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Ketamine Suppresses Endotoxin-induced Tumor Necrosis Factor Alpha Production in Mice

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***Background*: The cytokines play important roles in the pathophysiologic alterations associated with sepsis, but there are no reports about the effect of anesthetics on their production. Therefore, the authors examined the effect of ketamine on lipopolysaccharide (LPS)-induced and calcium ionophore A23187-induced tumor necrosis factor alpha (TNF-α) production in thioglycolate (TGC)-elicited peritoneal macrophages (MPs) in ddY mice.**

***Methods*: Ketamine was added to TGC-elicited MPs at various times after the stimulation with LPS or A23187. After the MPs were stimulated by LPS or A23187 and incubated, TNF-α activities in the supernatant of MPs were determined by an L929 cytotoxic assay. *In vivo*, the ddY mice were injected intraperitoneally with TGC. Four days later, they were injected subcutaneously with ketamine and then injected intravenously with LPS. Two hours after the LPS challenge, TNF-α activities of the sera were determined.**

***Results*: Ketamine suppressed both LPS-induced and A23187-induced TNF-α production in a dose-dependent manner. The simultaneous addition of ketamine to LPS-stimulated and A23187-stimulated MPs resulted in a 50% inhibition of TNF-α production at 20 μg/ml and 12.5 μg/ml, respectively. Ketamine also caused a significant suppression of TNF-α production even when added to the MPs 2 h after the LPS challenge. There was a significant decrease in A23187-induced TNF-α production in TGC-elicited MPs in a calcium-depleted medium when compared with that in a calcium-containing medium. Conversely, LPS-induced TNF-α production did not cause such a result. In addition, ketamine could suppress LPS-induced TNF-α production in TGC-pretreated mice *in vivo*.**

***Conclusions*: Ketamine suppresses LPS-induced TNF-α production in both TGC-elicited MPs and TGC-pretreated mice.**

(Key words: Anesthetics, intraperitoneal: ketamine. Sepsis: lipopolysaccharide; tumor necrosis factor alpha.)

SEPTIC shock is associated with many pathophysiologic alterations, such as metabolic acidosis, potentially lethal hypotension, and widespread end-organ damage. It has been reported that these changes are induced by lipopolysaccharide (LPS), which is a component of the bacterial cell walls.[1,2] More recent investigations have shown that the production of cytokines plays an important role in LPS-induced pathophysiologic events with sepsis.[3] Tumor necrosis factor alpha (TNF-α) has been implicated as a main cytokine in the pathogenesis of sepsis[4,5] because of several studies that have cited the following findings: (1) Purified TNF-α induces the pathophysiologic alterations associated with sepsis.[6,7] (2) Antibodies to TNF-α protect against the lethality of sepsis.[8–10] (3) Serum levels of TNF-α are increased in patients with sepsis.[11]

Ketamine commonly has been used for the induction of anesthesia in patients with sepsis because of its cardiostimulatory properties.[12] On the other hand, it has been reported that in patients with shock, including septic shock, induction of anesthesia with ketamine produces marked cardiovascular depression.[13] The use of ketamine anesthesia in patients with sepsis is still controversial. However, there are no reports about the effect of ketamine on the production of TNF-α.

Macrophages stimulated by LPS play a principal role in secreting TNF-α in sepsis. Recently, in addition to LPS, calcium ionophore A23187, which causes influx of extracellular calcium, has been reported to be capable of stimulating macrophages to produce TNF-α.[14] Despite the importance of LPS or calcium ionophore–macrophage interaction, this mechanism for the stimulation of macrophages is not well understood.

Therefore, we performed this study to examine the following: (1) the role of extracellular calcium influx on LPS-induced and A23187-induced TNF-α production *in vitro*; (2) the effects of ketamine on TNF-α produc-

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tion induced by two different stimulators, LPS and calcium ionophore A23187, *in vitro*; and (3) the effect of ketamine on LPS-induced TNF-α production *in vivo*.

Methods and Materials

Mice

Male ddY mice were obtained from Seiwa Experimental Animal (Oita, Japan). All mice used were 6–8 weeks of age. This study was approved by the Institutional Animal Care and Use Committee.

