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# Midazolam suppresses the lipopolysaccharide-stimulated immune responses of human macrophages via translocator protein signaling

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ABSTRACT

*Background:* Benzodiazepines are widely used for anesthesia and sedation and have immunomodulatory properties that may negatively influence clinical outcomes; however, the cellular targets and intermediary signaling pathways involved are unclear. We examined the immunomodulatory effects of the benzodiazepine midazolam on human macrophages and associated molecular mechanisms.

*Methods:* We analyzed effects of midazolam pretreatment on lipopolysaccharide (LPS)-induced upregulation of the costimulatory molecule CD80 and secretion of the pro-inflammatory factors interleukin-6 (IL-6), tumor necrosis factor-α, interleukin-10, and nitric oxide (NO) in the human monocyte–macrophage cell line THP-1 and in peripheral monocyte-derived macrophages (PMDMs). The effects of midazolam on NF-κB, IκBα protein, and mitogen-activated protein kinase (MAPK) activation were analyzed in THP-1 cells. We analyzed the involvement of translocator protein (TSPO) in the immunomodulatory effects of midazolam using TSPO ligands. The role of TSPO was investigated using THP-1 cells overexpressing TSPO and THP-1 cells with TSPO knockdown through transfection with small interfering RNA for TSPO.

*Results:* Midazolam suppressed LPS-induced upregulation of CD80 and release of IL-6 and NO in THP-1 cells and PMDMs. Additionally, midazolam suppressed the activation of NF-κB/AP-1 and MAPKs in human THP-1 cells. The assessed synthetic TSPO ligands showed the same inhibitory effects on macrophage activation as midazolam. Macrophages overexpressing TSPO exhibited enhanced susceptibility to immunosuppression by midazolam, and macrophages lacking TSPO expression exhibited reduced effects of midazolam.

*Conclusion:* Midazolam inhibits LPS-stimulated immune responses in human macrophages by activating TSPO signaling. Suppression of macrophage activity may contribute to deleterious side effects of benzodiazepines reported in critically ill patients.

## 1. Introduction

Benzodiazepines are some of the most commonly used drugs for sedation and anesthesia. Recently, several studies suggested that benzodiazepine use can contribute to poor outcomes in critically ill patients [1,2], possibly because of the disruption of immune responses. Although the immunomodulatory signaling pathways disrupted by benzodiazepines have not yet been identified, emerging evidence has suggested that these drugs interfere with macrophage activity [3]. Macrophages play important roles in antigen presentation, immunomodulation through cytokine secretion, and phagocytosis following activation by various physiological signals associated with injury or infection [4]. One such signal is lipopolysaccharide (LPS) that is found in the outer membrane of certain bacteria. LPS treatment is a standard experimental approach to induce macrophage activation in vitro [4]. Activated macrophages show upregulated expression of costimulatory molecules, such as CD80 and CD86, as well as secretion of nitric oxide (NO) and pro-inflammatory cytokines, such as interleukin-6 (IL-6). It has been reported that the benzodiazepine midazolam can interfere with the inflammatory functions of activated macrophages. For instance, the production of inflammatory mediators, including IL-6 and NO, by macrophages and neutrophils was shown to be suppressed by benzodiazepines, such as midazolam [3,5].

The sedative and hypnotic effects of benzodiazepines depend primarily on the modulation of $GABA_A$ receptors, which were previously referred to as central benzodiazepine receptors [6–8]. Translocator protein (TSPO), which was previously known as the peripheral benzodiazepine receptor, is a transmembrane protein expressed ubiquitously

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in peripheral tissues throughout the body. As the name suggests, TSPO binds to benzodiazepines. Although TSPO has been implicated in multiple biological functions, including steroid biosynthesis, apoptosis, and cell proliferation, its activity in immune regulation remains unknown [9].

