Differential production of TNF by Kupffer cells after phagocytosis of E. coli and C. albicans

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Olynyk, John K., George M. Matuschak, Andrew J. Lechner, Robert S. Britton, Trevor L. Tredway, Rosemary O’Neill, and Bruce R. Bacon. Differential production of TNF by Kupffer cells after phagocytosis of E. coli and C. albicans. Am. J. Physiol. 267 (Gastrointest. Liver Physiol. 30): G213–G219, 1994.—Tumor necrosis factor (TNF) of hepatic origin is thought to play a pivotal role early in the genesis of the septic shock syndrome, regardless of microbial etiology. To determine if production of TNF by Kupffer cells varies with microbial taxonomic class, we measured TNF secretory responses in primary cultures of rat Kupffer cells to numerically equivalent gram-negative bacterial or fungal phagocytic challenges. After a 30-min exposure to media, latex beads, soluble Escherichia coli lipopolysaccharide (LPS; serotype 055:B5), live or Formalin-fixed E. coli (serotype 055:B5), live or Formalin-fixed yeast-phase Candida albicans, or live hyphal-phase Candida, samples of culture supernatant were assessed at 30, 60, 120, and 240 min and at 24 h for TNF bioactivity by L929 cell cytotoxicity. Compared with media and latex bead controls, TNF levels progressively increased for up to 240 min after either LPS and equivalently in live E. coli or Formalin-fixed E. coli groups (P < 0.05). Formalin-fixed yeast-phase and live extracellular hyphal-phase C. albicans failed to stimulate production of TNF at any time point (6.9 ± 0.7 and 8.7 ± 0.4 U/ml, respectively, at t = 240 min; P < 0.05 vs. E. coli). In contrast, internalization of live yeast-phase C. albicans with subsequent hyphal formation and growth within Kupffer cells was accompanied by a rise in supernatant TNF levels (14.5 ± 1.8 U/ml at t = 240 min). We conclude that soluble or particulate gram-negative bacterial signals strongly induce TNF expression by rat Kupffer cells irrespective of viability, whereas cytokine production induced by yeast-phase Candida is critically dependent on germination and intracellular yeast-mycelial transformation.

Since production and export of TNF by the liver are appreciable early after endotoxemia (5, 8), phagocytic uptake of intact microbial cells is also believed to generate significant amounts of TNF, regardless of the taxonomic class of the organism or its viability (7, 23, 32, 33). In support of this hypothesis, induction of high levels of TNF occurs during bacterial, viral, mycobacterial, and protozoal infection, resulting in the cardiopulmonary and hematological manifestations of sepsis, directly or indirectly, via generation of eicosanoid mediators (1, 4, 11, 14, 23, 29). However, several recent reports have demonstrated that the kinetics and magnitude of TNF production are differentially regulated in intact animals during gram-negative bacterial sepsis compared with fungal sepsis (17, 20). In these studies, circulating TNF levels after lethal Escherichia coli bacteremia were markedly increased compared with those during candidiasis with disseminated candidiasis, in which peak TNF concentrations were < 1% of peak values during bacterial sepsis despite equivalent intravascular microbial clearance (20). Within the liver, phagocytic uptake of gram-negative bacterial products similarly led to large increases in cell-associated TNF within 90 min compared with values after Candida albicans fungemia (20). Although indicating that hepatic expression of TNF is dissimilar early after E. coli sepsis in relation to changes after C. albicans infection, the kinetics of pathogen-specific uptake by Kupffer cells and its relationship to TNF production over time have not been systematically examined.

The incidence of nosocomial candidiasis has increased 20-fold over the last 10 years and continues to be associated with significant mortality, especially when accompanied by hepatic candidiasis (9). We reasoned that characterization of the relationship between phagocytic uptake of C. albicans by Kupffer cells and cytokine production is important to clarify the role of the liver in antifungal host defense and fungal septic shock syndrome. Accordingly, we performed these studies to characterize TNF production by Kupffer cells in vitro in response to challenges with soluble and particulate gram-negative bacterial products compared with dimorphic forms of human pathogenic C. albicans.

