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• *BASIC RESEARCH* •

Lethal effect and apoptotic DNA fragmentation in response of D-GalN-treated mice to bacterial LPS can be suppressed by pre-exposure to minute amount of bacterial LPS: Dual role of TNF receptor 1

Bing-Rong Zhou, Marina Gumenscheimer, Marina A. Freudenberg, Chris Galanos

Bing-Rong Zhou, Department of Microbiology, Second Military Medical University, Shanghai 200433, China
Marina Gumenscheimer, Marina A. Freudenberg, Chris Galanos, Max-Planck-Institut für Immunbiologie Stübeweg 51 D-79108 Freiburg in Br. Germany
Supported by a fellowship (to Zhou B) from Max-Planck-Society, Germany, and partially supported by the National Key Basic Research and Development Program (973 Program) of China, No. 2002CB513006 (to Zhou B)
Correspondence to: Dr. Chris Galanos, Max-Planck-Institut für Immunbiologie Stübeweg 51 D-79108 Freiburg in Br. Germany. galanos@immunbio.mpg.de
Telephone: +49-761-5108400 Fax: +49-761-5108403
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Abstract

AIM: To investigate whether induction of tolerance of mice to lipopolysaccharide (LPS) was able to inhibit apoptotic reaction in terms of characteristic DNA fragmentation and protect mice from lethal effect.

METHODS: Experimental groups of mice were pretreated with non-lethal amount of LPS (0.05 μg). Both control and experimental groups simultaneously were challenged with LPS plus D-GalN for 6-7 h. The evaluations of both DNA fragmentations from the livers and the protection efficacy against lethality to mice through induction of tolerance to LPS were conducted.

RESULTS: In the naive mice challenge with LPS plus D-GalN resulted in complete death in 24 h, whereas a characteristic apoptotic DNA fragmentation was exclusively seen in the livers of mice receiving LPS in combination with D-GalN. The mortality in the affected mice was closely correlated to the onset of DNA fragmentation. By contrast, in the mice pre-exposed to LPS, both lethal effect and apoptotic DNA fragmentation were suppressed when challenged with LPS/D-GalN. In addition to LPS, the induction of mouse tolerance to TNF also enabled mice to cross-react against death and apoptotic DNA fragmentation when challenged with TNF and/or LPS in the presence of D-GalN. Moreover, this protection effect by LPS could last up to 24 h. TNFR1 rather than TNFR2 played a dual role in signaling pathway of either induction of tolerance to LPS for the protection of mice from mortality or inducing morbidity leading to the death of mice.

CONCLUSION: The mortality of D-GalN-treated mice in response to LPS was exceedingly correlated to the onset of apoptosis in the liver, which can be effectively suppressed by brief exposure of mice to a minute amount of LPS. The induced tolerance status was mediated not only by LPS but also by TNF. The developed tolerance to either LPS or TNF can be reciprocally cross-reacted between LPS and TNF challenges, whereas the signaling of induction of tolerance and promotion of apoptosis was through TNFR1, rather than TNFR2.

Key words: LPS tolerance; DNA fragmentation; Apoptosis; TNF receptor

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INTRODUCTION

Bacterial lipopolysaccharide (LPS), also known as endotoxin, is an integral component of outer membrane located in the cell wall of Gram-negative bacteria, and is one of the main causative agents of septic shock in the duration of Gram-negative bacterial infection in humans[1]. It has been found *in vivo* that the pathogenic role of LPS is not directly through LPS molecule itself, but rather mediated by a group of endogenous mediators including TNF[1-3], IL-6[1,3], and IL-12[1,2], which, when in excess, leads to serious systemic disorders with a high mortality rate[1]. Among these, TNFα is regarded as a reputed agent involved in the development of LPS pathogenesis[1], it binds to corresponding surface receptors exerting its biological effects. These receptors are termed as TNFR1 and TNFR2[5-9], respectively.

However, increasing evidence showed that by prior exposure of animals like mice to a minor amount of LPS (even a non-lethal dose of LPS was applied), a hypo-responsiveness or refractoriness to subsequent challenge of LPS was developed[10-15]. This phenomenon has been described as tolerance of host to LPS[10-15]. LPS tolerance can be observed with a decreased febrile response and escape from death as well as a reduced production of inflammatory endogenous cytokines in response to a secondary stimulation with LPS[10-15]. In this case, tolerant mice towards LPS actually protect themselves

from lethality due to pretreatment with LPS. Furthermore, it has also been found that the tolerant mice to LPS are cross-reaction towards the pathogenic role of TNF[10,12,13] in terms of protection of host. In some reports[11,12], tolerance of mice to TNF was also able to alleviate and counteract deadly suffering in the affected mice while challenged with a lethal combination of TNF and D-GalN[13].

