, Japan), was grown in Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI, USA) containing 10% Middlebrook ADC Enrichment (Becton Dickinson, Cockeysville, MD, USA) and 0.05% Tween 80. *M. tuberculosis* Aoyama B was grown on Sauton medium. Bacterial cells were autoclaved at 121°C for 15 min and harvested by centrifugation. Heat-killed bacilli of *M. tuberculosis* Aoyama B and *M. bovis* BCG strain Pasteur were prepared by washing with distilled water, grinding and freeze-drying.

Preparation of glycolipids

Crude lipids were extracted successively from bacilli of *M. tuberculosis* Aoyama B with chloroform:methanol (4:1; v/v), chloroform:methanol (3:1; v/v) and chloroform:methanol (2:1; v/v). Glycolipids (TDM and SL) were isolated from the crude lipids by repeating thin-layer chromatograpy using silica gel plates developed with chloroform:methanol:acetone:acetic acid (90:10:6:1; v/v/v/v) and subsequently with chloroform:methanol:water (90:10:1; v/v/v) as described previously.[11] Intact TDM and SL were identified by FAB/mass spectrometry. After hydrolysis and methyl esterification, mycolic acid esters (C_{76-88} α-, methoxy- and keto-esters) from TDM and C_{40} phthioceranic and hydroxy phthioceranic acid methyl esters from SL were identified mass-spectrometrically.

LPS and MDP-Lys(L18)

A highly purified LPS preparation from *Salmonella abortus equi*, donated by Dr C. Galanos (Max-Planck-Institut für Immunbiologie, Freiburg in Breisgau, Germany), was dissolved in pyrogen-free water (Otsuka Pharmaceutical Co., Tokushima, Japan) as a 1 mg/ml stock solution and diluted with pyrogen-free saline (Otsuka) before use. MDP-Lys(L18), kindly provided by Daiichi Pharmaceutical Co. (Tokyo, Japan), was dissolved in chloroform:methanol (3:1, v/v) as a stock solution (3 mg/ml). After evaporation of the solvent, the sample was suspended in 0.15M phosphate buffered saline (PBS).

Preparation of water-in-oil-in-water(w/o/w) emulsion

After dissolving or suspending glycolipid, heat-killed bacteria and MDP-Lys(L18), respectively, in Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI, USA) in a Teflon grinder, an equal volume of PBS was added to the grinder. The mixture was ground to prepare a water-in-oil emulsion, then saline containing 0.2% Tween 80 was added. The resulting mixtures were ground again to produce a w/o/w emulsion. The final oil concentration in the emulsion was 3.2%. As a vehicle control, a w/o/w emulsion without any test samples was prepared similarly.

Lethal toxicity

Mice were injected i.p. with 400 μg test samples (heat-killed bacteria, TDM, SL and MDP-Lys(L18)) in a w/o/w emulsion at a volume of 200 μl unless described otherwise. At various intervals after priming, all mice were challenged intravenously (i.v.) with 30 μg *S. abortus equi* LPS at a volume of 200 μl except for estimating the dose effect. Lethality was assessed by counting numbers of dead mice 72 h after challenge.

Mice (LPS/GalN) were injected i.v. with 0.1 μg *S. abortus equi* LPS at a volume of 200 μl and simultaneously i.p. with 20 mg D-galactosamine-HCl (GalN; Wako Pure

Chemical Industries, Ltd, Osaka, Japan) in a volume of 0.5 ml.

Collecting serum samples for TNFα assay or IFN-γ ELISA

Following deep anesthesia with diethyl ether, blood was taken from mice by cardiac puncture using a 1 ml syringe with 26 G needle either for measuring levels of serum IFN-γ at various intervals after priming or for estimating serum TNFα titers 60 min after LPS challenge. All sera were stored at –80°C until use.

TNF assay

Serum TNF titers were estimated by a cytotoxicity test using TNFα sensitive L929 (C5F6) cells in the presence of actinomycin D as described previously.[18,19] At 4 h before the termination of cultures, aliquots (10 µl) of WST-1 reagents (Dojindo, Kumamoto, Japan) were added to each well of 96-well microplates. The resultant formation of formazan by viable cells was estimated by measuring absorbance at 450 nm (reference at 690 nm). The TNFα titer in serum samples was determined by multiplying the sample dilution and the value of a recombinant murine TNFα (Genzyme Inc., Boston, MA, USA) which were able to give the 50% cytotoxicity.

