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TNF/Lymphotoxin-α Double-Mutant Mice Resist Septic Arthritis but Display Increased Mortality in Response to Staphylococcus aureus

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To evaluate the importance of the proinflammatory cytokines TNF and lymphotoxin-α (LTα) in an experimental model of Staphylococcus aureus sepsis and arthritis, we used TNF/LTα-double-deficient mice raised on the C57BL/6 background. Mice were i.v. inoculated with a toxic shock syndrome toxin-1 (TSST-1)-producing S. aureus strain, LS-1. Intravenous inoculation of a high dose of bacteria (1 × 10⁷/mouse) resulted in 67% mortality in TNF/LTα-deficient mice, whereas none of the controls died (p = 0.009). Those results correlated to a significantly decreased phagocytosis in vitro and inefficient bacterial clearance in vivo in mice lacking capacity to produce TNF/LTα. Thus, at day 6 after inoculation, S. aureus could not be found in the bloodstream of controls, but bacteremia developed in all TNF/LTα-deficient mice examined (p = 0.02). Interestingly, upon infection with a lower dose of staphylococci (3 × 10⁶/mouse) the mortality was overall low, but the frequency of arthritis was clearly higher in the wild-type group as compared with the TNF/LTα-deficient mice (40% vs 13%). Histopathologic examination revealed a lower frequency of synovitis (38% vs 90%, p < 0.05) and erosivity (25% vs 60%, NS) in TNF/LTα-deficient mice as compared with wild-type counterparts. Our results show the importance of TNF/LTα in defense against systemic S. aureus infections and point out the detrimental role of these cytokines as mediators of inflammatory response in S. aureus arthritis. The Journal of Immunology, 1998, 161: 5937–5942.

Bacterial arthritis is a serious disease with high mortality ranging from 5–56% depending on patient category and extent of joint manifestations, the higher values representing rheumatoid arthritis (RA) patients with polyarticular septic arthritis (1–4). The rapid progression and destruction of affected joints often results in sequelae, despite eradication of bacteria, and imply that manipulation of immune system/inflammatory responses might be of benefit in parallel with antibiotic treatment. In this respect, we have recently shown beneficial effects of systemic corticosteroid administration along with antibiotic therapy on the outcomes of Staphylococcus aureus arthritis (5). The most common pathogen in bacterial arthritis is S. aureus (2, 3). In our mouse model, we use an i.v. inoculum of a S. aureus strain LS-1, secreting toxic shock syndrome toxin-1 (TSST-1), an exotoxin with superantigenic properties. This bacterial strain originates from a spontaneously arthritic NZB/W mouse (6). We have previously shown S. aureus arthritis to be a T and B cell-dependent and superantigen-mediated disease (7–10). Using in situ hybridization techniques, we have recently demonstrated an early up-regulation of TNF in synovium, later (14–21 days after bacterial inoculation) followed by a peak of lymphotoxin-α (LTα) (11). TNF has been shown in various infection models to be a cytokine of critical importance in defense against bacteria (12–14). Simultaneously, TNF plays a detrimental role as one of the mediators of septic shock (15, 16). Depletion of TNF in RA patients diminishes the influx of inflammatory cells to the joint and thus reduces joint inflammation (17–19). LTα can engage the same cell surface receptors as TNF, p55 and p75 TNF-R (20–22), and, therefore, might have similar action as TNF though produced at different time periods during S. aureus-induced arthritis. Using TNF-single-mutant and TNF/LTα-double-mutant mice in our S. aureus arthritis model, we provide evidence for the beneficial action of TNF alone in host defense to generalized S. aureus infection and demonstrate the detrimental role of these cytokines in development of septic arthritis.