Apoptotic reaction has been extensively studied in cell models and/or in special animal models. Cells undergoing apoptosis exhibit specific morphological changes[17,19,24], including membrane blebbing, cytoplasmic and chromatin condensation, nuclear breakdown, DNA fragmentation, assemble of membrane-enclosed vesicles termed apoptotic bodies. All these are eventually subjected to phagocytosis. Among these, DNA fragmentation has been regarded as a classic mark[16-24]. We have found that a characteristic apoptotic DNA fragmentation in the liver of the affected mice was intimately correlated to lethality in mice that were challenged with a tremendously low amount of LPS (0.05 μg) in combination with D-GalN[27]. In this study, we addressed that whether the tolerance of mice to LPS would inhibit the apoptotic DNA fragmentation, and which TNF receptors were involved in the induction of lethality and tolerant status formation.

MATERIALS AND METHODS

Chemicals

Recombinant human tumor necrosis factor (rhTNFα) was provided by the BASF Company (Ludwigshafen, Germany), the activity was 5×10^6 U/mg protein. LPS was prepared from *Salmonella abortus equi* as described previously[28]. D-GalN hydrochloride was purchased from C. Roth Karlsruhe, Germany.

Mice

Mouse strains were kept under specific pathogen-free conditions in the animal facilities. In this study, C57BL10 ScSn (ScSn) and TNFR1-/-, and TNFR2-/- (genotypic background BL6´129sv) were used, respectively. Ten-week-old mice weighing approximately 22-25 g of both sexes were housed in stainless steel wire cages with free access to food and water. There was no difference in either male or female mice. After about a 1-wk equilibration period, the animals were randomly divided into different experimental groups according to the protocol. Mice were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals in Germany, as legally required. The experimental protocol was carried out in compliance with German regulations and local ethical committee guidelines for animal research.

Experimental design

All reagents used in these experiments included D-GalN, LPS, and TNFα. They were first dissolved in pyrogen-free phosphate-buffered saline (PBS, pH 7.2). Combination of LPS and/or TNFα with D-GalN (each dose of reagent used was indicated in the figure legend) in a total volume of 200 μL was injected into the mice. This volume was also used throughout the study. Injections were usually performed intravenously (i.v.) in the lateral tail vein of the mice.

Induction of tolerance and lethal test[29]

For the induction of tolerance, mice were pretreated with LPS (usually a concentration of 0.05 μg) or TNFα (usually a concentration of 2 μg) for different time lengths, thereafter the mice were challenged with the required reagent in combination with D-GalN for indicated time periods (usually for 6-7 h). As controls, mice received LPS and/or TNF in combination with D-GalN for an indicated time length. At certain time point, the livers were removed under ether anesthesia and DNA extraction was carried out immediately. Blood was collected under ether anesthesia by puncturing the axillary vessels, plasma was prepared and stored at -80 ℃ until use. For the lethal effect of the mice after challenged with the indicated reagent, usually the mice were observed for 24 h.

Removal of organs from mice and homogenization[27,30]

Organs of interest (livers either from experimental mice or from control mice) were removed under ether anesthesia of the animals, and placed in 1 mL lysis buffer solution (10 mmoL Tris-HCl, pH 7.5, 100 mmoL EDTA, 0.5% SDS, proteinase K 100 μg/mL, proteinase K was added shortly before use) and immediately pressed with the flat end of a sterile syringe piston through a metal mesh placed on ice. Alternatively, the organs were placed in a glass homogenizer containing 1 mL of lysis buffer, and homogenized at a high-speed vortexing (3 000 r/min) for 5 min on ice. The resulting suspensions were kept at -40 ℃ until extraction of DNA.

