Modulation of TNF Release by Choline Requires α7 Subunit Nicotinic Acetylcholine Receptor-Mediated Signaling

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The α7 subunit-containing nicotinic acetylcholine receptor (α7nAChR) is an essential component in the vagus nerve-based cholinergic anti-inflammatory pathway that regulates the levels of TNF, high mobility group box 1 (HMGB1), and other cytokines during inflammation. Choline is an essential nutrient, a cell membrane constituent, a precursor in the biosynthesis of acetylcholine, and a selective natural α7nAChR agonist. Here, we studied the anti-inflammatory potential of choline in murine endotoxemia and sepsis, and the role of the α7nAChR in mediating the suppressive effect of choline on TNF release. Choline (0.1–50 mM) dose-dependently suppressed TNF release from endotoxin-activated RAW macrophage-like cells, and this effect was associated with significant inhibition of NF-κB activation. Choline (50 mg/kg, intraperitoneally (i.p.)) treatment prior to endotoxin administration in mice significantly reduced systemic TNF levels. In contrast to its TNF suppressive effect in wild type mice, choline (50 mg/kg, i.p.) failed to inhibit systemic TNF levels in α7nAChR knockout mice during endotoxemia. Choline also failed to suppress TNF release from endotoxin-activated peritoneal macrophages isolated from α7nAChR knockout mice. Choline treatment prior to endotoxin resulted in a significantly improved survival rate as compared with saline-treated endotoxemic controls. Choline also suppressed HMGB1 release *in vitro* and *in vivo*, and choline treatment initiated 24 h after cecal ligation and puncture (CLP)-induced polymicrobial sepsis significantly improved survival in mice. In addition, choline suppressed TNF release from endotoxin-activated human whole blood and macrophages. Collectively, these data characterize the anti-inflammatory efficacy of choline and demonstrate that the modulation of TNF release by choline requires α7nAChR-mediated signaling.

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INTRODUCTION

The excessive production of the pro-inflammatory cytokines TNF, high mobility group box 1 (HMGB1), and other inflammatory molecules by immune cells and their subsequent release into the circulation are associated with unrestrained inflammation: a hallmark of septic shock, sepsis, and other disorders (1,2). Exacerbated release of TNF and other pro-inflammatory cytokines, and lethality during endotoxemia and sepsis, can be controlled by the efferent vagus nerve-based cholinergic anti-inflammatory pathway (3–7). Recent research has demonstrated that the α7 subunit-containing nicotinic acetylcholine receptor (α7nAChR) is an important component of the mechanism underlying the anti-inflammatory efficacy of the cholinergic anti-inflammatory pathway (4,8). Activation of this pathway by stimulation of the vagus nerve suppresses serum TNF levels in endotoxemic animals (3,4), but fails to cause statistically significant effects in mice lacking the α7nAChR (4). Accordingly, α7nAChR agonists, including GTS-21, reduce systemic pro-inflammatory cytokine levels during murine endotoxemia, sepsis (9), and other inflammatory conditions (10,11), and improve survival (9).

Choline is a selective and endogenous α7nAChR agonist (12–14). Choline also has other important physiological functions; this essential nutrient is a major donor of methyl groups, a cell membrane constituent, and a precursor in the biosynthesis of the neurotransmitter acetylcholine (15–17). Although previous studies have shown protective effects of choline against endotoxin-induced shock and organ damage (18–20), the mechanism of the anti-inflammatory action of

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this compound is not well understood. A particularly important question is whether the α7nAChR, which is an essential component of the cholinergic anti-inflammatory pathway, mediates the anti-inflammatory action of choline during endotoxemia. Another relevant question is whether choline suppresses the pro-inflammatory cytokine response and affects the survival rate during polymicrobial sepsis.

In this study, we provide evidence that choline functions as an anti-inflammatory molecule through an α7nAChR-dependent mechanism. In contrast to its anti-inflammatory effect in wild type mice, choline failed to reduce endotoxin-induced serum TNF levels in α7nAChR KO mice and TNF release from peritoneal macrophages isolated from these mice. These findings represent the first direct experimental evidence that the anti-inflammatory activity of a cholinergic agonist is mediated *in vivo* through an α7nAChR-dependent mechanism. We also show that choline suppresses the release of HMGB1, and choline treatment initiated within a clinically relevant time frame significantly improves survival in mice with severe sepsis.

MATERIALS AND METHODS

Animals

Male mice (BALB/c at 25–28 g [Taconic], and wild type [WT] or α7nAChR knockout [KO] C57BL/6 at 8–12 wk old) were used for *in vivo* studies, and WT or α7nAChR KO C57BL/6 female mice at 8–12 wk old were used in *ex vivo* peritoneal macrophage studies. All C57BL/6 animals were bred on site from heterozygous α7nAChR KO animals obtained from Jackson Laboratories (Bar Harbor, ME, USA). The genotype of the α7nAChR locus (*CHRNA7*) of all progeny was determined by genomic PCR using the Extract and Amp kit (Sigma, St. Louis, MO, USA). Animals were housed in standard conditions (room temperature 22° C with a 12-h light:dark cycle) with free access to regular chow and water. Animals were allowed to acclimate for at least 14 d before the corresponding experiment. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, New York, United States of America.