Materials and Methods

Mice, bacteria, and infection

Inbred male, 8–15 wk old, C57BL/6 mice, intact or defective with respect to TNF gene or TNF/LTα genes, were used throughout the experiments. Procedure of gene disruptions has been detailed in earlier reports (23, 24). At the time of experiments, TNF/LTα-deficient mice had undergone five backcrosses to C57BL/6 strain, while TNF⁻/⁻ mice were generated using C57BL/6 embryonic stem cells. Mice were maintained in the animal facility of the Department of Rheumatology, University of Göteborg, Sweden, kept under standard conditions of temperature and light, and fed laboratory chow and water ad libitum. A TSST-1 producing S. aureus strain LS-1, originally isolated from a spontaneously arthritic NZB/W mouse, was prepared according to a routine described previously (7) and injected into one of the tail veins. Two different doses of bacteria, 1 × 10⁷ and 3 × 10⁸ S. aureus per mouse, were used in this study. Viable counts were used to check the number of S. aureus injected.
Experimental protocol

In the first set of experiments, a high dose of bacteria, $1 \times 10^7$ S. aureus per mouse, was given to nine TNF/LTα-double-mutant mice and nine C57BL/6 control animals. Mice were checked at regular intervals for evaluation of arthritis and weight change. The experiment was completed at day 28 and kidneys were subjected to bacterial examination. In addition, six TNF/LTα-deficient mice and eight controls were injected i.v. with $1 \times 10^7$ S. aureus and sacrificed after 2 or 6 days to estimate early bacterial clearance from blood, joints, and kidneys.

In the second set of experiments, a lower dose of bacteria, $3 \times 10^6$ mouse, was given to nine TNF/LTα-knockout mice and 10 controls to permit evaluation of septic arthritis. Mice were examined at regular intervals and sacrificed 21 days after the inoculation. All four limbs were used for histopathologic examination of the joints.

In a further experiment, seven TNF-deficient mice and six wild-type controls were inoculated i.v. with $7 \times 10^6$ staphylococci/mouse. Mice were sacrificed 10 days after inoculation of bacteria, and bacterial clearance in liver and kidneys was estimated. The limbs were obtained for histopathologic examination.

Determination of bacterial load

Bacterial growth in talocrural and radiocarpal joints was checked using charcoaled sticks. A joint harboring 15 or more CFU was defined as infected. Bacterial growth in kidneys was estimated by removing both kidneys aseptically, homogenizing them on ice, and serially diluting them in PBS before culturing on blood agar plates. Colonies were tested for catalase and coagulase activity.

Histopathologic examination

Limbs were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Tissue sections (knee, ankle, tarsal joints, and toes; elbow, wrist, carpal joints, and fingers) were cut and stained with hematoxylin and eosin. Sections were examined by a blinded observer with regard to synovial hypertrophy (membrane thickness of more than two cell layers), pannus formation (joint cartilage covered with synovial tissue) and destruction of cartilage and bone, and scored 0–3 for each joint and each parameter studied, depending on the severity of inflammation and erosivity.

In vitro stimulation of spleen cells

Spleens were passed through a nylon mesh, and erythrocytes were depleted by hypotonic lysis. Splenocytes were cultured at $2 \times 10^6$/ml in Iscove’s medium supplemented with 10% FCS, 5 $\times$ 10$^{-5}$ M 2- ME, 2 nM 3-glutamine, and 50 $\mu$g/ml gentamicin, and incubated with 10 $\mu$g/ml TSST-1 (Toxin Technology, Sarasota, FL), 1 $\times$ 10$^{-3}$/ml of formalin-killed S. aureus LS-1 strain, or 1.25 $\mu$g/ml of Con A (ICN Biochemicals, Cleveland, OH) for 48 h. To study proliferative responses, $[^3]$H]thymidine was added after 48 h, and cultures were harvested after 4 h onto filters and counted in a beta counter. Supernatants from cell cultures incubated for 48 h were used for determination of cytokine levels.

Cytokine analysis

Microtiter plates were coated with 2 $\mu$g/ml of rat anti-mouse IFN-γ mAb (PharMingen, San Diego, CA) dissolved in sodium bicarbonate, pH 9.6 and blocked with 1% BSA dissolved in 0.05 M Tris, pH 7.4, for 1 h. Reombinant mouse IFN-γ (Genzyme, Cambridge, MO) was used to create a standard curve. Biotinylated rat anti-mouse IFN-γ, 2 $\mu$g/ml (PharMingen), was employed as the capture Ab. The plates were kept for 2 h at 37°C, and then incubated with streptavidin alkaline phosphatase 0.5 mg/ml (ICN Bio- medicals) and alkaline phosphatase substrate 1 mg/ml (Sigma, St. Louis, MO). Absorbance was measured at 405 nm in Titertec multispec photometer (Flow Laboratory, McLean, WA).