METHODS

Animals. Kupffer cells were isolated from pathogen-free male Sprague-Dawley rats (450–550 g, Harlan, Indianapolis, IN). Animals were given food and water ad libitum before the experiments. All studies conformed to National Institutes of Health guidelines for the use and care of experimental animals.
and were approved by the Animal Care Committee of Saint Louis University Health Sciences Center.

**Kupffer cell isolation.** Excised rat livers were sequentially perfused with pronase (0.25%, Boehringer Mannheim, Indianapolis, IN) and collagenase B (0.033%, Boehringer Mannheim, Indianapolis, IN) in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 (DMEM, Gibco BRL, Grand Island, NY). Livers were then cut into small pieces, suspended in DMEM containing 10 μg/ml deoxyribonuclease (DNase, Sigma, St. Louis, MO), and agitated at 250 revolutions/min (rpm) for 30 min at 37°C. The cell suspension was washed 3 times (150 g for 3 min) in DMEM containing 10 μg/ml DNase, after which the cells were washed, resuspended in the same medium, and layered on a sterile step-gradient of purified arabinogalactan (Lacoll, Sigma) with densities of 1.045, 1.055, 1.065, and 1.080. Centrifugation was performed at 20,000 rpm for 30 min at 25°C in a Beckman SW28 rotor. Kupffer cells were recovered at the interface of the 1.065 and 1.080 density layers, washed in buffer at 150 g for 3 min, and resuspended in medium 199 with Earle’s salts (Gibco BRL) containing penicillin (100 U/ml, Gibco BRL), streptomycin (100 μg/ml, Gibco BRL), insulin (4 μU/ml, Gibco BRL), corticosterone (1 μg/ml, Sigma), and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (10 mM, Sigma; termed stock M199) and supplemented with 10% calf serum and 10% horse serum. The Kupffer cells were plated at a density of 3x10^6 cells/well in 12-well tissue culture dishes, and the cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air. After 24 h, the medium was removed, adherent Kupffer cells were washed with sterile phosphate-buffered saline, and 1.0 ml of fresh medium was added. After the first change of medium, the viability of Kupffer cells from each isolate was determined by trypan blue exclusion, and their purity was assayed by phagocytosis of 3-μm latex beads (Sigma): Kupffer cells were defined by uptake of at least five beads per cell. The isolation procedure described above yielded Kupffer cell cultures of high viability (>95%) and purity (>85%). On day 3 after isolation, Kupffer cells were washed, and the medium was changed to stock M199 with 5% calf serum and 10% horse serum for the phagocytosis experiments. The endotoxin concentrations were measured in all reagents [except live E. coli and Formalin-fixed (FF) E. coli] coming in contact with the cell preparations by a quantitative chromogenic Limulus amebocyte lysate assay (QLC-1000, Whittaker M.A. Bioproducts, Walkersville, MD). All reagents (including live, FF, and hyphal-phase C. albicans) had endotoxin concentrations of <0.2 ng/ml (final concn) except the pronase (1.3 ng/ml) and collagenase (0.9 ng/ml) solutions. Exposure of the cells to these latter two solutions was limited to ~20 min, and this would not be expected to affect the responsiveness of the Kupffer cells, which were challenged on day 3 after isolation.

**E. coli.** E. coli serotype 055:B5 [no. 12014, American Type Culture Collection (ATCC), Rockville, MD] was from 18- to 24-h log-phase cultures in trypticase soy broth that was sedimented (1,000 g for 10 min at 4°C), washed twice with sterile 0.9% NaCl, and resuspended in stock M199 with 5% calf serum at a density of 15×10^6 bacteria/ml, and 5 ml were dispensed in 60-mm culture plates (Corning, Corning, NY). The culture plates were then scraped, and the hyphal-phase fungi were sedimented (400 g for 10 min at 4°C) and washed twice with sterile 0.9% NaCl, after which inocula were resuspended in 5 ml of stock M199 with 5% calf serum immediately before use. As our intent was to characterize the TNF secretory responses to viable extracellular hyphae, FF hyphae were not used in the current study.