RAW Cells, Drug Treatment, TNF, and HMGB1 Determination

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC TIB-71, Manassas, VA, USA) and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine (Biowhittaker, Walkersville, MD, USA) and 100 U/mL penicillin, 100 μg/mL streptomycin (both Gibco, Carlsbad, CA, USA). Cell cultures were maintained at 37° C, 5% CO_2. Cells were seeded in 48-well tissue culture plates at 5×10^5 cells per well, and were allowed to adhere for 24 h. Prior to adding compounds, media were removed and replaced with serum-free Optimem media (Gibco). Cells were exposed to lipopolysaccharide (LPS; endotoxin) (*Escherichia coli*, L4130 0111:B4; Sigma) (4 mg/mL) in the presence or absence of choline at the concentrations indicated. Cell culture media were harvested 4 h after LPS addition and centrifuged at 800*g* for 5 min to sediment cell debris. Secreted TNF was assayed from the media by Enzyme-Linked ImmunoSorbent Assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. For HMGB1 determination, cell culture media were collected 24 h after endotoxin treatment and centrifuged at 800*g* for 5 min to remove cellular debris. HMGB1 was analyzed from cleared media by Western blot as described previously (9). Briefly, cleared medium was filtered through Centricon YM-100 (Millipore, Billerica, MA, USA) diluted 1:2 with 2 × Laemmli sample buffer (BioRad, Hercules, CA, USA), and subjected to electrophoresis through 10%–20% Tris-HCl acrylamide gels (BioRad). Proteins were immobilized onto PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) and probed with polyclonal anti-HMGB1 antibodies. Membranes were developed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Autoradiograph films were scanned and densitometric analyses performed using Quantity One Software (BioRad). Standard curves of human recombinant HMGB1 were constructed and used to interpolate HMGB1 levels in the samples. Cell viability was monitored using trypan blue exclusion.

Nuclear Protein Extraction for NF-κB Activity Determination and Electrophoretic Mobility-Shift Assay (EMSA)

RAW 264.7 macrophages were treated with the indicated concentrations of choline followed by LPS (4 ng/mL). Two h after LPS stimulation, cells were processed for nuclear protein extraction as described previously (9). EMSA was performed using the Nushift NF-κB p65 kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions, as described previously (9).

Endotoxemia and Drug Treatment

Endotoxemia was induced by injecting mice intraperitoneally (i.p.) with 6 mg/kg endotoxin, which caused ~80% mortality. Mice were treated i.p. with the indicated dose of choline or vehicle (sterile saline) at 6 h, and at 30 min prior to endotoxin administration. Animals were euthanized by CO_2 asphyxiation 1.5 h after endotoxin injection, and blood was collected by cardiac puncture. Blood was centrifuged at 1,500*g* for 15 min to isolate serum. Sera were used for TNF analysis by ELISA (R&D Systems) according to the manufacturer's recommendations. In survival experiments, mice were treated i.p. with choline (50 mg/kg or 5 mg/kg) or sterile saline (controls) at 6 h and at 30 min prior to endotoxin injection. Survival was monitored for 2 wks.

Isolation and Treatment of Mouse Peritoneal Macrophages

WT or α7nAchR KO C57BL/6J female mice were injected with 2 mL of 9% thioglycollate broth i.p. to elicit peritoneal macrophages. Animals were euthanized by CO_2 asphyxiation 42–48 h later, and cells were collected by lavage of the peritoneal cavity three times with 5 mL ice-cold 11.6% sucrose. Cells were washed three times with phosphate-buffered saline (PBS) and once with RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine (Biowhittaker) and 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco). Cells were seeded at 1.5×10^6 per well into 24-well Falcon Primaria tissue culture dishes and were allowed to adhere for 2 h at 37° C under 5% CO_2. Then, cells were washed twice with PBS and supplied with fresh medium as described above, returned to the incubator, and allowed to rest for 18–24 h. Prior to treatment, media were removed and replaced with serum free Optimem media. Cells were incubated with the indicated concentration of choline for 10 min prior to LPS (100 ng/mL) exposure. Cell culture media were harvested 4 h after LPS treatment and TNF was assayed by ELISA (R&D Systems) according to the manufacturer's recommendations.