Extraction and preparation of RNA-free DNA

Usually 0.4 mL of the homogenized tissue suspension as described above was used for DNA extraction. The extraction procedure used was basically the same as described previously[27,30]. DNA from each sample was extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol (PCI, 25:24:1; V:V:V) and once with chloroform. The procedure was detailed as follows. An equal volume of phenol (0.4 mL) was added to 0.4 mL suspension of homogenized tissues, and mixed well by vortexing vigorously for 5 min. After centrifugation at 17 300 *g* at 4 ℃ for 10 min, the supernatant (aqueous phase) containing DNA was pipetted into a clean microcentrifuge tube. To this an equal volume of phenol was added and extraction was repeated as described above. The resulting supernatant was extracted once with PCI and once with chloroform to remove residual phenol. After centrifugation at 17 300 *g* at 4 ℃ for 10 min, the supernatant was transferred to a clean tube and DNA was precipitated by adding 2.5 volumes of absolute ethanol and sodium acetate to a final concentration of 0.3 mol/L and kept at -20 ℃ for 30 min (or overnight). Next it was spun by centrifugation (17 300 *g* at 4 ℃ for 10 min) and pellet was dissolved in TE buffer (Tris-HCL 10 mmol/L, pH 7.4, ethylene diaminotetraacetic acid 1 mmol/L, pH 8.0) containing RNase (0.1 mg/L) at 37 ℃ for 1 h. Subsequently, the suspension was re-extracted with phenol, PCI and chloroform, and precipitated as described above. Finally, the concentration of DNA was measured by a spectrophotometer (PM6, Zeiss, Jena, Germany).

Analysis of DNA ladder by agarose gel electrophoresis[20,23,27,30]

Before electrophoresis, the DNA concentration of the samples

was adjusted to approximately equivalent concentration. About 2-3 μg of DNA sample was mixed with loading buffer (40% sucrose, 0.25% xylene cyanol, 0.25% bromophenol blue) and placed in the wells of agarose gel. The agarose gel (15 mL/L) in electrophoresis buffer TBE (0.5×) was run at 100 V until the purple tracer marker migrated to approximately 2 cm before the end of the gel. The gels were photographed under UV light using a Mitrubishi video copy processor (Gibcol BRL, Heidelberg, Germany), graphs were stored in a magnetic disc. Alternatively photos were printed out directly for immediate analysis of results.

TNFα bioassay

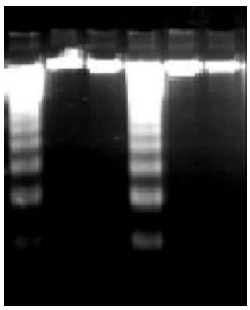
TNFα in plasma was measured in a cytotoxicity test using a TNF-sensitive L929 cell line in the presence of actinomycin D as described previously[31]. The detection limit of the assay was 50 pg TNF/mL plasma.

RESULTS

Pre-exposure to LPS effectively suppressed apoptotic DNA fragmentation

We found that the mortality in mice when challenged with LPS at the dose of 0.05 μg and in the presence of D-GalN (20 mg) was paralleled well to the characteristic apoptotic hallmark, DNA fragmentation in the liver of the affected mice, which was also shown elsewhere[27]. We further explored whether pre-exposure of mice (ScSn) to LPS (0.05 μg) was able to inhibit the onset of apoptotic DNA fragmentation and protect host mice from lethal challenge as well as which receptor signaling pathway in the mice was pre-exposed to a minute dose of LPS.

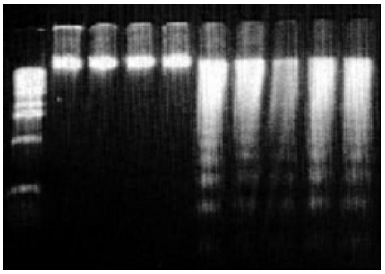
The results showed that when mice were pre-exposed to LPS for as long as 75 min and then challenged with a combination of LPS (0.05 μg) in the presence of D-GalN (20 mg) for 6 h, the distinctive apoptotic DNA fragmentation was suppressed (Figure 1, lanes 2 and 3). In contrast, in the naïve mice that had no pre-exposure to LPS, the apoptotic DNA fragmentation from the homogenate liver of mice was clearly seen in agarose gel electrophoresis (Figure 1, lane 1 and Figure 2, lanes 8 and 9). Therefore, pre-exposure of mice to LPS was able to suppress the onset of apoptotic DNA fragmentation.



Lanes	1	2	3	4	5	6
Pre-LPS	-	+	+	-	+	+
LPS/GaIN	+	+	+	-	-	-
TNF/GIaN	-	-	-	+	+	+

Figure 1 Suppression of LPS and/or TNF-induced apoptotic DNA fragmentation in D-GalN-treated mice by pre-exposure to LPS.