Reagents

Phenol-extracted *Escherichia coli* (0127: B8) LPS was purchased from Difco Laboratories (Detroit, MI). The A23187 was purchased from Sigma Chemical (St. Louis, MO). The ketamine was purchased from Sankyo Pharmaceutical (Tokyo, Japan).

Experimental Approach In Vitro

The 6–8-week-old ddY mice were injected intraperitoneally with 2 ml 3% thioglycolate (TGC). The mice were maintained under standard care and given food and water *ad libitum*. Four days later, peritoneal exudate macrophages were lavaged, washed twice in phosphate-buffered saline, and suspended in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum (GIBCO Laboratories, Grand Island, NY). For the calcium-depletion experiments, minimal essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) was used, because it was available with and without calcium (MEM-D). After the concentration of peritoneal macrophages (MPs) was adjusted to 2×10^6 cells/ml, 0.5 ml of the cell suspension was transferred to flat-bottomed, 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) and incubated at 37° C for 2 h. After nonadherent cells were removed by washing the plates three times in phosphate-buffered saline, the cells were found to contain > 98% macrophages, as determined by morphology with a Giemsa stain.

Ketamine was added to TGC-elicited MPs at the time of the stimulation with LPS or A23187. After the MPs were incubated for 16 h at 37° C in a carbon dioxide incubator, TNF-α activities in the supernatant of the MPs were determined by an L929 cytotoxic assay.

Experimental Approach In Vivo

The ddY mice were injected intraperitoneally with 2 ml 3% TGC. Four days later, they were injected subcutaneously with 5 mg ketamine 15 min before the LPS challenge. LPS (50 μg in 0.5 ml of saline) was injected intravenously into the tail vein. Control mice were injected subcutaneously with 0.5 ml saline before the LPS challenge. Two hours after the LPS injection, the sera were obtained to measure the TNF-α activity.

TNF Assay

TNF-α activity in the supernatant of macrophages cultures was determined by the L929-cells cytotoxic assay described previously.[15] Briefly, L929 cells in an RPMI 1640 medium containing 5% fetal calf serum were seeded at 3×10^4 cells/well in 96-well, flat-bottomed microtiter plates (Becton Deckinson, Lincoln Park, NJ) and incubated overnight at 37° C in an atmosphere of 5% CO_2 in air. Serial 1:2 dilutions of either samples or supernatants were made in the aforementioned medium, containing 1 μg/ml actinomycin D (Banyu Pharmaceutical, Tokyo, Japan), and 0.1 ml of each dilution was added to the wells. On the following day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), and 0.1 ml of 1% sodium dodecyl sulfate was added to each well to solubilize the stained cells. A microplate reader (Bio-Rad Laboratories, Richmond, CA) determined the absorbance of each well to be 490 nm. One unit of TNF-α activity was defined as the amount of TNF-α giving 50% cell survival. In our assay, 1 U/ml was equivalent to 0.63 pg/ml of recombinant murine TNF-α.

Statistics

Student's *t* test was used to compare groups of data expressed as mean values ± SEM. A *P* value of less than 0.05 was considered statistically significant.

Results

Effects of Lipopolysaccharide or A23187 on TNF-α Production

TGC-elicited MPs were stimulated by various doses of LPS (0.01–10 μg/ml) or A23187 (0.1–10 μg/ml). LPS induced TNF-α production in the MPs in a dose-dependent manner at a concentration between 0.01 μg/ml and 1 μg/ml. LPS concentrations greater than 1 μg/ml were found to induce maximum TNF-α production (data not shown). A23187 induced TNF-α production in the MPs in a dose-dependent manner at a concentration between 0.1 μg/ml and 1 μg/ml. Peak

TNF-α production occurred after A23187 administration at a concentration of 1 μg/ml (data not shown).

Effects of Time Intervals between Ketamine Treatment and Lipopolysaccharide Challenge on TNF-α Production

Ketamine (100 μg/ml) was added to TGC-elicited MPs at various times (−2, −1, 0, +1, and +2 h) after the LPS (1 μg/ml) challenge (fig. 1). Ketamine significantly suppressed LPS-induced TNF-α production at each time interval, even when added to MPs 2 h after LPS, when compared with the nonketamine-treated MPs.