Cecal Ligation and Puncture Surgery and Drug Treatment

Severe polymicrobial sepsis was induced by cecal ligation and puncture (CLP). Mice were anesthetized using ketamine (100 mg/kg) and xylazine (8 mg/kg) administered intramuscularly. Abdominal access was gained via a midline incision. The cecum was isolated and ligated with a 6-0 silk ligature below the ileocecal valve and then punctured once with a 22 G needle. Stool (approximately 1 mm) was extruded from the hole, and the cecum placed back into the abdominal cavity. The abdomen was closed with two layers of 6-0 Ethilon sutures. An antibiotic (Imipenem-Cilastatin, 0.5 mg/kg, subcutaneously, in a total volume of 0.5 mL/mouse) was administered immediately after CLP as part of the resuscitation fluid. Sham-operated animals had the cecum isolated and then returned to the peritoneal cavity without being ligated or punctured. Sham animals also received an antibiotic treatment and resuscitative fluid as described above. For HMGB1 determination, mice were randomized 24 h after CLP and were injected i.p. with either sterile saline or choline (25 mg/kg). Mice received additional treatments at 30 h and 44 h after CLP. Blood was collected by cardiac puncture at 45 h after CLP, and serum HMGB1 levels were determined by quantitative Western blot analysis as described above. For survival studies, 24 h after CLP, mice were randomized and injected i.p. with either sterile saline or choline (25 mg/kg or 5 mg/kg). This treatment was repeated 6 h later (30 h after CLP) and then twice daily for 2 d for a total of six treatments. Survival was routinely monitored for 2 wks.

Isolation and Treatment of Human Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient fractionation from whole blood that was obtained from anonymous donors through the Long Island Blood Services (Westbury, NY, USA), and were differentiated to macrophages in culture as described previously (4). Briefly, PBMCs were harvested from the plasma/Ficol-Hypaque interface after centrifugation for 30 min at 550*g*. Cells were washed twice with PBS, once with RPMI 1640 medium, and resuspended in RPMI 1640 supplemented with 10% heat-inactivated human serum, 2 mM glutamine (Biowhittaker), and 100U/mL penicillin, 100 μg/mL streptomycin (Gibco). Cells were then seeded at 5×10^7 per 10 cm Falcon Primaria tissue culture plate and incubated for 2 h at 37° C in 5% CO_2 to allow attachment. Plates were then washed twice with PBS lacking Ca^{2+} and Mg^{2+}, and adherent cells were detached by gentle scraping in PBS lacking Ca^{2+} and Mg^{2+} and containing 1mM EDTA. Cells were washed once and resuspended in medium supplemented with human recombinant macrophage colony-stimulating factor (hrMCSF), and were seeded into 24-well Falcon Primaria tissue culture plates at 1×10^6 cells per well. Cultures were incubated for 6 d in the presence of hrMCSF to promote macrophage differentiation. Macrophages were rested for 24 h in the absence of hrMCSF prior to use. Media were removed and replaced with serum free Optimem media prior to treatment. Cells were incubated with the indicated concentration of choline for 10 min prior to exposure to LPS (20 ng/mL). Cell culture media were harvested 4 h after LPS treatment and secreted TNF was assayed by ELISA (R&D Systems) as described above.

Statistical Analysis

Data are expressed as mean ± SEM. Significant differences were assessed by using one way analysis of variance (ANOVA) followed by a Student *t* test. The statistical significance of differences between groups of animals in survival experiments was analyzed by the log-rank test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Choline Inhibits Endotoxin-Induced TNF Release and NF-κB Activation

The anti-inflammatory efficacy of cholinergic agonists has been tested previously by using RAW-264.7 mouse macrophage-like cells (9), a well-established cell culture system for *in vitro* studies of innate immune cell inflammatory responses. We studied the efficiency of choline in inhibiting TNF release from endotoxin-activated RAW cells. The cells were pre-incubated for 10 min with increasing choline concentrations prior to endotoxin activation. As shown in Figure 1A, choline dose-dependently suppressed endotoxin-stimulated TNF release from RAW cells. NF-κB is a key transcription factor that is activated in