Two previous studies suggested that TSPO signaling can reduce pro-inflammatory cytokine production in microglia, which are resident macrophages of the central nervous system [10,11]. In the current study, we tested the hypothesis that sedative and anxiolytic midazolam can suppress the inflammatory responses of human macrophages through TSPO signaling. We designed in vitro cell culture experiments using the human monocyte–macrophage cell line THP-1 and peripheral monocyte-derived macrophages (PMDMs) to clarify the effects of midazolam and other TSPO ligands on macrophages. We also attempted to demonstrate the immunomodulatory effects of TSPO signaling using THP-1 cells stably overexpressing TSPO and using THP-1 cells without TSPO expression.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Recombinant human macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Dimethyl sulfoxide (DMSO) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA), LPS from *Escherichia coli* 055:B5, and the $GABA_A$ receptor/TSPO ligands vigabatrin, muscimol, etifoxine, FGIN1-27, and Ro5-4864 were purchased from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) for detecting the expressions of CD11b (M1/70) and CD80 (C3H), respectively, were purchased from BD Biosciences (San Diego, CA, USA). In order to detect the protein levels of mitogen-activated protein kinases (MAPKs), anti-p38 MAPK (D13E1), anti-phospho-p38 MAPK (D3F9), anti-SAPK/JNK (56G8), anti-phospho-SAPK/JNK (81E11), anti-p44/42 MAPK (137F5), and anti-phospho-p44/42 MAPK (D13.14.4E) antigens were purchased from Cell Signaling Technology (Danvers, MA, USA). IκBα Antibody was purchased from Cell Signaling Technology to detect protein levels of IκBα. Additionally, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was purchased from Cell Signaling Technology. Midazolam was purchased from Astellas Pharma Inc. (Tokyo, Japan). Midazolam was dissolved in DMSO and was diluted with DMEM. The Quanti-Blue substrate to detect and quantify secreted embryonic alkaline phosphatase (SEAP) was purchased from Invivogen (San Diego, CA, USA).

### 2.2. THP-1 macrophage culture

Human monocytic THP-1 cells were purchased from Riken BioResource Center (Tsukuba, Japan). THP-1 cells were cultured for 24 h with 10 ng/mL PMA to trigger differentiation into macrophages and were then maintained in 24-well plates with complete medium (DMEM supplemented with 10% fetal bovine serum albumin, 100 U/mL penicillin, and 100 μg/mL streptomycin; $10^6$ cells/mL) at 37 °C under a 5% $CO_2$ atmosphere. The viability of the cultured cells was assessed by trypan blue exclusion, and more than 90% viability was noted for all cultures used in the experiments.

THP-1 macrophages were cultured in the presence and absence of midazolam or other TSPO ligands for 24 h, followed by stimulation with LPS (1000 ng/mL) for 24 h. After stimulation with LPS, the expression levels of surface molecules were assessed by flow cytometry, and culture supernatants were collected for IL-6, tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and NO measurements.

### 2.3. NF-κB activity assay using THP-1 Xblue NF-κB/AP-1 CD14 reporter cells

THP-1 Xblue NF-κB/AP-1 CD14 reporter cells stably expressing SEAP driven by the NF-κB/AP-1 promoter were purchased from Invivogen. Activation of the NF-κB/AP-1 promoter induces SEAP expression, which is measured in the extracellular medium as an index of NF-κB/AP-1 activity. Cells were seeded in 96-well plates at $10^6$ cells/mL and were cultured in DMEM supplemented with 10% fetal bovine serum albumin, 100 μg/mL G418, and 200 μg/mL Zeocin at 37 °C under a 5% $CO_2$ atmosphere. Pretreatment with midazolam, $GABA_A$ receptor, or TSPO ligands for 24 h and subsequent stimulation with LPS for 24 h were performed as described above. Promoter activity of NF-κB/AP-1 was measured by detecting accumulation of SEAP in the culture medium. The amount of SEAP in culture supernatants was analyzed by 2 h exposure to Quanti-Blue alkaline phosphatase substrate, and it was measured at an optical density of 600 nm.