Effects of Ketamine on Lipopolysaccharide-induced TNF-α Production

Various doses (1–400 μg/ml) of ketamine were added to TGC-elicited MPs at the time of the LPS (1 μg/ml) challenge. Ketamine suppressed LPS-induced TNF-α production in a dose-dependent manner at a concentration between 5 μg/ml and 200 μg/ml. The 50% inhibitory concentration was observed with 20 μg/ml of ketamine. More than 400 μg/ml ketamine suppressed the TNF-α production to undetectable levels. However, less than 5 μg/ml ketamine had no effect (fig. 2).

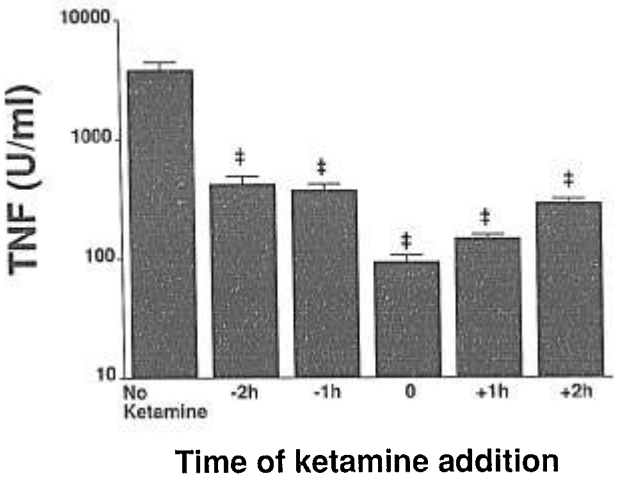

Fig. 1. Inhibitory effects of time intervals between ketamine treatment and LPS challenge on TNF-α production. Ketamine (100 μg/ml) was added to the peritoneal macrophages at various times (−2, −1, 0, +1, and +2 h) after the LPS (1 μg/ml) challenge. Ketamine significantly suppressed the TNF-α production at each time interval. Each point represents means values ± SEM for seven experiments. ‡ = $P < 0.01$ *versus* nonketamine-treated one.

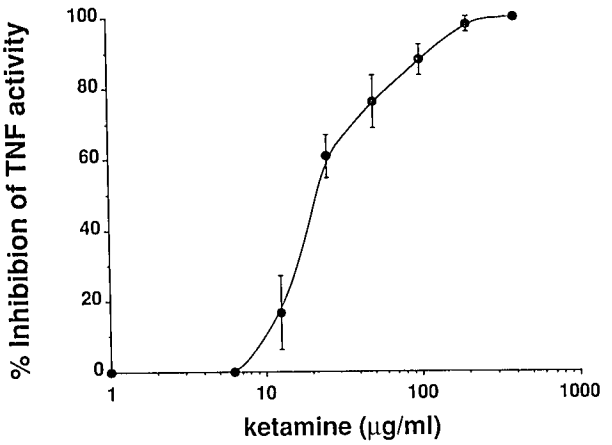

Fig. 2. Inhibitory effects of ketamine on LPS-induced TNF-α production. The peritoneal macrophages were treated with various doses (1–400 μg/ml) of ketamine at the time of the LPS (1 μg/ml) challenge and incubated for 16 h. The data are percentages of control values (*i.e.*, for macrophages not treated with ketamine). Each point represents mean values ± SEM for seven experiments.

Effects of Ketamine on A23187-induced TNF-α Production

Various doses (1–400 μg/ml) of ketamine were added to TGC-elicited MPs at the time of the A23187 (1 μg/ml) challenge. Ketamine suppressed A23187-induced TNF-α production in a dose-dependent manner. The 50% inhibitory concentration was observed with 12.5 μg/ml of ketamine. At a concentration above 200 μg/ml, ketamine suppressed the TNF-α production to undetectable levels (fig. 3).

Effects of Calcium on Lipopolysaccharide-induced TNF-α Production

Adherent MPs were washed three times in phosphate-buffered saline and incubated for 2 h at 37° C in a carbon dioxide incubator in either MEM or MEM-D. After either fresh medium was replaced, various doses (200 or 400 μg/ml) of ketamine were added to the MPs in each medium at the time of the LPS (1 μg/ml) challenge. There were no significant differences between LPS-induced TNF-α production in MEM and that in MEM-D. Ketamine also significantly suppressed LPS-induced TNF-α production in both media (fig. 4).