Total and S. aureus-specific Ig production

Serum levels of total IgG1, IgG3, and IgM were measured by the radial immunodiffusion technique (25). Antisera and Ig standards were purchased from Sigma. IgG1 and IgG2a levels of Abs to S. aureus cells and TSST-1 were measured by an ELISA technique. Coating and blocking procedures are described in detail elsewhere (10). Goat anti-mouse IgG1 and IgG2a were purchased from Sigma and biotin-conjugated rabbit anti-goat from Cooper Biomedical (Malvern, PA). Finally, samples were incubated with 0.5 $\mu$g/ml of avidin-horseradish peroxidase (Sigma), followed by the addition of 2.5 mg/ml of enzyme substrate 2,2-azino-bis(3-ethylbenzoazoline sulfonylic acid) (Sigma), and OD was measured in a spectrophotometer.

Phagocytosis and intracellular killing

Intraperitoneal macrophages from noninfected mice were extracted, adjusted to $2 \times 10^6$ cells/ml, and incubated in a 24-well plate (Nunc, Roskilde, Denmark) according to previously detailed procedure (26, 27). Adherent macrophages were incubated with 500 $\mu$L of S. aureus at a concentration of $5 \times 10^6$ bacteria/ml for 30 min at 37°C, and subsequently washed three times in Iscove’s medium. Macrophage content of bacteria was then measured after two incubation intervals, 0 and 4 h, to study phagocytosis and intracellular killing capacity, respectively. To avoid extracellular bacterial growth in the intracellular study, incubation medium contained 10 $\mu$g/ml of gentamicin. Antibiotics were washed away before lysing macrophages with distilled water.

Statistics

The Student’s t test, Mann Whitney U test, and Fisher’s exact test were used. Values are expressed as mean ± SEM.

Results

TNF/LTα play an important role in survival during S. aureus infection

All C57BL/6 control mice survived an i.v. inoculation of $1 \times 10^7$ S. aureus, whereas 67% of TNF/LTα-deficient mice succumbed to
infection \((p = 0.009, \text{Fig. 1A})\). Death occurred quite late during infection, thus 44% of mice died within the third week after infection. No difference in weight decrease could be seen during the early phase of infection, despite the suggested role of TNF as a major mediator of cachexia \((28)\) and TNF production early in the immune response to \(S. \text{aureus} \) \((11)\). In contrast, 14 days after the bacterial inoculation, TNF/LTα-deficient mice had 24.3 \pm 4.0% weight decrease vs 10.9 \pm 1.8% in control group \((p = 0.005, \text{Fig. 1B})\). Thus, whereas control mice regained most of their initial weight decrease, TNF/LTα-knock-out mice did not. Later on during the experiment, differences in weight decreased as a result of death of the most severely affected TNF/LTα-deficient mice. Inoculation with a lower dose of \(S. \text{aureus}, 3 \times 10^6 \) per mouse, resulted in the death of one of nine TNF/LTα-deficient mice, while all ten C57BL/6 controls survived. To evaluate the importance of TNF alone in systemic staphylococcal infection, TNF-single-deficient mice were compared with wild-type controls after the i.v. inoculation of \(7 \times 10^6 \) staphylococci per mouse. A significantly more pronounced weight decrease was seen in TNF-deficient mice from 4 days after inoculation and through the end of the experiment. When the experiment was finished 10 days later, TNF-deficient mice displayed a weight decrease of 19.3 \pm 3.1%, compared with 6.7 \pm 1.6% in controls \((p = 0.005)\). This experiment was performed with a lower infectious dose. Having this in mind, it is plausible to conclude that the main effects on weight development and sepsis-induced mortality seen in TNF/LTα-deficient mice are caused by the absence of TNF.