### 2.4. Preparation of monocyte-derived macrophages

Peripheral blood mononuclear cells were isolated from whole peripheral blood samples of healthy volunteers by density-gradient centrifugation using LeucoSep tubes (Greiner Bio-One, Frickenhausen, Germany). Monocytes were further purified by magnetic-activated column sorting for $CD14^+$ cells (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched human $CD14^+$ monocytes were seeded in 24-well plates at $10^6$ cells/mL and were cultured in DMEM supplemented with 10% fetal bovine serum albumin, 100 U/mL penicillin, 100 μg/mL streptomycin, and recombinant human M-CSF (20 ng/mL) at 37 °C under a 5% $CO_2$ atmosphere for 4 days to obtain PMDMs. The viability of cultured cells was assessed by trypan blue exclusion, and more than 90% viability was noted for all cultures used in the experiments. On day 4, PMDMs were cultured in the presence and absence of midazolam, $GABA_A$ receptor ligands, and TSPO ligands for 24 h, followed by stimulation with 100 ng/mL of LPS for 24 h. After stimulation with LPS, the expression levels of surface molecules and the secretion of IL-6 and NO were measured.

### 2.5. Establishment of THP-1 cells stably overexpressing TSPO

TSPO cDNA was introduced into the pLVSIN-EF1alpfa mammalian expression vector (Takara Bio Inc., Shiga, Japan). To generate lentiviruses, pLVSIN-EF1alfa Neo/TSPO was co-transfected with Lentiviral High Titer Packaging Mix (Takara Bio Inc.) in Lenti-X 293T cells (ATCC, Manassas, VA, USA) using TransIT-293 transfection reagent (Mirus Bio LCC., Madison, WI, USA). Culture supernatants of infected packaging cells were harvested on day 2. THP-1 cells were cultured with the virus supernatants and 8 μg/mL of polybrene (Sigma-Aldrich) for 24 h, and then, cells were selected for stable TSPO overexpression by passage in G418 (Thermo Fisher Scientific). Overexpression of TSPO in THP-1 cells was confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

### 2.6. Knockdown of the TSPO gene in THP-1 cells

Silencer Select small interfering RNA (siRNA) against the *TSPO* gene (s224728) and negative control siRNA were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The sequence of siRNA against TSPO was 5′-UCUGCAUGCUUAGAGCAUGtt-3′. THP-1 cells were differentiated into macrophages by PMA as described above. The transfection of 1 μM duplexes of siRNA was performed using Nucleofector 2b (Lonza, Basel, Schweiz) under the condition X001. After transfection, cells were quickly transferred to 24-well plates at $10^6$ cells/mL with the complete medium and were incubated at 37 °C under a 5% $CO_2$ atmosphere for 24 h. Knockdown of the *TSPO* gene in THP-1 cells was analyzed by qRT-PCR.

### 2.7. Flow cytometric analysis of cell-surface marker expression

The expression level of the costimulatory surface molecule CD80 for THP-1 macrophages and PMDMs was analyzed by flow cytometry. To prevent nonspecific binding of antibodies at each staining step, cells ($2–5 \times 10^5$) were incubated in staining buffer containing FITC-labeled anti-CD11b and PE-labeled anti-CD80 mAbs on ice for 20 min. Flow cytometric analysis was performed using a BD FACSVerse flow cytometer and BD FACS Suite software (BD Biosciences, San Diego, CA, USA). Data were analyzed using FlowJo (FlowJo LLC., Ashland, OR, USA).

### 2.8. IL-6, TNF-α, and IL-10 enzyme-linked immunosorbent assay

IL-6, TNF-α, and IL-10 released into the culture supernatant by LPS stimulation was measured using an enzyme-linked immunosorbent assay kit following the manufacturer's instructions (R&D Systems, Minneapolis, MI, USA).