Effects of Calcium on A23187-induced TNF-α Production

Adherent MPs were washed three times in phosphate-buffered saline and incubated for 2 h at 37° C in a

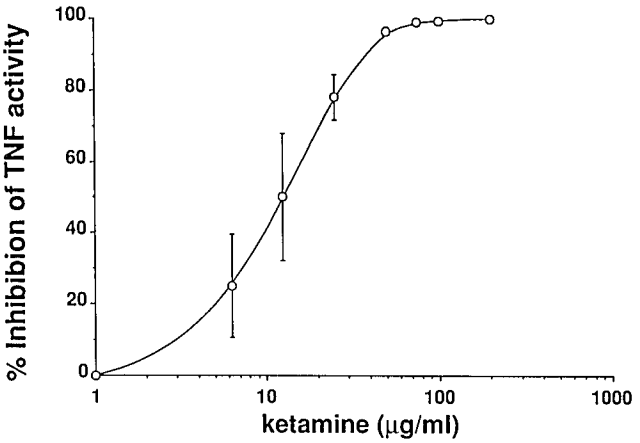

Fig. 3. Inhibitory effects of ketamine on A23187-induced TNF-α production. The peritoneal macrophages were treated with various doses (1–400 μg/ml) of ketamine at the time of the A23187 (1 μg/ml) challenge and incubated for 16 h. The data are percentages of control values (*i.e.*, for macrophages not treated with ketamine). Each point represents mean values ± SEM for seven experiments.

carbon dioxide incubator in either MEM or MEM-D. After either fresh medium was replaced, various doses (200 or 400 μg/ml) of ketamine were added to the MPs in each medium at the time of the A23187 (1 μg/ml) challenge. There was a significant decrease in A23187-induced TNF-α production in MEM-D when compared with that in MEM. Ketamine significantly suppressed A23187-induced TNF-α production in both media (fig. 5).

In Vivo *Effects of Ketamine on Lipopolysaccharide-induced TNF-α Production in TGC-pretreated Mice*

LPS (50 μg/mouse)-induced TNF-α activities in sera of control mice and ketamine (5 mg/mouse)-treated mice were 167 ± 54 U/ml and 6.5 ± 4.8 U/ml, respectively. Ketamine significantly suppressed LPS-induced TNF-α production *in vivo* (fig. 6).

Discussion

The present study demonstrates that the calcium ionophore A23187 induced TNF-α production in TGC-elicited MPs, and ketamine suppressed both LPS-induced and A23187-induced TNF-α production in TGC-elicited MPs.

Our data show that A23187-induced TNF-α production in a calcium-depleted medium is less than that in a calcium-containing medium. These findings indicate that calcium influx into macrophages can be a trigger for A23187-induced TNF-α production in TGC-elicited MPs. However, extracellular calcium influx is not the only mechanism of A23187-induced TNF-α production, because the MPs in a calcium-depleted medium were shown to produce TNF-α in response to A23187. Previous studies have suggested that A23187 is the molecule that mimics the action of inositol triphosphate.[16] We assumed that A23187-induced TNF-α production in a calcium-depleted medium was caused by A23187 that had the mimic action of inositol triphosphate. Further study will be needed to clarify the mechanism of A23187-induced TNF-α production.

In contrast, LPS-induced TNF-α production was not influenced by the presence of extracellular calcium. These findings indicate that extracellular calcium influx is not required for LPS-induced TNF-α production. Other investigators have suggested that A23187 caused an uptake of radiolabeled extracellular calcium by macrophages, but LPS did not.[14] Taken together, the mechanism of LPS-induced TNF-α production was dif-