Moreover, in mice pre-exposed to TNF (2 μg) instead of LPS for 3 h, the same results as those in the mice pre-exposed to LPS were shown and that it effectively suppressed apoptotic DNA fragmentation when mice were challenged with TNF (1 μg) plus D-GlaN (20 mg) for 6 h (Figure 2, lanes 1 and 2). However, in the control mice receiving TNF (1 μg) and D-GalN (20 mg), DNA fragmentation occurred (Figure 1, lane 4 and Figure 2, lanes 5-7). As a result, pre-exposure to either TNF was both able to inhibit the characteristic apoptotic DNA fragmentation when challenged with TNF in the presence of D-GalN.



Lanes	M	1	2	3	4	5	6	7	8	9
Pre-TNF		+	+	+	+	-	-	-	-	-
TNF-GaIN		+	+	-	-	+	+	+	-	-
LPS-GaIN		-	-	+	+	-	-	-	+	+

Figure 2 Inhibition of LPS and/or TNF-induced DNA fragmentation in D-GalN-treated mice by pre-exposure to TNF, M: DNA marker.

Cross-reaction in the suppression of apoptotic DNA fragmentation

In addition to the suppression of apoptotic DNA fragmentation by pre-exposure of the corresponding homogenous agents and then challenge with LPS and/or TNF in the presence of D-GalN, DNA fragmentation could also be suppressed by the corresponding heterogeneous agents. In this study, we found that when pre-exposure to LPS (0.05 μg) for 75 min, apoptotic DNA fragmentation was dramatically suppressed in mice when challenged with TNF (1 μg) in the presence of D-GalN (20 mg) (Figure 1, lanes 5 and 6). Furthermore, this also took place in the mice that were pre-exposed to TNF (2 μg) instead of LPS for 3 h and subsequently challenged with LPS (0.05 μg) other than TNF, in the presence of D-GalN. Therefore, the suppression of characteristic apoptotic DNA fragmentation seemed to be a cross-reaction (Figure 2, lanes 3 and 4).

Induction of LPS tolerance and onset of apoptotic DNA fragmentation

Biological roles of LPS were mediated by endogenous TNF molecules produced from macrophage cells and other cell types[1-2,9]. TNF molecules must bind to the cognated TNF receptors responsible for signals in the biological roles of TNF molecules[5,8,9]. Here, we used the mice that were knocked out of corresponding TNF receptor genes. These mice were directly challenged with LPS and D-GlaN under the same experimental conditions as those performed in ScSn mice aforementioned. The results showed that administration of LPS (0.05 μg) and D-GalN (20 mg) resulted in apoptotic

DNA fragmentation only in the livers of TNFR2-/-mice (Figure 3, lanes 1 and 3), while the DNA fragmentation was absent in the livers of TNFR1-/-mice (Figure 3, lanes 5 and 7). In addition, in the same experimental situation, the DNA fragmentation in the spleens from TNFR1-/- and TNFR2-/- mice, respectively, was also examined. The results showed that neither in the spleens of TNFR1-/-mice (Figure 3, lanes 6 and 8) nor in those of TNFR2-/-mice (Figure 3, lanes 2 and 4), apoptotic DNA fragmentation was detectable. These results depicted in Figure 3 strongly suggested that the induction of apoptotic DNA fragmentation in mice by LPS was mediated by TNFR1 rather than TNFR2. TNFR2 did not seem to be involved in the induction of apoptotic DNA fragmentation by LPS, since its absence in TNFR2-/-mice had no influence on the development of apoptotic DNA fragmentation. Still in this situation, the host liver, not the spleen, was a target organ when challenged with LPS in the presence of D-GalN.

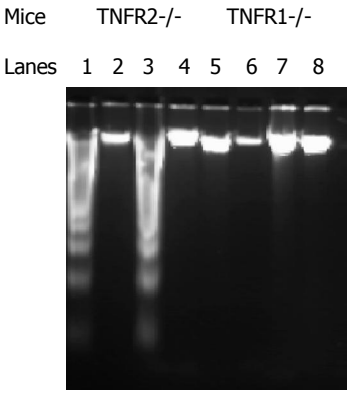


Figure 3 Requirements of TNFR1 for the induction of DNA fragmentation by LPS in D-GlaN-treated mice.