### 2.9. Nitrite/nitrate assay

The amount of nitrites plus nitrates in the culture supernatant was assessed as an indicator of NO release, using a commercially available colorimetric assay kit (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

### 2.10. Quantitative qRT-PCR

For the estimation of TSPO expression, total RNA from THP-1 macrophages and monocyte-derived macrophages was prepared using the RNeasy Plus Mini kit (Quiagen, Hilden, Germany). Genomic DNA was removed by treatment with DNAse. Complementary DNA was synthesized from total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio Inc.). qRT-PCR was conducted using the TaqMan Gene Expression System (Applied Biosystems, Foster, CA, USA) and the ViiA 7 Real-time PCR System (Applied Biosystems) as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Ready-made primer pairs were utilized for the amplification of GAPDH (internal control, Hs0275899_g1) and TSPO (Hs00559362_m1). Gene expression was quantified using the ΔT method [12]. Threshold cycle (CT) values were calculated, and they are expressed as fold induction defined by the comparative CT (ΔCT) method.

### 2.11. Western blot analysis

Cultured cells were lysed with 200 μL of mammalian protein extraction reagent (Thermo Fisher Scientific). Cell lysates were diluted with 4× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) supplemented with 2-mercaptoethanol (Bio-Rad). Protein samples were denatured at 95 °C for 10 min. Protein (30 μg/well) was separated by SDS-PAGE and was electro-transferred onto nitrocellulose membranes. Blots were initially incubated in blocking solution (Block Ace, DS Pharma Biomedical, Osaka, Japan) for 1 h and were then incubated overnight at 4 °C with anti-p38 MAPK, anti-phospho-p38 MAPK, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK (ERK1/2), and anti-IκBα antibodies (1:1000 dilution). Membranes were washed and were incubated with HRP-conjugated anti-rabbit IgG antibody (1:1000 dilution) (Cell Signaling Technology). HRP-conjugated antibody to GAPDH (1:1000; Cell Signaling Technology) was used for the detection of the endogenous control. Blots were detected using the ECL detection system (GE Healthcare, Little Chalfont, UK). Blot images were acquired using the ChemiDo XRS + System (Bio-Rad).

### 2.12. Statistical analysis

In a preliminary study, power analysis showed that a treatment group size of 6 (6 independent culture treatments) was sufficient to detect a 50% reduction in surface molecule expression by midazolam, with a power of 0.8 at an α level of 0.05. Data are expressed as mean ± standard deviation (SD). Group means were compared using Student's *t*-test or ANOVA with a post hoc Tukey test as appropriate. All tests were two tailed. A *P*-value of $< 0.05$ was considered statistically significant. All statistical analyses were conducted using JMP Pro Version 11.2 (SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. Midazolam suppressed the LPS-induced pro-inflammatory responses of THP-1 macrophages

We first examined whether midazolam could influence macrophage activation via LPS. Stimulation of the human monocyte–macrophage cell line THP-1 with LPS upregulated the expression of costimulatory CD80, as determined by fluorescence-activated cell sorting, and increased the media concentrations of IL-6, TNF-α, IL-10 and NO products (nitrates and nitrites) (Fig. 1), whereas pretreatment with midazolam significantly suppressed CD80 upregulation and the medium concentrations of IL-6, TNF-α, IL-10 and nitrates/nitrites. In addition, the inhibitory effects of midazolam on LPS-induced upregulation of pro-inflammatory molecules (activation) increased as the midazolam dose increased (Fig. 1).

### 3.2. Midazolam inhibited LPS-induced activation of NF-κB/AP-1 and prevented the proteolysis of IκBα

The transcription factor NF-κB and AP-1 are master regulators of inflammatory changes in immune cells, including changes associated with the expression of genes encoding inflammatory mediators (cytokines and chemokines) and costimulatory surface molecules. Activation of Toll-like receptors by various pathogen-associated molecules, such as binding of TLR4 to LPS, enhances NF-κB and AP-1 transactivational activities, resulting in various pro-inflammatory changes in macrophages. We thus analyzed the effects of midazolam on LPS-induced activation of NF-κB and AP-1 in THP-1 Xblue CD14 cells, which express SEAP driven by the NF-κB/AP-1 promoter. The detection of SEAP by addition of Quanti-Blue substrate reagents revealed strong activation of NF-κB/AP-1 by LPS treatment and dose-dependent inhibition by midazolam pretreatment (Fig. 2A). Thus, midazolam suppressed the inflammatory cascade, at least in part, by inhibiting the transduction of LPS–TLR4 signaling to the nucleus associated with NF-κB and AP-1, thereby reducing the expression of genes with NF-κB/AP-1 promoters (including those encoding CD80, IL-6, TNF-α and NO).