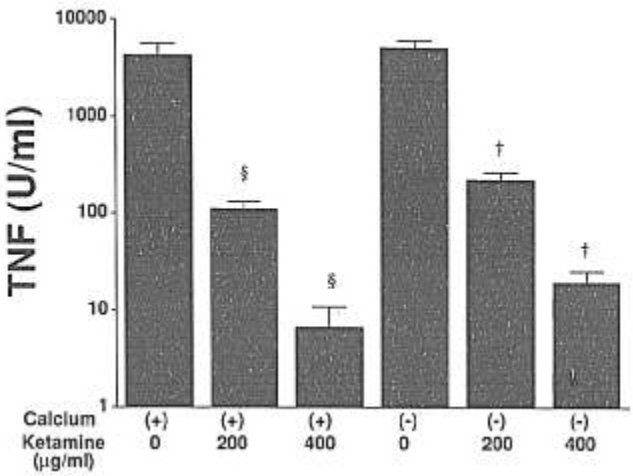

Fig. 4. Effects of calcium on LPS-induced TNF-α production. Adherent peritoneal macrophages were washed three times in phosphate-buffered saline and incubated for 2 h at 37° C in a carbon dioxide incubator in either MEM or MEM-D. After either fresh medium was replaced, the peritoneal macrophages were treated with various doses (200 or 400 μg/ml) of ketamine at the time of the LPS (1 μg/ml) challenge and incubated for 16 h. There were no significant differences between the TNF-α production in MEM and that in MEM-D. Ketamine significantly suppressed the TNF-α production in both media. The results are expressed as mean values ± SEM for seven experiments. § = $P < 0.01$ *versus* nonketamine-treated group in MEM; † = $P < 0.01$ *versus* nonketamine-treated group in MEM-D.

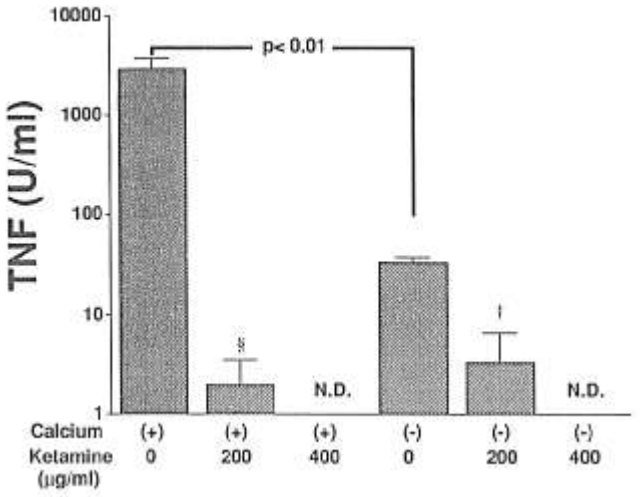

Fig. 5. Effects of calcium on A23187-induced TNF-α production. Adherent peritoneal macrophages were washed three times in phosphate-buffered saline and incubated for 2 h at 37° C in a carbon dioxide incubator in either MEM or MEM-D. After either fresh medium was replaced, the peritoneal macrophages were treated with various doses (200 or 400 μg/ml) of ketamine at the time of the A23187 (1 μg/ml) challenge and incubated for 16 h. There was a significant decrease in A23187-induced TNF-α production in MEM-D when compared with that in MEM ($P < 0.01$). Ketamine significantly suppressed the TNF-α production in both media .The results are expressed as mean values ± SEM for seven experiments. § = $P < 0.01$ *versus* nonketamine-treated group in MEM; † = $P < 0.05$ *versus* nonketamine-treated group in MEM-D; N.D. = not detected.

ferent from that of A23187-induced TNF-α production. We believe that LPS-induced TNF-α production occurs via the extracellular calcium influx independent signal transduction pathway.

We demonstrated that ketamine suppressed TNF-α production in TGC-elicited MPs under three different conditions: (1) LPS-induced TNF-α production, (2) A23187-induced TNF-α production in a calcium-containing medium, and (3) A23187-induced TNF-α production in a calcium-depleted medium. We do not know by what mechanism ketamine suppresses the TNF-α production; however, our results suggest that ketamine acts on both extracellular calcium-influx–dependent and extracellular calcium-influx–independent signal transduction pathways. It has been reported that agents that suppress LPS-induced TNF-α production include prostaglandin E_2,[17] corticosteroids such as dexamethasone,[18] cyclic adenosine 3′:5′ monophosphate (cAMP) analogs such as dibutyryl cAMP and 8-bromo cAMP,[19] phosphodiesterase inhibitors such as pentoxifylline,[20] β-adrenergic agonists such as epinephrine,[21] and platelet-activating factor antagonists.[22] When dexamethasone and prostaglandin E_2 are given after an LPS injection, they do not suppress LPS-induced TNF-α production.[17,18,23] These indicate that they are incapable of suppressing LPS-induced TNF-α production once the gene expression of TNF-α is initiated. Dexamethasone and prostaglandin E_2 regulate LPS-induced TNF-α production predominantly at a transcriptional level.[23] In contrast, our findings show that the addition of ketamine 2 h after the LPS stimulation was effective in suppressing the TNF-α production. TNF-α messenger ribonucleic acid levels have been noted to rise detectably minutes after the LPS injection and peak 2 h after the LPS injection.[24] We therefore assume that ketamine may regulate LPS-induced TNF-α production at a posttranscriptional level.