ELISA for IFN-γ

Serum IFN-γ titers were estimated by ELISA as described previously.[20,21] Briefly, microplates (Nunc, Roskilde, Denmark) were coated with aliquots (50 µl) of anti-IFN-γ mAb (R4-6A2; 17 µg/ml) in 0.1 mM $NaHCO_3$ (pH 8.2) and then with 3% BSA in PBS to block remaining uncoated sites. Aliquots (50 µl) of test samples and standard recombinant murine IFN-γ (PharMingen, San Diego, CA, USA) were diluted serially at 1:2 and added to each well of the plates. After incubation for 1 h at 37°C and washing 3 times with PBS-0.05% Tween 20, aliquots (50 µl) of biotinylated anti-IFN-γ mAb (AN-18.17.20; 3 µg/ml) were added and incubated for 1 h at 37°C. After washing, aliquots (50 µl) of a substrate solution containing 1 mg/ml of *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.8) were added. After incubation, the absorbance at 405 nm (reference at 492 nm) was estimated. The level of IFN-γ in serum was calculated from the value of the standard.

Histology

Samples from the liver of C57BL/6 mice 6 h after LPS challenge were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using the *in situ* apoptosis detection kit (MK503) from Takara Shuzo Co. Ltd (Kyoto, Japan). Staining of liver sections was performed according to the manufacturer's recommendation. Briefly, sections were deparaffinized and then treated with proteinase K (20 µg/ml) in PBS. Endogenous peroxidase was blocked by a subsequent incubation with 3% H_2O_2 in methanol for 5 min. After washing with PBS, TUNEL reaction mixture was dropped on slides and incubated for 90 min at 37°C. Apoptotic cells were then detected with horseradish peroxidase-conjugated anti-FITC antibodies and 0.26 mg/ml diaminobenzidine (Sigma-Aldrich Japan, Tokyo, Japan) followed by counterstaining with 1% methylgreen.

Electrophoresis and Western blotting

Serum samples were heated with the sample buffer and analyzed using a 12.5% gel by SDS-PAGE according to the method of Laemmli.[22] After blotting, sCD14 was visualized by use of anti-mouse CD14 mAb (rmC5-3, PharMingen) and a Vectastain ABC-PO kit (Vector Laboratories Inc., Burlingam, CA, USA).

Statistical analysis

Statistical significance of the data was determined by Student's *t*-test. A *P* value of less than 0.05 was taken as significant.

RESULTS

Comparison of killed Mycobacterium, *TDM, SL, and MDP-Lys(L18) to induce hypersensitivity to LPS lethality when administered as a w/o/w emulsion*

To compare the inducing activity of test samples, female 8-week-old C57BL/6 mice were injected i.p. with 400 µg test samples in 200 µl of a w/o/w emulsion as vehicle. After 14 days, mice were challenged i.v. with 30 µg of *S. abortus equi* LPS. As shown in Table 1, priming with heat-killed *M. tuberculosis* Aoyama B increased sensitivity to LPS lethality (100%), compared with that of vehicle controls. In the case of heat killed BCG, the activity was weaker than that of Aoyama B (40%). A clear difference in the ability to induce hypersensitive conditions was observed between mycobacterial glycolipids, TDM and SL. Priming with TDM induced lethal shock to all mice, whereas all mice survived in the case

Table 1 Induction of hypersensitivity to LPS by priming with killed *Mycobacterium* and TDM but not with SL and MDP-Lys(L18)

Priming with[a]	Challenge with	Dead/ tested	Lethality (%)[b]
Vehicle alone	LPS	0/4	0
+ Killed BCG	LPS	2/5	40
+ Killed Aoyama B	LPS	5/5	100
+ TDM	Saline	0/4	0
+ TDM	LPS	5/5	100
+ SL	LPS	0/4	0
+ MDP-Lys(L18)	LPS	0/4	0

[a]Female 8-week-old C57BL/6 mice were injected i.p. with 400 μg test samples as the w/o/w emulsion in a volume of 0.2 ml vehicle 14 days before an i.v. challenge with 30 μg *S. abortus equi* LPS in saline. Purified TDM (trehalose 6,6′-dimycolate) and SL (sulfolipid, 2,3,6,6′-tetraacyl trehalose 2′-sulfate) prepared from *M. tuberculosis* Aoyama B were used.
[b]Lethality was assessed by counting numbers of dead mice 72 h after challenge.

of SL. Similarly, MDP-Lys(L18) did not show any detectable activity in this experimental system.