In inactivated conditions, IκBα is bound to NF-κB and inhibits the activation of NF-κB as the negative regulator of NF-κB. Stimulation by LPS induces the ubiquitination of IκBα and the degradation of IκBα by proteasome. The release of NF-κB from IκBα and the translocation of NF-kB into nucleus results in turning on the target genes of proinflammtory mediators [13]. To examine whether midazolam affects these pathways, we investigated the levels of IκBα level in midazolam-treated THP-1 macrophages. The administration of LPS to THP-1 cells induced the reduction of cellular IκBα. Treatment with midazolam prevented the degradation of IκBα induced at 30 min after LPS treatment (Fig. 2B).

### 3.3. Midazolam inhibited the phosphorylation of MAPKs

MAPK activation has been shown to be involved in the activation pathway induced by LPS [14]. Thus, we assessed the activation status of MAPKs, including p38 MAPK, SAPK/JNK, and p44/42 MAPK. We

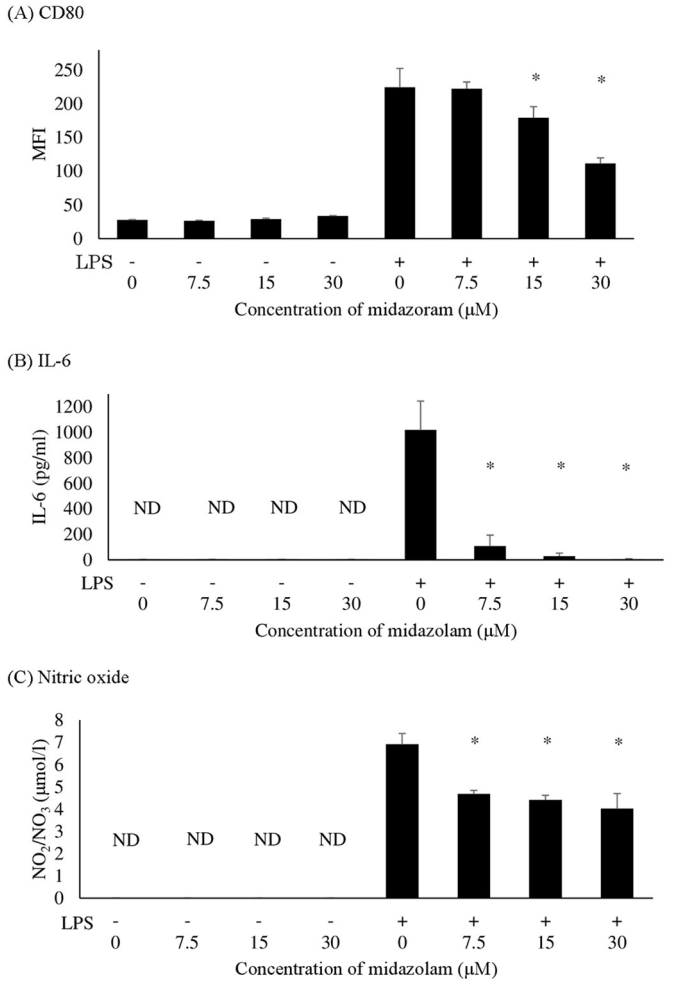



**Fig. 1.** The effects of midazolam on the LPS-induced pro-inflammatory responses of THP-1 macrophages.
The effects of midazolam on the expressions of (A) CD80, (B) IL-6, (C) nitrite/nitrate, (D) TNF-α, and (E) IL-10 in THP-1 macrophages. THP-1 cells are incubated with midazolam or vehicle control for 24 h and are subsequently stimulated with or without LPS for 24 h. After stimulation with LPS, phenotypic changes are analyzed. All results are presented as mean ± SD ($N = 6$). THP-1 cells treated with midazolam show less expression of