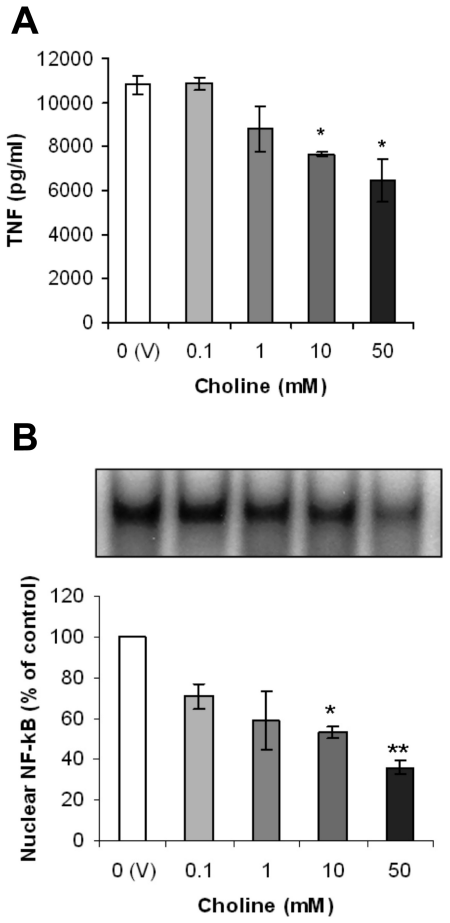

Figure 1. Choline inhibits TNF release (A) and NF-κB activation (B) in endotoxin-stimulated RAW-264.7 mouse macrophage-like cells. (A) RAW cells were exposed to the indicated concentration of choline 10 min prior to the addition of endotoxin (4 ng/mL). Culture supernatants were harvested 4 h later and TNF was determined by ELISA. Data represent the mean ± SEM of two representative experiments conducted in duplicate (*$P < 0.04$ as compared, with vehicle (V) treated controls). (B) RAW cells were exposed to the indicated concentrations of choline 10 min prior to the addition of endotoxin (4 ng/mL) and cells were harvested 2 h after endotoxin challenge for determination of NF-κB activation by EMSA. Autoradiographs were subjected to densitometry by using Quantity One software (Biorad). Data represent the mean ± SEM of three independent experiments (*$P < 0.04$, **$P < 0.006$ as compared with the lowest choline concentration tested).

response to endotoxin for the production of inflammatory mediators such as TNF. Therefore, we tested whether the choline-induced suppression of TNF was associated with inhibition of NF-κB activation. RAW cells were incubated for 10 min in the absence or presence of increasing concentrations of choline prior to exposure to endotoxin. Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) were conducted to measure activated NF-κB. Choline dose-dependently suppressed NF-κB activation in response to endotoxin (Figure 1B).

Choline Suppresses Systemic TNF Levels During Endotoxemia Through an α7nAChR-Mediated Mechanism

Based on our *in vitro* data, we next tested whether choline reduces systemic TNF during endotoxemia. Choline (5 mg/kg or 50 mg/kg) or vehicle (saline) was injected into BALB/c mice at 6 h and at 30 min prior to endotoxin (6 mg/kg i.p.) administration. This dose of endotoxin previously was shown to cause about 80% mortality (9). The higher choline dose (50 mg/kg) significantly suppressed serum TNF levels (Figure 2A). The lower choline dose (5 mg/kg) failed to alter serum TNF levels (see Figure 2A). In light of the recently discovered role for the α7nAChR in mediating the cholinergic suppression of systemic TNF during endotoxemia (4), and the fact that choline is a selective α7nAChR agonist, we tested the efficacy of choline in suppressing systemic TNF levels during endotoxemia in WT and α7nAChR KO mice. WT and age-matched α7nAChR KO mice were injected i.p. with choline (50 mg/kg) or vehicle (saline) at 6 h and at 30 min prior to endotoxin (6 mg/kg, i.p.) administration. Choline significantly suppressed systemic TNF levels in endotoxemic WT mice as compared with saline-treated endotoxemic controls (Figure 2B, $P < 0.05$). In contrast, choline administration in α7nAChR KO mice did not alter systemic TNF levels significantly, as compared with controls (see Figure 2B). Macrophages represent a major source of TNF during endotoxemia (21,22), and the α7nAChR expressed on macrophages plays a critical role in mediating cholinergic anti-inflammatory signaling (4). Therefore, we reasoned that choline may suppress macrophage TNF release through an α7nAChR-dependent signaling mechanism. Accordingly, we examined the effect of choline on TNF release from endotoxin-activated peritoneal macrophages collected from WT mice and α7nAChR KO mice. Peritoneal macrophages were exposed to the indicated concentrations of choline 10 min prior to the addition of endotoxin, and TNF levels were measured in media supernatants collected 4 h later. As shown in Figure 2C, choline dose-dependently suppressed TNF release by macrophages from WT mice. However, choline treatment did not suppress TNF release by macrophages isolated from α7nAChR KO mice (see Figure 2C).

Choline Improves Survival in Lethal Endotoxemia

We have shown previously that the α7nAChR agonist GTS-21 significantly improves survival of BALB/c mice during endotoxemia (9). We next studied whether choline improves survival in lethal endotoxemia. Choline (5 mg/kg or 50 mg/kg, i.p.) or vehicle (saline, i.p.) was injected into BALB/c mice at 6 h and at 30 min prior to the i.p. administration of endotoxin (6 mg/kg, i.p.). Vehicle-treated mice showed a 27% survival rate that was not improved by 5 mg/kg choline (Figure 3). In contrast, treatment with 50 mg/kg choline resulted in a significantly improved survival rate of 63% (see Figure 3).