The optimal dose of TDM and LPS for inducing lethal toxicity

To clarify the challenge dose of LPS, female C57BL/6 mice were injected i.p. with either 200 or 400 μg TDM together with a vehicle 14 days before an i.v. challenge with the indicated dose of LPS. As shown in Table 2, a challenge with 3 μg LPS did not induce lethal shock, as values are similar to vehicle controls. One out of four mice succumbed when challenged with 10 μg LPS. At a dose of 30 μg, none of the mice survived. Even after a challenge with 30 μg LPS all mice survived when primed with 200 μg TDM, indicating that a combination of priming with 400 μg TDM and a challenge with 30 μg LPS is critical for inducing lethal shock in this experimental system.

The optimal period to induce hypersensitive conditions by TDM

To determine the optimal interval, female C57BL/6 mice were injected i.v. with 30 μg of LPS at the indicated day after priming with 400 μg TDM. As shown in Table 3, induction of hypersensitivity by TDM was not yet observed on days 2 and 4 after priming. Seven days later, the survival rate in TDM-primed mice was 50% (two out of four). The maximal lethality (100%) was seen in mice 14 days after TDM-priming. The increased sensitivity lasted at least until day 21 days after priming.

Table 2 Challenge dose of LPS for inducing lethal shock in TDM-primed C57BL/6 mice

Priming with[a]	Challenge with	Dead/ tested	Lethality (%)
Saline	100 μg LPS	0/6	0
Vehicle alone	30 μg LPS	0/4	0
+ 400 μg TDM	3 μg LPS	0/4	0
+ 400 μg TDM	10 μg LPS	1/4	25
+ 400 μg TDM	30 μg LPS	4/4	100
+ 200 μg TDM	30 μg LPS	0/4	0

[a]Female 8-week-old C57BL/6 mice were injected i.p. with either 200 or 400 μg TDM in a volume of 0.2 ml vehicle 14 days before an i.v. challenge with the indicated dose of *S. abortus equi* LPS. See footnotes of Table 1 for other details.

Table 3 Sensitization period with TDM for inducing lethal shock in C57BL/6 mice

Priming with[a]	Period (days)	Dead/ tested	Lethality (%)
Vehicle alone	14	0/4	0
+ TDM	2	0/4	0
+ TDM	4	0/4	0
+ TDM	7	2/4	50
+ TDM	14	4/4	100
+ TDM	21	3/3	100

[a]Female 8-week-old C57BL/6 mice were injected i.p. with 400 μg TDM in a volume of 0.2 ml vehicle on the indicated day before an i.v. challenge with 30 μg *S. abortus equi* LPS. See footnotes of Table 1 for other details.

Mouse strain differences in sensitization with TDM

To clarify whether mouse strain differences exist in priming with TDM to induce the hypersensitivity, different strains of mice were injected i.p. with 400 μg of TDM before an i.v. challenge with 30 μg of LPS. As shown in Table 4, BALB/c as well as C57BL/6 mice became hypersensitive to LPS lethality by TDM injection. However, C3H/HeN mice were less sensitive to the lethality (two out of eight). All BALB/c IFN-$\gamma^{-/-}$ mice were resistant to the challenge with LPS after priming with TDM.

Inhibitory effect of SL on sensitization with TDM

To estimate how SL influences the sensitization to LPS lethality, mice were primed with 400 μg TDM and/or SL. As shown in Table 5, none of mice died when challenged with LPS even at a dose of 100 μg 14 days after priming with 400 μg SL. Additionally, a failure of induction of

Table 4 Strain difference in sensitization with TDM for LPS-induced lethal shock

Mouse strains[a]	Dead/tested	Lethality (%)
C57BL/6	4/4	100
C3H/HeN	2/8	25
BALB/c	4/4	100
BALB/c IFN-$\gamma^{-/-}$	0/4	0

[a]Female 8-week-old mice were injected i.p. with 400 μg TDM in a volume of 0.2 ml vehicle 14 days before an i.v. challenge with 30 μg *S. abortus equi* LPS. See footnotes of Table 1 for other details.