**TNF/LTα mediate development of septic arthritis**

C57BL/6 control mice receiving a high dose of \(S. \text{aureus} \) \((1 \times 10^7)\) developed a higher frequency of septic arthritis from day 3 (44% vs 22%) to day 28 (89% vs 67%) as compared with TNF/LTα-deficient mice. Because so few of the TNF/LTα-deficient mice survived, we repeated the experiment using a lower inoculum size, \(3 \times 10^6 \) \(S. \text{aureus} \) per mouse. Seven days after the inoculation, 30% (3/10) of control mice had arthritis, compared with none (0/8) of the TNF/LTα-deficient ones. At the peak of disease (day 14–21), 40% of wild-type animals compared with 13% of TNF/LTα-deficient mice displayed joint swelling. Histopathologic examination (Fig. 2A) confirmed the in vivo observation of a lower frequency of arthritis in TNF/LTα-deficient mice. Only 38% of double-deficient mice had visible synovitis compared with 90% of control mice \((p < 0.05)\).  Importantly, bone/cartilage destruction was also less pronounced in mice lacking TNF/LTα, 25% vs 60% in controls. Erosivity score showed a similar outcome: 0.5 \pm 0.4 vs 2.8 \pm 1.2 (NS). In the TNF-single-knock-out mice, a somewhat lower frequency (43% vs 50%) and severity (1.1 \pm 0.6 vs 2.2 \pm 1.4) of arthritis were noted as compared with C57BL/6 controls, although neither of these differences were statistically significant, at least with the number of mice tested.

**Impaired bacterial clearance in TNF/LTα-deficient mice**

Only three mice in the TNF/LTα-deficient group survived the high bacterial inoculum \((1 \times 10^7 \text{ } S. \text{aureus/mouse})\), while none of the wild-type mice died. The median bacterial count in kidneys 28 days after the inoculation was clearly higher in TNF/LTα-double-deficient mice, \(1.5 \times 10^6 \) as compared with \(2 \times 10^6 \) in wild-type group. Because the most severely affected mice in the TNF/LTα-deficient group had died, the difference was not significant. To assess bacterial clearance \(1 \times 10^6 \text{ } S. \text{aureus} \) per mouse was inoculated i.v., and bacterial growth was studied in kidneys, joints, and blood on days 2 and 6. At day 2 no significant differences were found in any tissue, but surprisingly there were more bacteria in blood of the wild-type mice compared with TNF/LTα-mutant mice. However, after 6 days TNF/LTα-deficient mice had higher bacterial counts in all tissues studied compared with wild-type mice: in blood, \(1900 \pm 1600 \text{ vs none} \,(p = 0.02)\); in kidneys, \(1.2 \pm 1.0 \times 10^7 \text{ vs } 5 \pm 4 \times 10^6 \) (NS); the percentage of mice

![FIGURE 2](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
Importance of TNF for phagocytosis of S. aureus

Because the bacterial eradication was diminished in vivo in the absence of TNF/LTα, we extracted peritoneal macrophages from TNF/LTα-deficient mice and their wild-type counterparts to study their phagocytic and intracellular killing capacity. When exposed to S. aureus for 50 min, macrophages from TNF/LTα-deficient mice had a significantly lower uptake of bacteria compared with wild-type controls, 1585 ± 189 S. aureus/10^7 macrophages vs 2720 ± 178 (p < 0.01). In contrast, no major difference in macrophage content of bacteria could be seen after 4 h incubation, because in all cases very few viable bacteria were found (TNF/LTα−/− macrophages: 73 ± 14 vs controls; 52 ± 2; NS). The decreased phagocytic activity seen in macrophages deficient in TNF/LTα may provide a plausible explanation for bacterial accumulation seen in those double-mutant mice, as well as in TNF-deficient mice.

Decreased IFN-γ response in splenocytes of TNF/LTα double-deficient mice

Stimulation of spleen cells from noninfected mice with Con A, TSST-1, and S. aureus cells gave rise to significantly lower IFN-γ production in mice lacking TNF/LTα (p < 0.05; Table I). Proliferation in response to Con A, TSST-1, and S. aureus cells was, in contrast, not affected by lack of TNF/LTα production (data not shown). No significant differences in numbers of splenic CD3, Mac-1, and B cells could be detected between the groups (not shown). Serum IFN-γ in response to S. aureus infection was similar in both groups at day 2 and 6; however, 28 days after the bacterial inoculation C57BL/6 wild-type mice had 446 ± 128 U/ml compared with 245 ± 204 U/ml in TNF/LTα-double-mutant mice (NS). The latter outcome may obviously be due to profound differences in the clinical spectrum of infection between the two substrains.