Choline Suppresses HMGB1 Release and Improves Survival in Mice with Severe Sepsis

HMGB1 is a late pro-inflammatory cytokine mediator of inflammation during experimental sepsis and an important therapeutic target in the treatment of this disorder (23–25). We studied whether choline suppresses HMGB1 release *in*

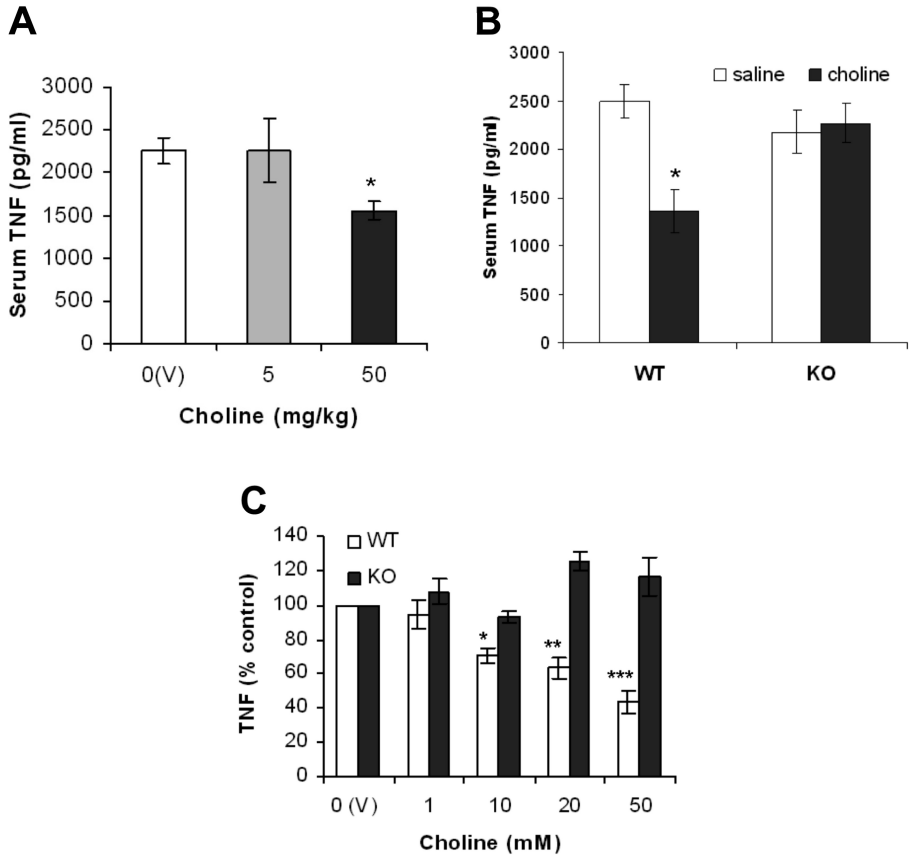

Figure 2. Choline suppresses systemic TNF levels during endotoxemia through an α7nAChR-dependent mechanism. (A) Choline or vehicle (V, saline) was injected i.p. in BALB/c mice (n = 10 per group) at 6 h, and at 30 min, prior to endotoxin (6 mg/kg, i.p.) administration. Serum TNF was analyzed by ELISA in blood obtained 90 min after endotoxin administration. Results show the mean ± SEM for each group (*$P < 0.05$ as compared with vehicle (V) administered controls). (B) Choline (50 mg/kg, i.p.) or saline was injected i.p. in age-matched WT and α7nAchR KO mice (WT n = 8–9/group, α7nAChR KO n = 7–9/group) at 6 h, and at 30 min, prior to endotoxin (6 mg/kg, i.p.) administration. Serum TNF was analyzed by ELISA in blood obtained 90 min after endotoxin administration. Results show the mean ± SEM for each treatment group (*$P < 0.001$ as compared with saline administered controls). (C) Peritoneal macrophages from age-matched WT and α7nAChR KO mice were incubated with the indicated concentrations of choline or vehicle (V) for 10 min prior to exposure to endotoxin (100 ng/mL). TNF in cell culture media was determined by ELISA 4 h after endotoxin addition. Results represent the mean ± SEM of three independent experiments conducted in duplicate (*$P < 0.04$, **$P < 0.02$, ***$P < 0.002$ as compared with lowest choline concentration tested).