Table 5 Inhibition of sensitization with TDM for LPS-induced lethal shock in C57BL/6 mice

Priming with[a]	Challenge with	Dead/ tested	Lethality (%)
Saline	100 μg LPS	0/4	0
Vehicle alone	30 μg LPS	0/4	0
+ TDM	30 μg LPS	4/4	100
+ SL	30 μg LPS	0/4	0
+ SL	100 μg LPS	0/4	0
+ TDM + SL	30 μg LPS	0/4	0

[a]Female 8-week-old C57BL/6 mice were injected i.p. with 400 μg TDM and/or 400 μg SL in a volume of 0.2 ml vehicle 14 days before an i.v. challenge with either 30 or 100 μg *S. abortus equi* LPS. See footnotes of Table 1 for other details.

hypersensitivity to the LPS lethality was observed by priming with SL even at higher dosages (800 and 1,200 μg; data not shown). Interestingly, simultaneous injection of SL with TDM for priming resulted in the complete inhibition of hypersensitivity induction.

Increases in serum IFN-γ levels by priming with TDM

To estimate whether IFN-γ levels in serum increase by priming with TDM, C57BL/6 mice were given an i.p. injection with 400 μg of TDM. As shown in Figure 1, significant production of serum IFN-γ was observed in mice primed with TDM but not with vehicle alone. The serum IFN-γ levels reached a peak (5.95 U/ml) on day 4, gradually declined by day 7 and still kept significant levels (2.66 U/ml) by 14 days after priming. On day 14 the level of serum IFN-γ was higher in mice primed with TDM than with heat-killed *M. tuberculosis* Aoyama B (0.95 U/ml).

Enhancement of TNFα production by priming with TDM

To demonstrate whether the priming effect of TDM is mediated by enhanced production of TNFα in serum,

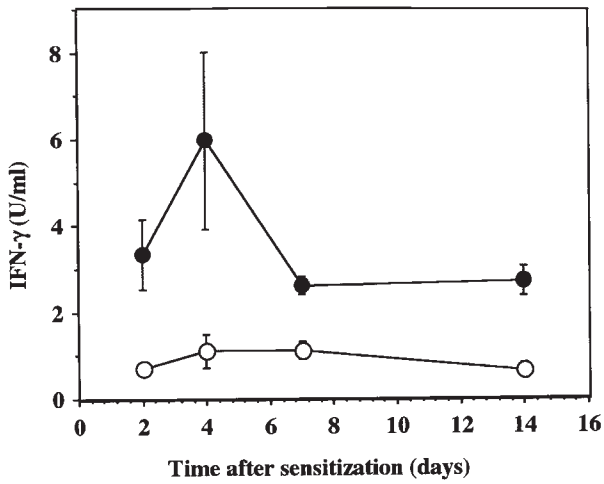

Fig. 1. Kinetics of serum IFN-γ production after priming with TDM and vehicle alone. C57BL/6 mice were injected i.p. with either 400 μg TDM (closed circles) in a w/o/w emulsion or vehicle alone in a volume of 200 μl (open circles). At the indicated days, serum samples were collected from mice. The levels of serum IFN-γ were estimated by ELISA as described in Materials and Methods. Results are shown as arithmetic mean ± SD of 5 mice per each.

Table 6 Enhanced TNF production by priming with TDM and inhibitory activity of SL

Priming with[a]	Serum TNF titer (ng/ml) after LPS challenge	Significance
Vehicle alone	28.7 ± 6.2 (5)	
+ killed Aoyama B	252.8 ± 63.7 (5)	
+ TDM	438.8 ± 99.5 (4)	
+ SL	59.8 ± 16.7 (5)	
+ TDM + SL	181.4 ± 47.5 (4)	vs (+ TDM, $P < 0.01$)

[a]Female 8-week-old C57BL/6 mice were injected i.p. with 400 μg TDM and/or 400 μg SL in a volume of 0.2 ml vehicle 14 days before an i.v. challenge with 30 μg *S. abortus equi* LPS. Results show arithmetic mean ± SD. Numbers in parentheses show mice numbers per group. Significance was assessed by the Student's *t*-test.