Our data demonstrate that an intraperitoneal injection of 5 mg ketamine also results in the suppression of LPS-induced TNF-α production *in vivo*. However, less than 2 mg of ketamine cannot suppress it (data not shown). We also have observed in a carrageenan–endotoxin shock model[25] of rats that the continuous intravenous infusion of ketamine at 10 $mg \cdot kg^{-1} \cdot h^{-1}$ prevents LPS-induced TNF-α production, lethality, and an early cardiovascular depression.§ A possible explanation for the

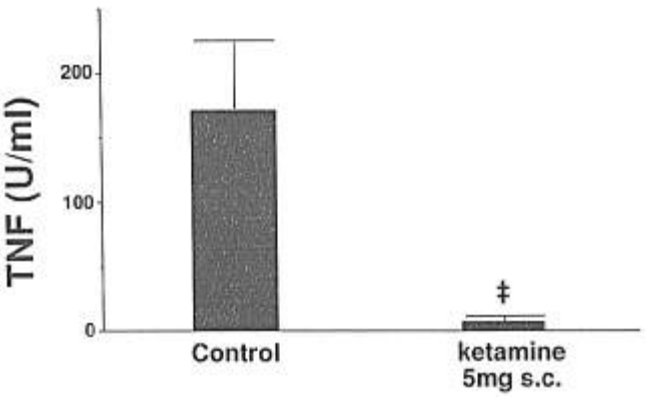

Fig. 6. Inhibitory effects of ketamine on LPS-induced TNF-α production in TGC-pretreated mice. Each mouse was given an intraperitoneal injection of 2 ml 3% TGC 4 days before LPS (50 μg per mouse) was injected intravenously. The TNF-α production of mice treated with ketamine (5 mg per mouse) subcutaneously 15 min before the LPS challenge was significantly suppressed when compared with that of control mice, which were injected subcutaneously with 0.5 ml saline before the LPS challenge. The results are expressed as mean values ± SEM. Each group consisted of 10 ddY mice. ‡ = $P < 0.01$ *versus* control.

§ Koga K, *et al:* Unpublished data. 1993.

large dose of ketamine required to suppress TNF-α production in mice is that a large dose bolus intraperitoneal injection of ketamine may be necessary to maintain the plasma ketamine concentration, which suppresses the TNF-α production, during a period of TNF-α production after the LPS injection. It has been shown that serum levels of TNF-α peak at 2 h and disappear 6 h after the LPS injection.[25]

Recently, some reports have shown that TNF-α alone cannot explain the complex pathophysiologic alterations with sepsis, because LPS induces other cytokines and inflammatory mediators such as interleukin-1, interleukin-6, platelet activating factor, and leukotrienes, which may participate in these alterations with sepsis.[10,26] The pathophysiologic alterations with sepsis can continue to deteriorate even in absence of detectable serum levels of TNF-α and anti–TNF-α antibodies have little protective effect when given after the LPS administration.[8] Once the cascade of events that causes various pathophysiologic alterations with sepsis have been set fully in motion, TNF-α alone may be incapable of explaining this complex disease. However, because TNF-α is the first cytokine to appear in the circulation in response to LPS administration and activates characteristic cascades of other cytokines and inflammatory mediators, TNF-α plays an important role in the hemodynamic and metabolic alterations in the early phase of sepsis.[27]

In summary we have shown that ketamine suppresses both A23187-induced and LPS-induced TNF-α production in TGC-elicited mouse MPs; ketamine inhibits TNF-α production in TGC-elicited mouse MPs *via* both extracellular calcium-influx–dependent and extracellular calcium-influx–independent signal transduction pathways; and ketamine suppresses LPS-induced TNF-α production in TGC-pretreated mice *in vivo*.

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