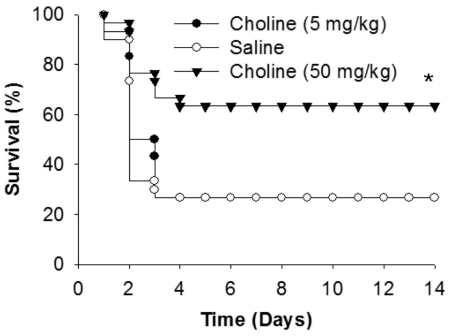

Figure 3. Choline improves survival in lethal endotoxemia. BALB/c mice (n = 30/ group) were injected i.p. with either vehicle (saline) or choline at 6 h, and at 30 min, prior to endotoxin (6 mg/kg, i.p.) administration. Survival was monitored for 14 d (*$P < 0.002$).

vitro and *in vivo* and improves survival of mice with polymicrobial sepsis. Based on our data that choline suppresses TNF release and NF-κB activation in RAW cells (see Figure 1A,1B), we tested whether choline also attenuates HMGB1 release from RAW cells. Choline dose-dependently reduced HMGB1 release from endotoxin-stimulated RAW cells (Figure 4A). We next studied whether choline suppresses serum levels of HMGB1 in mice with CLP-induced sepsis. Choline (25 mg/kg) or saline were injected i.p. to septic mice 24 h after the CLP surgery. Mice received additional treatments 30 h and 44 h after CLP, and serum HMGB1 levels were determined in blood obtained at 45 h after CLP. Serum HMGB1 levels were reduced by 85% in the animals receiving choline as compared with the saline-administered controls (Figure 4B). It is noteworthy that the mortality rate at the 45-h time point was higher in saline-treated mice (5/12) compared with the choline-treated group (1/12) (data not shown). We then specifically tested whether choline improves survival when therapeutically administered to septic mice. Choline (25 mg/kg, or 5 mg/kg) or vehicle (saline) was administered i.p. to mice with CLP-induced sepsis 24 h after surgery. This treatment was repeated 6 h later (30 h after surgery) and twice daily for 2 d more. The survival rate for choline-treated (25 mg/kg) mice (64%) was improved significantly when compared with control animals (23%) (Figure 4C). The 42% survival rate of septic mice treated with the lower choline dose (5 mg/kg) was not significantly different as compared with control mice (see Figure 4C). These data show that therapeutically administered choline attenuates systemic HMGB1 levels and improves survival in polymicrobial sepsis.

Choline Suppresses TNF Production from Endotoxin-Stimulated Human Whole Blood and Cultured Macrophages