C57BL/6 mice were injected i.p. with 400 μg TDM 14 days before an i.v. injection with 30 μg LPS. As shown in Table 6, administration of TDM showed strongly enhanced TNFα production in mice compared with that of vehicle control. As similar to the result of IFN-γ production, the levels of serum TNFα were much higher in mice which had been administered TDM (438.8 ng/ml) than in mice administered heat-killed bacilli of *M. tuberculosis* Aoyama B (252.8 ng/ml). Simultaneous injection of TDM and SL resulted in suppression of TNFα production compared with that of TDM alone.

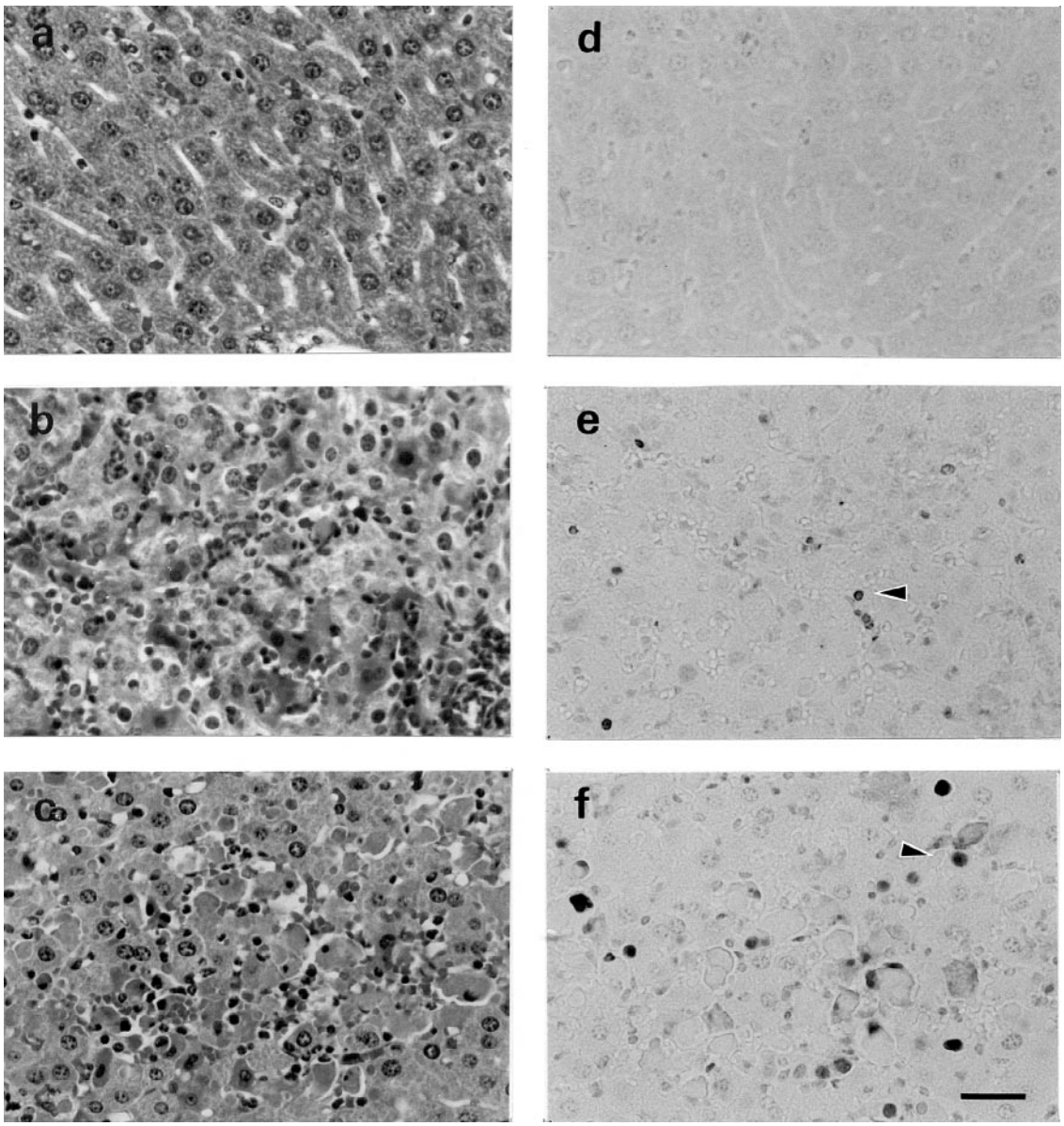

Fig. 2. Histological changes in livers of mice after LPS challenge. C57BL/6 were primed with either 400 μg TDM or vehicle alone 14 days before an i.v. injection with 30 μg *S. abortus equi* LPS. As a positive control, mice received an i.v. injection with 0.1 μg LPS followed by an i.p. injection with 20 mg GalN. Livers were removed from the mice 6 h after the challenge, fixed with 10% formalin and embedded in paraffin. Successive sections were stained with either hematoxylin-eosin (HE) or TUNEL method. (a–c) and (d–f) show the results of HE staining and TUNEL method, respectively. The groups of mice received: LPS alone (a,d); TDM/LPS (b,e); or LPS/GalN (c,f). The arrowhead and the black bar in figures show apoptotic cells and 30 μm length, respectively.

Histological change in liver

To show histological changes in the liver after priming with TDM followed by LPS challenge (TDM/LPS), liver specimens removed from TDM/LPS and LPS/GalN mice 6 h after challenge were fixed with 10% formalin and stained with HE. Local infiltration of mononuclear cells was observed in the liver of mice on day 14 after priming with 400 μg TDM (Fig. 2b) but not with vehicle alone (Fig. 2a). GalN-sensitized mice showed massive cell infiltration in wide areas of liver and appearance of apoptotic cells (Fig. 2c). To confirm apoptosis in liver cells, successive sections were stained using a detection kit for apoptotic cells according to the TUNEL method. The appearance of apoptotic liver cells was observed distinctively in LPS/GalN mice but slightly in TDM/LPS mice on day 14 after priming with 400 μg TDM. Apoptotic cells were mainly seen in the sinus of TDM/LPS mice (Fig. 2e).

Increased release of sCD14 to the circulation