In the following experiments, we still investigated the role of TNFR1 in the induction of tolerance to LPS. The TNFR2-/-mice were pretreated with LPS (0.1 μg) for 75 min, the property was retained that is the tolerance to the induction of apoptotic DNA fragmentation by a challenge with LPS (0.05 μg) in the presence of D-GalN (20 mg) (Figure 4, lanes 1-3). In contrast, the control mice that had no pretreatment with LPS (Figure 4, lanes 4-6) and were directly challenged with LPS (0.05 μg) in the presence of D-GalN, exhibited the expected DNA fragmentation. These data indicated that the development of tolerance to LPS could lead to the inhibition of DNA fragmentation in the livers of affected mice when challenged with LPS and involved TNFR1 only.

Suppression of apoptotic DNA fragmentation activity in the liver of mice pre-exposed to LPS

We found that the ScSn mice pre-exposed to LPS (0.05 μg) for 75 min were able to suppress the apoptotic DNA fragmentation when challenged with LPS (0.05 μg) plus D-GlaN (20 mg) (Figure 1). In the following experiments, we studied the time course of development and duration of tolerance induced by LPS pretreatment and the apoptotic

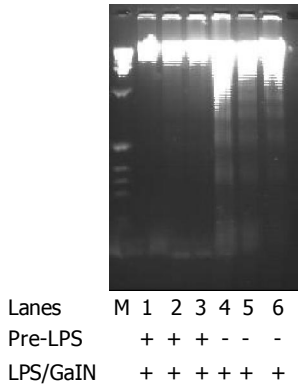


Figure 4 Induction of tolerance to the apoptotic activity by LPS in TNFR2-/-mice, M: DNA marker.

effect of LPS on D-GalN-treated mice. When pretreated with LPS (0.1 μg) for different time periods (Figure 5), the TNFR2-/-mice were challenged with a lethal combination of LPS (0.05 μg) and D-GalN (20 mg). Seven hours after challenge, the livers were removed under ether anesthesia and DNA was extracted and analyzed for the presence of fragmentation (Figures 4, 5 and Table 1). The results showed that the suppressive effect on the onset of apoptotic DNA fragmentation was detectable after preexposure of mice to LPS for 75 min (Figures 1 and 4) and this effect was maintained for up to 24 h (Figure 5 and Table 1).

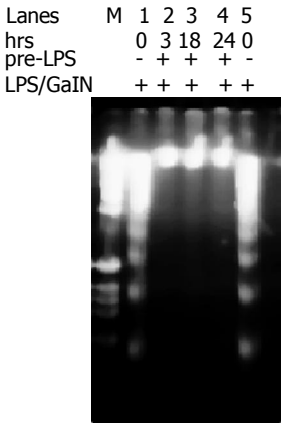


Figure 5 Time course of DNA fragmentation inhibition pretreated by LPS in TNFR2-/-mice, M: DNA marker.

Table 1 Effect of LPS pretreatment on the lethality and DNA fragmentation induced by LPS/D-GalN in TNFR2-/- mice

LPS pretreatment (0.1 μg) before challenge (h)	Challenge: LPS (0.05 μg) + D-GalN (20 mg)	
	Lethality (dead/total)	DNA fragmentation
None	5/5	+
24	0/5	-
18	0/5	-
3	0/5	-
1.25	0/5	-

+, fragmentation present; -, fragmentation absent.

Lethal effects of LPS on D-GlaN-treated mice correlated with characteristic apoptotic DNA fragmentation

In the following experiments, we investigated whether a correlation existed between the apoptotic DNA fragmentation in the livers of affected mice and lethal outcome. Mouse strains of TNFR2-/- were pretreated with 0.1 μg of LPS for different time periods, and then challenged with LPS (0.05 μg) in the presence of D-GalN. Mice with no pretreatment served as controls. The results indicated that DNA fragmentation in the liver of affected mice was highly correlated with the mortality of mice, which was due to the onset of apoptosis in the liver of affected mice (Table 1). Likewise a similar correlation between suppression of DNA fragmentation and suppression of mouse death was also seen in mice after pretreatment with TNFα and challenged with TNFα in combination with D-GalN (data not shown).