**Experimental protocol.** Kupffer cells were inoculated for 30 min with medium alone (medium controls), 3-μm latex beads (15×10^6 beads/well, phagocytosis controls), 400 ng/well LPS, or 15×10^6 organisms/well of live E. coli, FF E. coli, C. albicans, FF C. albicans, or live hyphae (1 ml) in separate experiments. This inoculum was based on a 5:1 target/Kupffer cell ratio, similar to that utilized in previous studies in the isolated perfused liver (22). After completion of incubations (t = 0), Kupffer cells were washed with sterile phosphate-buffered saline to remove residual organisms or latex beads, 1.0 ml of fresh stock M199 with 5% serum was added, and 200-μl samples of medium were taken at t = 5, 20, 60, 120, and 240 min, and at 24 h. Aliquots were immediately centrifuged (150 g for 3 min at 4°C) and stored at −20°C until assayed for TNF. After each sample, 200 μl of fresh medium were added to each well. Latex bead and yeast phagocytosis was confirmed by phase-contrast microscopy (Diaphot-TMD, Nikon, Japan). Determination of yeast and latex bead clearance was performed by counting the number of phagocytosed particles per 100 Kupffer cells, calculating the mean number of particles phagocytosed per Kupffer cell, multiplying the result by the total number of Kupffer cells per well (3×10^6), and, finally, dividing the total number of phagocytosed particles by the number of particles initially inoculated into the well (15×10^6) and converting this to a percentage. After 30 min, Kupffer cells from the wells receiving FF C. albicans were harvested by trypsinization (0.5% trypsin and 0.02% EDTA for 1 min) followed by immediate fixation in 2% glutaraldehyde for electron microscopic documentation of phagocytosis. The viability of Kupffer cells for the first 4 h after inoculation with both viable and nonviable E. coli, C. albicans, or latex beads was >95% as determined by propidium iodide-fluorescein diacetate in vitro staining of separate wells. At 24 h, Kupffer cell viability of >95% was maintained after exposure to E. coli, latex beads or, FF yeast-phase C. albicans. In the wells exposed to either live yeast-phase or mycelial-phase C. albicans, overgrowth of yeast prevented direct assessment of cell viability at 24 h. Separate

**C. albicans.** C. albicans was a clinical isolate confirmed by taxonomic criteria (25, 26) and generously supplied as serotype A, strain MFN by Dr. J. F. Ryley, IC1 Pharmaceuticals, Cheshure, UK. The yeast (5-μm diam) was maintained on Sabouraud D agar at 28°C, transferred to Sabouraud’s broth, and incubated at 37°C for 48–72 h before experiments. Yeast-phase blastospores were sedimented (400 g for 10 min at 4°C), washed twice with sterile 0.9% NaCl, and resuspended at 4°C in stock M199 with 5% calf serum at 15×10^6 blastospores/ml for experiments. Inocula were free of germ tubes or hyphae, and viability was confirmed by culture and trypan blue exclusion. FF yeast-phase C. albicans were prepared (24) and were suspended in stock M199 with 5% calf serum at 15×10^6 blastospores/ml.
experiments were performed for each group in duplicate, using Kupffer cells isolated from different animals.

**TNF bioassay.** Actinomycin D-treated murine L929 cells (CCL1; NCTC clone 929, ATCC) were used to quantitate TNF bioactivity, as described previously (17, 18, 20). Duplicate samples from each time point were assayed at 1:2, 1:4, 1:8, and 1:16 dilutions, and cytotoxicity was quantitated at 550 nm (EL 311, Bio-Tek, Burlington, VT). Samples were calibrated against duplicate curves of murine recombinant TNF (rTNF) with a specific activity of 1.2 × 10^7 U/mg (Genentech, South San Francisco, CA) and rTNF internal controls on each plate.

**Statistical analysis.** Data are presented as means ± SE. Comparisons between groups were performed using Mann-Whitney's U test (Statgraphics, Statistical Graphics, Princeton, NJ). Longitudinal comparisons within groups over time were performed by analysis of variance. Significance was accepted for P < 0.05.