In previous studies, we demonstrated that the release of sCD14 to the circulation after LPS injection increases in the case of $AgNO_3$-induced inflammation[19] and *S. typhimurium* infection (unpublished data). Western blot analysis was carried out to determine whether sensitization with either 400 μg of TDM or SL 14 days before 30 μg LPS challenge enhances the release of sCD14. As shown in Figure 3, priming with TDM increased the levels of sCD14 in the serum when compared with that of SL, suggesting that TDM stimulates the responsiveness to LPS via formation of sCD14. The levels of sCD14 in a vehicle control stimulated with LPS was similar to that of mice primed with SL and then challenged with LPS (data not shown).

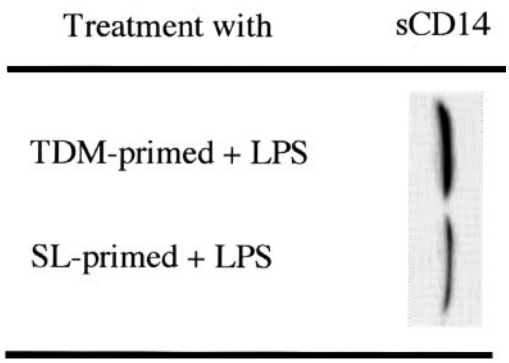

Fig. 3. Difference in sCD14 release between TDM and SL-primed mice. C57BL/6 mice were primed with 400 μg of either TDM or SL 14 days before LPS challenge. Serum samples were collected from 5 mice per group 6 h after an i.v. injection with 30 μg *S. abortus equi* LPS and pooled. After blotting, sCD14 in aliquots (2 μl) of the samples was visualized by using anti-mouse CD14 mAb and Vectastain ABC-PO kit.

DISCUSSION

In this study we demonstrated that priming with TDM as well as heat-killed bacilli of *M. tuberculosis* Aoyama B in a form of w/o/w emulsion was able to induce hypersensitive conditions to LPS lethality. Parant *et al*. showed that the chain length of mycolic acid was important for expressing the activity of TDM, since TDM molecules carrying the $C_{85\pm5}$ fatty acids were the most effective.[14] The chain length of mycolic acid in TDM isolated from *M. tuberculosis* Aoyama B is approximately C_{76-88}.[15] Therefore, the chain length of mycolic acid of TDM tested would be suitable. Yarkoni and Rapp reported that suitable conditions for making TDM emulsions were important for inducing hypersensitivity to LPS lethality,[16] *i.e*. increasing the oil concentration and decreasing the Tween 80 concentration needed for induction of hypersensitive condition by TDM. Concentrations of 0.2% Tween 80 and 5–10% oil were found to be optimal. In this study, a combination of approximately 0.2% Tween 80 and 3.2% Freund's incomplete adjuvant (containing a detergent at equal volume) was used. Thus even if a lower oil concentration was used, hypersensitivity to LPS lethality could be induced.