To examine the anti-inflammatory efficiency of choline in human cells, we

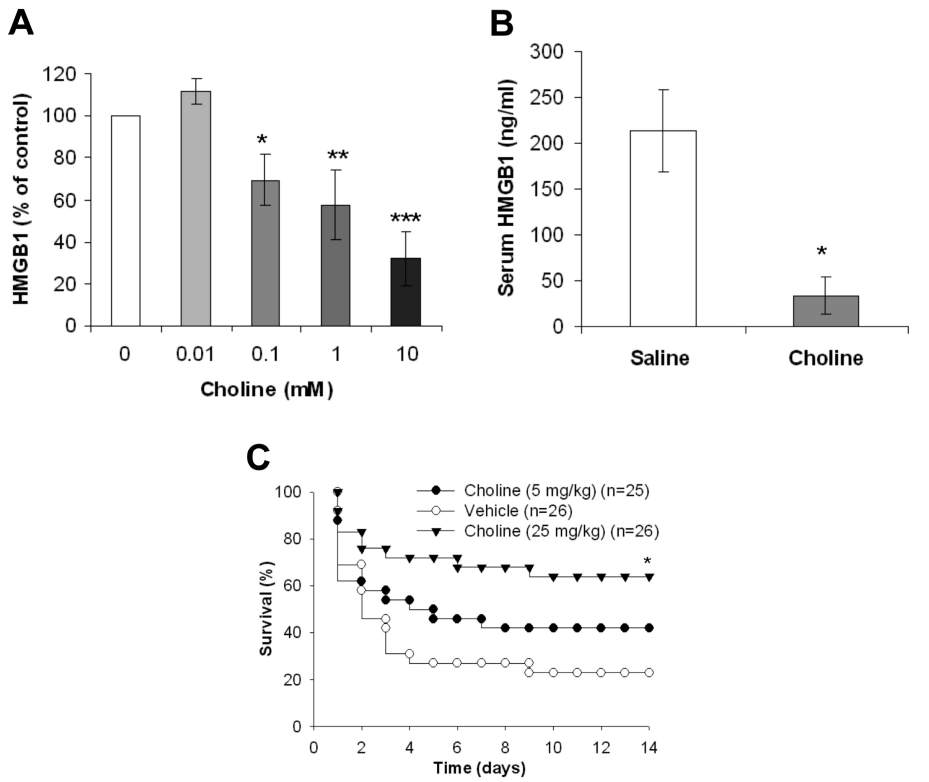

Figure 4. Choline treatment suppresses HMGB1 release and improves survival in severe sepsis. (A) Choline suppresses HMGB1 release from endotoxin-stimulated RAW cells. RAW cells were exposed to the indicated concentration of choline or vehicle 10 min prior to LPS (100 ng/mL) addition for 24 h. Culture supernatants were harvested, and secreted HMGB1 was detected by Western blot analysis. HMGB1 was not detected in the supernatant from cells that were not treated with LPS. Data represent the mean ± SEM of four experiments conducted in duplicate (*$P < 0.05$, **$P < 0.02$, ***$P < 0.001$ as compared with lowest choline concentration tested). (B) Mice (n = 12) were administered i.p. with either saline or choline (25 mg/kg) 24 h after CLP. Mice received additional treatments at 30 h and 44 h after CLP. Serum HMGB1 levels were determined in surviving mice (n = 11 for choline treatment, n = 7 for control treatment) in blood obtained at 45 h after CLP (*$P < 0.0008$). (C) Mice (n = 26 per group) were subjected to CLP surgery. 24 h after CLP, mice were randomized and injected i.p. with either saline or choline (25 mg/kg). This treatment was repeated 6 h later (30 h after CLP) and twice daily for 2 d more for a total of six treatments, and survival was monitored for 2 wks (*$P < 0.002$).

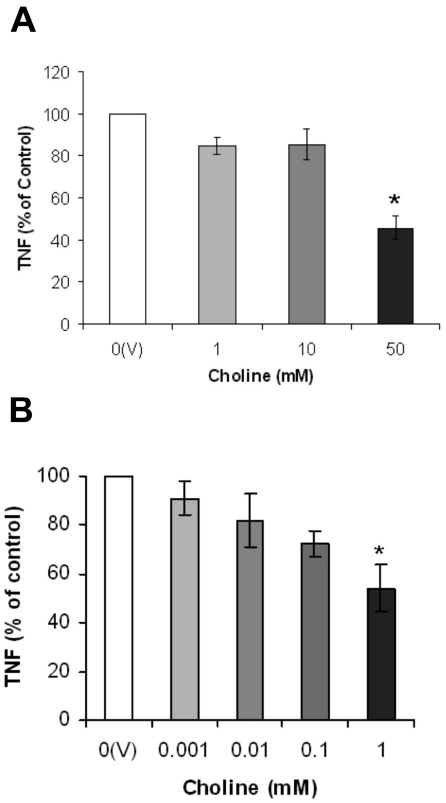

Figure 5. Choline suppresses TNF release from endotoxin-stimulated human whole blood (A) and human macrophages (B). (A) Whole blood was treated for 10 min with the indicated concentrations of choline prior to endotoxin (10 ng/mL) challenge at 37° C. Plasma TNF was determined by ELISA 4 h later. Data represent the mean ± SEM of duplicate determinations from five donors (*$P < 0.0001$) as compared with the lowest choline concentration tested). (B) Peripheral blood mononuclear cells (PBMCs) were isolated from adult donors and were differentiated into macrophages. Macrophages were treated with the indicated concentration of choline 10 min prior to the addition of endotoxin (20 ng/mL). Culture supernatants were harvested 4 h later and the level of TNF secreted into the media was determined by ELISA. Data represent the mean ± SEM of at least three experiments conducted in duplicate from independent donors (*$P < 0.02$) as compared with the lowest choline concentration tested.

tested whether choline suppressed TNF release from endotoxin-activated human whole blood and cultured human macrophages. Blood was collected from healthy volunteers and blood samples were exposed to increasing concentrations of choline or vehicle for 10 min prior to the addition of endotoxin. As shown in Figure 5A, choline (50 mM) significantly suppressed endotoxin-induced TNF release from human whole blood. In a parallel set of experiments, human macrophages that express the α7nAChR (4) were differentiated from peripheral blood mononuclear cells (PBMCs) and treated with various choline concentrations 10 min prior to the addition of endotoxin. As shown in Figure 5B, choline (1 mM) significantly reduced TNF release from endotoxin-stimulated human macrophages. The level of TNF suppression did not increase with higher concentrations of choline (up to 50mM) (data not shown).

DISCUSSION

In this study, we show that choline suppresses serum TNF levels in endotoxemic mice and this anti-inflammatory effect of choline is dependent on an α7nAChR-mediated signaling. In addition, we demonstrate the anti-inflammatory efficacy of choline in experimental polymicrobial sepsis and in human cells.

Choline suppressed systemic TNF levels in endotoxemic mice, but failed to reduce TNF levels in mice lacking the α7nAchR (α7nAchR KO mice) and these findings clearly indicate the α7nAChR dependence of this anti-inflammatory *in vivo* effect of choline. In contrast to its suppressive effect on TNF release from endotoxin-stimulated WT peritoneal macrophages, choline did not suppress TNF release from α7nAChR KO cells. These results strengthen the concept that α7nAChR expressed on macrophages and other immune cells plays a critical role in controlling inflammatory responses (4,26).