**RESULTS**

**Latex bead and microbial phagocytosis.** During the 30-min incubation period, Kupffer cells phagocytosed five to eight latex beads per cell. However, bacteria were almost totally cleared from the media, as judged by microscopy, when live or FF E. coli were coincubated with Kupffer cells over the same time period. Kupffer cells phagocytosed three to five live C. albicans or FF C. albicans per cell during the incubation period, resulting in ~75% clearance of the C. albicans inoculum per well. Electron microscopic examination of Kupffer cells demonstrated that almost 100% of Kupffer cells contained multiple phagocytosed FF C. albicans after the 30-min incubation (Fig. 1). After live C. albicans, significant hyphal formation commenced both within Kupffer cells and on the bare surfaces of wells during the incubation period (Fig. 2) that progressed over 24 h. Live hyphae, which were not phagocytosed, also continued to grow over 24 h. In contrast, no germ tubes or mycelia were observed in the FF C. albicans-exposed cultures over the 24-h experimental period.

**TNF production after soluble and particulate gram-negative microbial products.** Figure 3 shows sequential group-specific differences in TNF production by Kupffer cells exposed to medium and latex beads compared with those exposed to LPS and live and FF E. coli. Kupffer cells exposed to medium alone (control group) showed no significant TNF production over basal levels during the experimental period (5.1 ± 1.2 at t = 0 vs. 4.8 ± 0.9 U/ml at 24 h). Phagocytosis of particulate material per se was not accompanied by increases in TNF levels in Kupffer cell supernatants above baseline levels, since no changes were found after internalization of latex beads over the course of the experiment. TNF levels progressively increased after LPS, peaking at 240 min compared with the control group (27 ± 11 vs. 4.9 ± 0.9 U/ml, respectively; P < 0.05), and returning to baseline levels at t = 24 h. Similarly, uptake of live and FF E. coli increased TNF production to similar peak levels at 120 min (23 ± 5 and 21 ± 5 U/ml, respectively), compared

Fig. 1. Electron photomicrograph taken at end of 30-min coincubation period shows two Kupffer cells that have phagocytosed multiple Formalin-fixed (FF) yeast-phase C. albicans (C). Original magnification ×5,500.
with the control or latex bead groups (5.2 ± 1.0 and 3.6 ± 0.8 U/ml, respectively; P < 0.05).

**TNF production after C. albicans is dependent on viability and dimorphic form.** Figure 4 shows the kinetics of TNF production in the live C. albicans, FF C. albicans, and live hyphae groups compared with TNF levels observed in the control group. Uptake of live C. albicans followed by germination and yeast-mycelial transformation within Kupffer cells was associated with significantly increased TNF production at t = 240 min compared with the FF C. albicans group (14.5 ± 1.8 and 6.9 ± 0.7 U/ml, respectively; P < 0.01). These differences were maintained at 24 h (13.9 ± 1.2 and 8.6 ± 1.6 U/ml, respectively; P < 0.05). In contrast, phagocytosis of FF C. albicans by Kupffer cells failed to induce TNF production above control levels at any point during the experimental protocol. Similarly, contact of extracellular nonphagocytosed hyphae with Kupffer cells also did not lead to TNF secretion into the supernatant.

**DISCUSSION**

Recently, it has been recognized that the liver is a pivotal organ in host defense during severe microbial sepsis, owing to dual regulatory mechanisms controlling the vascular clearance of microbial pathogens and the secondary generation of inflammatory cytokine mediators such as TNF (5, 8). Although TNF production by the liver is thought to be central in the cascade of molecular events that result in sepsis-related tissue injury (4, 7, 23, 32), the temporal linkage between microbial phagocytosis and TNF production by Kupffer cells has not been critically examined. Thus it has been assumed, although never proven, that TNF generation
is equivalent after phagocytosis of gram-negative bacterial or nonbacterial pathogens by Kupffer cells (3, 19). Support for this thesis derives from the similarity of clinical manifestations of bacterial and fungal sepsis and from data showing induction of TNF by nonbacterial pathogens (4, 7, 23, 31).