This induction was dose-dependent and stronger than that induced by heat-killed bacilli of *M. tuberculosis* Aoyama B in terms of the production of IFN-γ and TNFα (Fig.1 and Table 6). On the other hand, SL, a glycolipid without mycolic acid, did not induce hypersensitive conditions (Table 1). In the case of priming with MDP-Lys(L18) in a w/o/w emulsion, the hypersensitivity was also not induced (Table 1), although others have shown that the injections of MDP and LPS in aqueous solutions elicited lethal shock.[4–6] These results suggest that the presence of mycolic acids in mycobacterial glycolipids is important for inducing hypersensitive conditions but not for eliciting the shock.

We also demonstrated that strain differences in induction of hypersensitivity by TDM exist (Table 4). BALB/c as well as C57BL/6 mice showed similar results in LPS lethal shock, but C3H/HeN mice were somewhat more resistant to lethality. It is known that C57BL/6 and BALB/c mice belong to the strain (Ity^s/Bcg^s) susceptible to infections with *Salmonella typhimurium*, *Listeria monocytogenes* and *M. bovis* BCG, while C3H/HeN mice belong to the strain (Ity^r/Bcg^r) resistant to these infections. Natural resistance to infection with intracellular parasites is controlled by a dominant gene (*Lsh/Ity/Bcg*) on mouse chromosome 1, designated *Nramp* (natural resistance-associated macrophage protein).[23] However, the induction of hypersensitivity does not correlate to the genetic difference between *Nramp*. Development of delayed-type hypersensitivity (DTH) to mycobacterial antigens differs among the mouse strains and relates to the H-2 loci: C57BL/6 ($H\text{-}2^b$) as a high responder, BALB/c ($H\text{-}2^d$) as an intermediate responder and C3H/HeN ($H\text{-}2^k$) as a low responder.[24,25] Therefore, the difference in induction of hypersensitivity by TDM seems to reflect the difference in the development of DTH.

Regarding the induction of hypersensitivity, SL, one of the virulence factors during mycobacterial infection,[17] exhibited not only inability but also the suppressive action to TDM-priming when injected together with TDM (Table 5). It has been reported that macrophage functions such as O_2^- and cytokine production were inhibited when SL was added to human monocyte cultures.[26] Administration of SL did not increase release of sCD14 after LPS challenge (Fig. 3) and also production of serum IFN-γ (data not shown). As shown in Figure 1, TDM stimulated the production of serum IFN-γ. Conversely, the priming effect of TDM was not observed in BALB/c IFN-$\gamma^{-/-}$ mice. During priming with TDM, therefore, it might be that TDM acts on macrophages to secrete IL-12 and

IL-18, and these cytokines activate NK cells to produce IFN-γ. In contrast, SL seems to suppress the production of cytokines and to act as the suppressive component comparable to the effect of lipoarabinomannan during mycobacterial infections.

It is known that sensitization with GalN results in hypersensitivity to TNFα which is produced by macrophages responding to a small amount of LPS.[27] Massive cell infiltration in wide areas and appearance of apoptotic cells were observed in the liver of GalN-sensitized mice after the challenge with LPS (Figs 2c and 2f). In mice primed with TDM followed by LPS challenge, the cell infiltration pattern was different from that in GalN/LPS mice. In flow cytometry, priming with TDM resulted in disappearance of liver $CD4^+$ $NK1.1^+$ T cells from the interface of Percoll-gradient centrifugation (unpublished data). The down-modulation of IL-4-producing $TCR\alpha\beta^{intermediate}$ $CD4^+$ $NK1.1^+$ T cells in the livers of mice infected with live *M. bovis* BCG is responsible for IL-12 production during infection.[28] We need further studies to clarify the mechanism by which TDM acts on various types of cells participating in the immune response.

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