DISCUSSION

LPS, as a very important pathogenic agent released from Gram-negative bacteria[1], exerts dual biological roles in animals and humans. In the experimental animal model, if the mice were administrated with a high dose of purified LPS, a commonly lethal effect on mice would ensue and severe injuries would occur in some vital organs documented as multi-organ failure. On the contrary, if the animals were pre-exposed to a minute amount of LPS, or to detoxified LPS[1,32,33], and/or derivatives of LPS, monophosphoryl lipid A[32], a hypo-reaction would develop, protecting the affected mice from later challenge with a lethal amount of LPS and even live bacteria[34]. These distinctively paradoxical effects[7, 35,36] induced by LPS are attributed to the pre-exposure of mice to LPS, therefore alternating the potential reactivity of mice to bacterial component LPS.

In the present study, pre-exposure of animals to a minute amount of LPS leading to the suppression of both apoptotic DNA fragmentation and lethality against the later lethal challenge of LPS in the presence of D-GalN, to some extent, might reflect the role of hosts in terms of instinctive protection of themselves[7,10, 12-14]. This phenomenon in mice and other relevant host animal models has been widely documented, particularly in the field of study on tolerance of hosts to lethal toxicity of LPS[10-14]. However, the actual mechanism underlying the tolerance of hosts to LPS still seems obscure.

The exploration of mechanism responsible for cell apoptosis and anti-apoptosis (suppression of apoptosis[7,18,25]) has become a hot spot. Appropriate pretreatment with LPS resulting in a prolonged survival when challenged with a high dose of LPS and others including a lethal combination of LPS (or TNF) and D-GalN, which is a special case in studying mechanisms of how a given host responds to LPS, has been so far reported to be ascribed to the inhibition of apoptosis[16,19,38]. Hence, the onset of apoptosis challenged by exposure to LPS and induction of tolerance through pre-exposure of host and/or cells to LPS leading to suppression of apoptosis consists of the interaction between the protection role in host cells and detrimental effect of causative agents from bacteria. The similarity between the tolerance to the lethal toxicity of LPS, and the suppression of apoptosis has greatly intrigued us to further explore the mechanism, which might be due to the existence of a common signaling pathway[7,26,38].

The results of this study showed that, by a brief pre-exposure to a minute amount of LPS alone, induction of apoptosis in terms of the characteristic apoptotic hallmark, DNA fragmentation[17-19,24] and lethal effect of LPS were effectively suppressed. The pre-exposure to LPS was able to prevent mice from the predestined death when challenged with a lethal combination of LPS and D-GalN. Moreover, this protective effect on lethality and apoptosis by pre-exposure to a causative agent other than LPS, even by TNF, was also quite apparent. The results of the present study indicated that this protection role in suppression of both apoptotic reaction and lethal effect was seemingly related to the action of either LPS or TNF. In fact, whether endogenous TNF production induced by LPS or recombinant human TNF, they were able to converge signals through TNFR1[4,8], which functions in signaling to apoptotic reaction, and induction of tolerance to LPS. In view of this, TNF is still a key molecule outside of cells.

TNF, as one of the most putative mediators[1-2,10-15] in the pathogenesis of LPS and Gram-negative bacterial infection, has been widely proved. However, in the present study, TNF activity in plasma was not detectable (data not shown). The possible reason might be the concentration (0.05 μg only) of LPS used was too low to induce a large amount of free TNF molecules released into blood circulation. However, in order to study the action of TNF, we used TNFR2-/- mice. When challenged with LPS (0.05 μg) plus D-GalN, the lethality and apoptotic DNA fragmentation and suppression of both in TNFR2-/- mice could be seen as those in ScSn mice. This clearly indicated that the TNF molecules produced by such a low amount of LPS were in action. On the other hand, the free TNF molecules in plasma were not detectable, this could not exclude the role of membrane-type TNF[8,9,43] despite that we did not detect the membrane-type TNF in the present study. However, membrane TNF binds to the cytoplasmic membrane carrying out its functions like free-type TNF molecules through local cell-cell interaction. Therefore, in inducing apoptotic reaction in the liver and lethality in mice, the membrane-type TNF might exert its biological role.