High polyclonal B cell activation but low Ag-specific IgG response in TNF/LTα-deficient mice

Total IgM and IgG1 levels were significantly higher in TNF/LTα-deficient mice compared with wild-type controls: IgM, 237 ± 32 μg/ml vs 126 ± 10 (p = 0.001); and IgG1, 16.1 ± 2.7 mg/ml vs 9.7 ± 0.9 (p = 0.012). However, total IgG3 was somewhat lower in double-deficient mice as compared with controls 953 ± 232 μg/ml vs 1513 ± 157 (NS). IgG1 and IgG2α Ab levels specific for TSS-1 and S. aureus cells were overall lower in TNF/LTα-deficient mice compared with control animals (Table II).

Discussion

In the present study, TNF-single- and TNF/LTα-double-mutant mice and their C57BL/6 wild-type counterparts were used to evaluate the potential impact of these two closely related cytokines on superantigen-mediated S. aureus sepsis and septic arthritis. Our results clearly show that TNF/LTα deficiency protects against infectious arthritis but aggravates sepsis-induced mortality, the latter outcome mainly caused by the absence of TNF.

The detrimental role of TNF in chronic aseptic joint diseases has been carefully investigated previously and found to be of importance in human rheumatoid arthritis (17–19). However, this is the first report describing TNF/LTα as important promoters for development of synovitis with bone/cartilage destruction in the case of septic arthritis. This finding is especially remarkable because mice lacking TNF/LTα had a higher bacterial load locally and systemically as compared with congenic controls. This emphasizes the critical role of TNF/LTα in the development of septic arthritis. TNF is a major cytokine in the proinflammatory cytokine cascade, and neutralization of TNF reduces production of IL-1, granulocyte-macrophage-CSF, and IL-6 (29–31). Both TNF and IL-1 up-regulate expression of E-selectin, ICAM-1, and VCAM-1 on endothelial cells (32, 33), increasing adhesion and transmigration of inflammatory cells. Due to TNF deficiency, low expression of adhesion molecules and consequently reduced extravasation of inflammatory cells to synovial tissue might have accounted for the decreased frequency of synovitis seen in mice lacking TNF/LTα. In fact, TNF/LTα-deficient mice display a marked reduction of ICAM-1, VCAM-1, and Mac-1 expression (23). However, results from another inflammatory model using TNF-deficient mice show normal up-regulation of VCAM-1 in absence of TNF, though a critical importance of TNF is shown in the early inflammatory process (34). This indicates not only that reduced up-regulation of adhesion molecules goes with TNF/LTα-deficiency, but also that additional effects can be expected by the absence of TNF alone on cell movement, maybe caused by decreased levels of TNF-inducible chemotactic factors, like chemokines (35). The lower erosivity score seen in TNF/LTα-deficient mice may be due to decreased osteoclastic activity and increased bone resorption, both regulated by TNF (36).