In this study, we have shown that this thesis is no longer tenable. As with soluble LPS, administration of both live and FF E. coli stimulated TNF production by Kupffer cells in vitro as expected. This finding supports the data of Natanson et al. (23), who found equivalent cardiovascular dysfunction after administration of live and FF E. coli, and contrasts strikingly with the rapid development of shock after administration of viable yeast-phase C. albicans but not after nonviable yeast-phase C. albicans (18). In the current study, nonviable yeast-phase C. albicans failed to stimulate TNF production by Kupffer cells despite avid phagocytosis. FF C. albicans was used to limit the organism to the yeast phase while maintaining surface antigenicity (20). Thus FF yeast-phase organisms were no more able than inert latex beads to induce TNF production, suggesting that phagocytosis per se is not sufficient cause for stimulating TNF secretion by Kupffer cells. The absence of TNF secretion by Kupffer cells after phagocytosis of latex beads supports the work of Decker (6) and provides further support for the hypothesis that specific microbial antigens may be required to induce TNF secretion. This belief is strengthened by our recent observations that Kupffer cell predominantly by absorptive pinocytosis whereas exogenously administered LPS enters the cell-associated TNF after C. albicans fungemia in vivo (20) or in the isolated perfused rat liver ex situ (22) makes this unlikely. Rather, our data are in agreement with the observation of Blasi et al. (2) that uptake of yeast-phase C. albicans failed to induce TNF gene expression in transformed murine macrophages (line ANA-1) over a 3-h period. However, in contrast to our results with the live hyphal phase, Blasi et al. (2) found that the extracellular hyphal phase of C. albicans was capable of inducing TNF gene expression and TNF secretion. There are several potential explanations for this difference. First, it is conceivable that ANA-1 macrophages and Kupffer cells intrinsically differ with respect to Candida-mediated induction of TNF, since the former is a transformed murine cell line, whereas our experiments were performed with fresh isolates from rats. A second potential explanation is that the experimental design in the two studies contributed to the observed difference in results. Not only were different strains of Candida used in two studies, but we co-incubated hyphal-phase Candida with Kupffer cells for 30 min followed by washing and serial sampling for up to 24 h, whereas Blasi et al. (2) co-incubated ANA-1 macrophages with hyphal-phase yeast for up to 3 h and then measured TNF levels in supernatants or TNF mRNA levels. Finally, it is likely that the concentrations of live hyphal-phase forms exposed to either Kupffer cells or ANA-1 macrophages in the two studies also differed, resulting in different hyphae/effecter cell ratios. Collectively, our data suggest that Kupffer cell production and export of TNF are critically dependent on viability, dimorphic phase, and intracellular vs. extracellular location of C. albicans.

We noted equivalent phagocytosis and resultant TNF production by Kupffer cells after incubation with live and FF E. coli. After phagocytosis and lysosomal digestion of intact organisms, unmasking of LPS must occur, whereas exogenously administered LPS enters the Kupffer cell predominantly by absorptive pinocytosis (27). However, both the phagocytosis of E. coli and uptake of LPS result in protein kinase C-mediated TNF gene transcription with subsequent protein synthesis and extracellular secretion of TNF (12). It is interesting to note the return of TNF production by Kupffer cells to baseline levels by 24 h after exposure to LPS and E. coli. It is unlikely that this decline resulted from dilution of TNF in supernatants by our sampling protocol, since high TNF levels were present at 4 h. Furthermore, dilution amounting to only 20% occurred as a result of significant reduction of virulence in experimental animals (18, 21), we would speculate that prevention of mycelial transformation might be beneficial in the prevention of septic shock and multiple organ failure.