Choline, a byproduct of acetylcholine degradation, is a stable, natural, and selective agonist on α7nAChR. Earlier studies indicated that acetylcholine suppresses TNF release from peritoneal mouse macrophages and human macrophages (3,4), but it was unknown previously whether choline also can regulate TNF in these cells. Interestingly, our data show that choline concentrations that suppress TNF release from these endotoxin-stimulated immune cells are significantly higher than acetylcholine concentrations (in the presence of an acetylcholinesterase inhibitor) that exert similar suppressive effects (3,4). These observations are in line with studies showing a lower agonistic efficacy of choline on neuronal α7nAChRs as compared with acetylcholine (14,27,28) and have implications for signaling after acetylcholine release, because choline can persist after acetylcholine degradation.

The expression of the α7nAChR in several non-neuronal cells, including macrophages, monocytes, and dendritic cells, has been documented (8,29,30). However, knowledge about the receptor function and pharmacological characteristics related to cytokine production is very limited. Previously, agonistic properties of choline have been studied on neuronal α7nAChRs and it has been shown that choline is a full agonist on the α7nAChR with an EC_{50} of 1.6 mM (12). These data were suggestive for the drug concentrations we used to study the effects of choline on TNF release, NF-κB activation, and HMGB1 release in response to endotoxin. Our results show that choline concentrations required to cause statistically significant suppression of TNF release and NF-κB activation *in vitro* are generally higher than those that suppress HMGB1 release. This observation may indicate that the cellular mechanisms governing the release of HMGB1 could be more sensitive to choline-stimulated α7nAChR signaling than those controlling the release of TNF. This *in vitro* difference also was extrapolated to the *in vivo* studies. While a choline dose of 50 mg/kg was required to suppress serum TNF significantly and to improve survival during endotoxemia, a lower choline dose of 25 mg/kg significantly inhibited serum HMGB1 levels and improved survival in mice with polymicrobial sepsis. A possible explanation could be related to differences between the underlying inflammatory mechanisms of endotoxemia and CLP-sepsis. The survival-improving effect of choline, administered i.p. 6 h and 30 min prior to endotoxin is in line with a previous study, demonstrating that a choline-rich diet improves survival in endotoxemic rats (18). Moreover, our results that choline treatment, initiated within a clinically relevant time frame, improves survival in polymicrobial sepsis indicate the potential for clinical development of choline.

Choline deficiency has been shown previously to induce liver injury in humans and in rodents, which is exacerbated upon endotoxin administration (31,32). In contrast, choline (20 mg/kg, intravenous [i.v.]) administration suppresses TNF release and attenuates inflammation during endotoxemia in dogs (19,20). Moreover, choline (60 mg/kg, i.v.) attenuates acid-induced lung injury in mice (33). The effective doses of choline used in the present study (25–50 mg/kg, i.p.) are within the dose range used in these other studies. It is important to note that we did not observe any adverse neurobehavioral effects of these choline doses, which are comparable with the recommended tolerable upper limit of dietary choline intake in humans (34). Unlike other synthetic α7nAChR agonists, choline is an endogenous molecule with important physiological functions, including its vital roles in maintaining the structural integrity of cell membranes and providing methyl groups for the synthesis of betaine, thus participating in methionine, folate, and homocysteine metabolism (34). While some of these metabolic functions have been linked previously to anti-inflammatory effects of choline (18,35) our data clearly show that α7nAChR signaling is required for the anti-inflammatory efficacy of this compound during endotoxemic shock. Our results (data not shown) also indicate that α7nAChR plays a role in mediating the anti-inflammatory efficacy of choline in polymicrobial sepsis. Therefore, our findings bring new light to these previous studies and suggest that endogenous choline may act on the α7nAChR and play an important role in regulating innate immune responses to maintain homeostasis. Choline also is a precursor for the synthesis of acetylcholine, which is the principle neurotransmitter of the efferent vagus nerve (36). It is possible that a portion of the exogenous choline is metabolized as a substrate for acetylcholine biosynthesis, which may contribute to anti-inflammatory effects *in vivo*. In the cholinergic synapse, acetylcholine is degraded rapidly by acetylcholinesterases into acetate and choline. Choline generated in this mode may act to prolong α7nAChR activation selectively (36). While indicating a critical anti-inflammatory role for choline as an α7nAChR agonist, we cannot entirely exclude the contribution of other effects of

choline to its anti-inflammatory activity *in vivo*, including the stimulation of cholinergic signaling in the central nervous system (CNS), which has been shown recently to play a role in controlling inflammation during endotoxemia (37). However, it is possible that these alternative pathways also culminate in α7nAChR-mediated signaling.

Choline also suppressed TNF release from endotoxin-activated human whole blood and macrophages effectively, demonstrating the anti-inflammatory efficacy of this compound in human cells. In conclusion, our data provide experimental evidence that the cholinergic agonist choline suppresses TNF release through an α7nAChR-dependent mechanism and has therapeutic potential in the treatment of sepsis and other inflammatory diseases.

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