However, signaling in inducing apoptotic reaction and lethality is through TNFR1[5,6,8], the signaling in which the induction of tolerance to LPS in mice leads to suppression of apoptotic effect and lethality is also through TNFR1[4,6,8,9]. The results in Figures 4 and 5 clearly indicated that induction of tolerance to apoptotic reaction and lethality involved only TNFR1. Therefore, TNFR1 plays a dual signaling role in inducing apoptotic reaction and lethality as well as host protection in terms of suppression of apoptotic reaction and lethality. Regarding the duality[7,38-42] of TNFR1 in signaling of either induction of apoptotic reaction or induction of suppression of apoptotic reaction due to tolerance, the latest progress of TNFR1 in signaling of cells still involves a complicated signaling circuit in post-receptors. The bifurcation and divergence point[26,38-42] in signaling is seemingly dependent upon the turn of the activation of signaling molecules tending to die (e.g., Caspase 8, Caspase 3 and others[17-19,21-24]) and to survive (e.g., activated NFκB and others[25,37-42] could protect cells).

The role and function of TNFR2 in the induction of apoptotic reaction and lethality are less clear and not as evidenced as TNFR1. Some studies reported that TNFR2 possessed the ability to pass TNF ligand[4] to TNFR1 leading to a relatively local high concentration of TNF in the vicinity of TNFR1 that accepts TNF ligand from TNFR2 and is then activated, signaling the TNFR1 apoptotic machinery. In the present study, the results showed that TNFR2 did not directly take part in the induction of apoptotic reaction. Despite that we used recombinant human TNF instead of mouse TNF, it was reported that in mice only TNFR1 could bind to human TNF molecules[8]. This also implicated that induction of apoptotic DNA fragmentation and lethality as well as suppression of both involved TNFR1[4-6,8,9] rather than TNFR2.

The fragmentation of DNA in an ongoing apoptotic reaction of host cells has been regarded as a gold standard[17,19,23,24]. In necrosis, DNA is degraded showing a smear in electrophoresis gel. However, in this study, when a combination of LPS and D-GalN was administered, a fragmented DNA pattern was distinguishable. In the control mice receiving LPS (0.05 μg), TNF (2 μg) and D-GalN (20 mg) respectively, DNA molecules were intact. The characteristic DNA fragmentation in the mice challenged with LPS plus D-GalN might involve a set of endogenous deoxyribonucleases, which have been described as a killer and/or executor [21] in ongoing apoptotic reaction in cells. Up to now, some of them have been characterized, such as DNase[17-19,21-24], Caspases family[21] and Caspase-activated DNase (CAD/DFF40)[18,19,23], which are activated and executed to degrade the corresponding substrates, ultimately leading to cell death. In fact, the degradation of nucleosomal DNA as one of the parameters in apoptotic reaction is based upon endogenous DNase that could recognize and cleave the special nucleic acid sequence between chromosomes[23-24] during apoptosis. However, it is not yet completely clear whether the inhibition of lethality and DNA fragmentation in response of D-GalN-treated mice to LPS (or TNFα) resulted from down-regulated endogenous DNase activity by unknown mechanism in the mice tolerant either to LPS or to TNFα.

The data obtained in this study suggested that the experimental sequence of D-GalN *vs* LPS or TNFα was a crucial factor, which could determine the death or survival of the affected mice. For instance, if mice were exposed to LPS (or TNFα) before D-GalN, they subsequently developed tolerance to lethality and DNA fragmentation. However, if exposure of mice to D-GalN was before exposure to LPS (or TNFα), it resulted in lethality in mice and apoptotic DNA fragmentation in the liver.

D-GlaN was originally identified as a hepatotoxic agent[29]. The underlying mechanism of its toxicity was shown to involve a strong depletion of uridine triphosphate in hepatocytes[29], which leads to an inhibition of synthesis of macromolecules, including RNA, membrane glycoproteins and glycogen. Inhibition of RNA synthesis induced by D-GalN could lead to a further decrease in protein synthesis[37]. Therefore, the crucial consequence of D-GalN action on the liver is an arrest of vital protein synthesis[37] at the transcriptional level. Although D-GalN could increase the sensitization of mice or cultured cells to LPS (or TNF), mice tolerant to LPS or TNFα could still effectively antagonize the lethality and apoptotic activity in the liver, suggesting that D-GalN is not able to alter or reverse this tolerant status to LPS or TNFα in mice. The role of D-GalN was reported to arrest transcription. However, more detailed experiments are needed to elucidate its mechanism.

In conclusion, pre-exposure of mice to a minute amount of LPS and/or TNFα can effectively suppress both apoptotic reaction and lethality. Induction as well as suppression of apoptotic reaction involves TNFR1. Thereby, mice will protect themselves from the detrimental effect even the lethal challenge with a high dose of LPS and bacteria.

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