The current study strongly suggests that the observed discrepancies in systemic and hepatic TNF production between lethal gram-negative and C. albicans sepsis (20) reflect differential host regulation of TNF biosynthesis in Kupffer cells. It might be argued that the dissimilar findings after C. albicans challenge could be accounted for by disproportionate intracellular sequestration of TNF or increases in the 26-kDa cell-associated form (12, 13, 15). However, the lack of increase of liver cell-associated TNF after C. albicans fungemia in vivo (20) or in the isolated perfused rat liver ex situ (22) makes this unlikely. Rather, our data are in agreement with the observation of Blasi et al. (2) that uptake of yeast-phase C. albicans failed to induce TNF gene expression in transformed murine macrophages (line ANA-1) over a 3-h period. However, in contrast to our results with the live hyphal phase, Blasi et al. (2) found that the extracellular hyphal phase of C. albicans was capable of inducing TNF gene expression and TNF secretion. There are several potential explanations for this difference. First, it is conceivable that ANA-1 macrophages and Kupffer cells intrinsically differ with respect to Candida-mediated induction of TNF, since the former is a transformed murine cell line, whereas our experiments were performed with fresh isolates from rats. A second potential explanation is that the experimental design in the two studies contributed to the observed difference in results. Not only were different strains of Candida used in two studies, but we co-incubated hyphal-phase Candida with Kupffer cells for 30 min followed by washing and serial sampling for up to 24 h, whereas Blasi et al. (2) co-incubated ANA-1 macrophages with hyphal-phase yeast for up to 3 h and then measured TNF levels in supernatants or TNF mRNA levels. Finally, it is likely that the concentrations of live hyphal-phase forms exposed to either Kupffer cells or ANA-1 macrophages in the two studies also differed, resulting in different hyphal/effecter cell ratios. Collectively, our data suggest that Kupffer cell production and export of TNF are critically dependent on viability, dimorphic phase, and intracellular vs. extracellular location of C. albicans.

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sampling between this measurement and the 24-h time point. It is more likely that the decreased TNF levels reflect cytokine metabolism and downregulation of TNF production by Kupffer cells (28).

The mechanisms responsible for the disparity between LPS-induced cytokine secretion and the failure of C. albicans to stimulate appreciable TNF production remain unclear. A possible explanation may involve the presence of surface proteins on yeast phase C. albicans that mimic receptors of the mammalian integrin superfamily (10, 30). These surface proteins may confer less antigenicity to the yeast-phase C. albicans, resulting in a weaker signal for TNF production compared with the E. coli- or LPS-stimulated pathways. Our data suggest that intracellular hyphal formation by phagocytosed live blastospores may result in differential antigen expression or unmasking within the Kupffer cell, which stimulates TNF production. Conceivably, extracellular presentation of this factor is insufficient to result in TNF production. This would explain the lack of effect of extracellular live hyphae that were not phagocytosed. Alternatively, disruption of Kupffer cells by germinating C. albicans may stimulate TNF release as an agonal event. It is also possible that differential production of proinflammatory and anti-inflammatory cytokines contributes to the observed differences in TNF production after exposure to E. coli or viable yeast-phase C. albicans. Exposure of Kupffer cells to viable and nonviable E. coli results in the production of both TNF and interleukin-1 (IL-1), as elevated levels of both these proinflammatory cytokines have been observed in bacterial sepsis (5, 6, 7, 20). Since TNF and IL-1 both stimulate the production of other cytokines and exhibit a bidirectional stimulatory interaction (7), TNF and IL-1 likely contributed to elevated TNF production after exposure to E. coli. Conversely, after phagocytic uptake and intracellular yeast-mycelial transformation, production of anti-inflammatory cytokines such as IL-4, IL-6, IL-10, or transforming growth factor-β could potentially inhibit TNF production (7), thereby accounting for the relatively small rise in TNF observed in this study. Overall, although our data differ from the observations of Blasi et al. (2) concerning the effect of mature hyphae on TNF secretion, they support the contention that the transformation from yeast to hyphal phase is associated with altered immunogenicity and potential for triggering TNF secretion (2, 20).

In conclusion, we have described a model for the in vitro study of microbial-induced TNF production by primary cultures of rat Kupffer cells. Kupffer cell stimulation by LPS and particulate gram-negative bacteria induces rapid and significant TNF production, independent of microbial viability. In contrast, both the nonviable yeast phase and the extracellular hyphal phase of C. albicans fail to stimulate TNF production by Kupffer cells. Our results with the live yeast phase support the hypothesis that TNF production induced by C. albicans is critically dependent on germination and intracellular yeast-mycelial transformation in Kupffer cells.

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