TNF as one of the most important inflammatory mediators in the cascade leading to sepsis and septic shock could be expected to exert harmful effects in S. aureus/TSS-1-induced pathologies. Indeed, higher bacterial counts in the blood of wild-type mice early during the infection (48 h postinoculation) support the notion that...
mice producing TNF/LTα may be more sick than the knock-out counterparts. In contrast, after the first 48 h of infection clearly higher mortality rates were noted in TNF/LTα-deficient mice compared with wild-type controls. The beneficial role of TNF/LTα seems to be exerted by improving bacterial clearance. The difference between experimental groups with respect to bacterial load was measurable 6 days after the infection, most clearly in blood but also in other tissues like kidneys and joints. During the second week after bacterial inoculation, TNF/LTα-deficient mice continued to lose weight while the wild-type controls recovered. Death occurred in general quite late during the infectious process, typically after 16–18 days. Even the most fit, surviving TNF/LTα-knock-out mice showed higher bacterial counts than control mice at sacrifice after 28 days. Thus, TNF/LTα-deficient mice display a clear defect in infection control from day 2 and on. Consistent with our findings, using another S. aureus strain for neutralization of TNF caused increased mortality and decreased bacterial clearance (37). Our results suggest that the reason for this mortality increase is decreased bacterial clearance due to inefficient phagocytosis as shown in vivo and in vitro in the present study. One may consider that another possible explanation of the high mortality rate might be the reduced or absent production of Ag-specific Abs to S. aureus cells and TSST-1. This defect in Ag-specific Ab production by TNF/LTα-deficient mice has also been shown recently, demonstrating deficient humoral immune responses toward T cell dependent (SRBC) and T-cell independent (TNF-LPS and DAGG-Ficoll) Ags (23, 38). The decreased Ag-specific immune responsiveness most likely depend on the lack of lymph nodes and complete splenic disruption in mice with LTα gene deficiency, rather than impaired development of germinal centers (39), because TNF-knock-out mice do not form germinal centers either, but raise good Ab responses (34). These lymphoid organ abnormalities result in decreased Ag presentation, possibly because of absence of follicular dendritic cells (23, 40–44). The fact that TNF-single-deficient mice do not carry the same defect in Ag-specific Ab production as TNF/LTα-double-mutant animals, but despite that fact display a severe defect in bacterial clearance, implies that the S. aureus-specific Ab production is not the main cause for the detrimental outcome of S. aureus infection in these cytokine deficient mice. Because LTα signals not only through TNF-R p55 and p75, but also as a heterotrimeric, membrane-associated complex with LTβ via the LTβ receptor (45–49), mice defective in LTα or TNF/LTα display abnormal development of peripheral lymphoid organs that depends upon membrane LTα,β2 interaction (23, 50, 51). Therefore, the experiment with TNF-single-knock-out mice show not only that many of the effects seen in TNF/LTα-double-deficient mice can be attributed to TNF alone but also that the major defects in lymphoid organ development of TNF/LTα-deficient mice do not seem to be responsible for the outcome of S. aureus infection in those double-deficient mice. Using TNF-single-deficient mice, the importance of TNF for resistance to other infectious agents, e.g., Candida albicans, has been shown (52). Though TNF-single-deficient mice show a somewhat lower frequency and severity of septic arthritis compared with wild-type controls, no significant differences are shown in our study encompassing a limited number of animals. However, having in mind the much higher bacterial load found in TNF-deficient mice, beneficial effects on the joint inflammation might be expected using TNF neutralization as part of a combinatory therapy with antibiotics. However, caution is required, especially if such a treatment is applied systemically, because harmful effects could be expected to follow this procedure due to 1) high TNF production resulting in efficient clearance of infectious agents (52), 2) the increased lethality of TNF+/− mice to endotoxin compared with TNF+/+ animals (52), i.e., an incomplete TNF neutralization may result in an increased tissue damage by bacterial toxins, and 3) the unexpected outcome of treatment of septic shock patients with the TNF receptor:Fc fusion protein, showing a trend toward higher mortality rates in subjects receiving the soluble TNF receptor (53).

TNF/LTα-deficient mice show a clear resistance in developing septic arthritis, and at the same time display a definitive defect in bacterial clearance, comparable to that seen in TNF-knock-outs. On the one hand, LTα does not compensate for the defective bacterial clearance shown in TNF-deficient mice, but on the other, the absence of both TNF and LTα is required for resistance to S. aureus arthritis. These results give several possible explanations for the development of septic arthritis: 1) a critical importance of LTα alone, e.g., due to some potential stimulatory properties on secretion of other Th1 cytokines, like IFN-γ; 2) effects mediated through TNF receptor p55 and/or p75, where soluble LTα homotrimer can compensate, to some extent, for the absence of TNF in the case of joint inflammation due to high local production by T cells or that only a low signaling through this receptor system is required at a critical step, e.g., up-regulation of adhesion molecules; 3) LTα/β2 signaling through LTβ receptor might have influence on the inflammatory process (54); and 4) developmental abnormalities in the immune system, responsible for the inability to mount Ag-specific Ab production, are the main cause for the observed resistance, rather than the specific actions of TNF/LTα, on the induction of septic arthritis.

In conclusion, our results show that TNF/LTα play an important role in host defense to S. aureus, with beneficial effects mainly attributed to TNF alone, and that TNF/LTα-double but not TNF-single deficiency leads to decreased joint inflammation and bone/cartilage destruction in S. aureus-mediated arthritis. Thus, neutralization of TNF/LTα could be an intraarticular target for immunomodulatory treatment in septic arthritis patients. Further work should address the potential role of LTα alone in septic arthritis.

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