CD80, IL-6, NO, TNF-α, and IL-10. (A) The effect of midazolam on CD80 expression is analyzed in CD11b-gated cell samples of THP-1 cells using flow cytometry. $*p < 0.05$, control vs. 15 or 30 μM midazolam. MFI = mean fluorescence intensity. (B) The effects of midazolam on IL-6 production are analyzed in the culture supernatant of THP-1 cells using cytokine-specific enzyme-linked immunosorbent assay. $*p < 0.05$, control vs. 7.5, 15, or 30 μM midazolam. (C) The effects of midazolam on NO expression are measured in the culture supernatant of THP-1 cells using a colorimetric assay. $*p < 0.05$, control vs. 7.5, 15, or 30 μM midazolam. (D) Effects of midazolam on TNF-α production are analyzed using the culture supernatant of THP-1 cells using cytokine-specific enzyme-linked immunosorbent assay. $*p < 0.05$, control vs. 30 μM midazolam. (E) Effects of midazolam on the production of IL-10 are analyzed using the culture supernatant of THP-1 cells using cytokine-specific enzyme-linked immunosorbent assay. $*p < 0.05$, control vs. 7.5, 15, or 30 μM midazolam.

analyzed the involvement of MAPKs in the suppressive effects of midazolam. To examine whether midazolam inhibits the activation of MAPKs induced by LPS, we investigated the phosphorylation levels of p38 MAPK, SAPK/JNK, and p44/42MAPK in midazolam-treated THP-1 cells. Phosphorylation was induced by LPS at 30 min. Treatment with midazolam suppressed activation of p38 MAPK, SAPK/JNK, and p44/42 MAPK induced by LPS (Fig. 3).

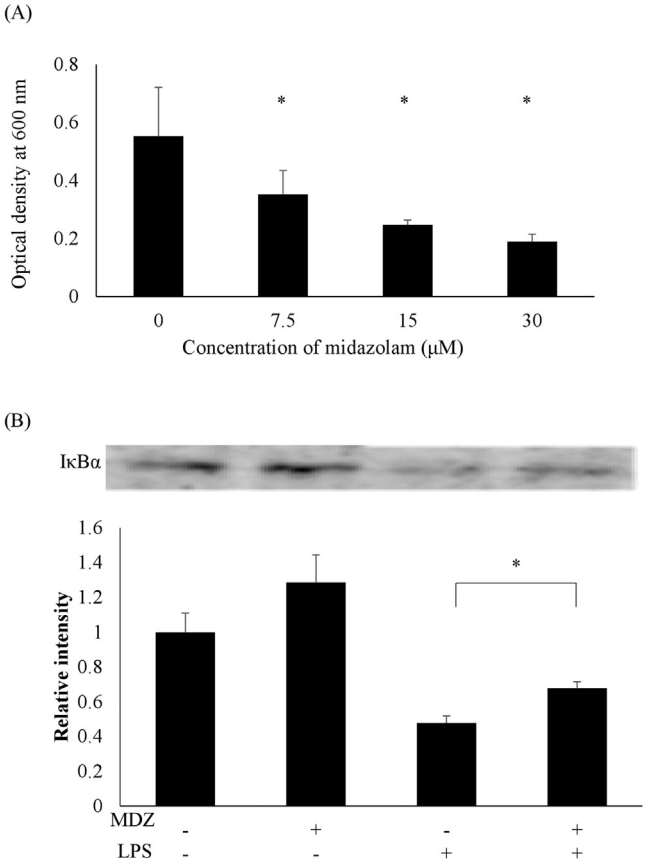


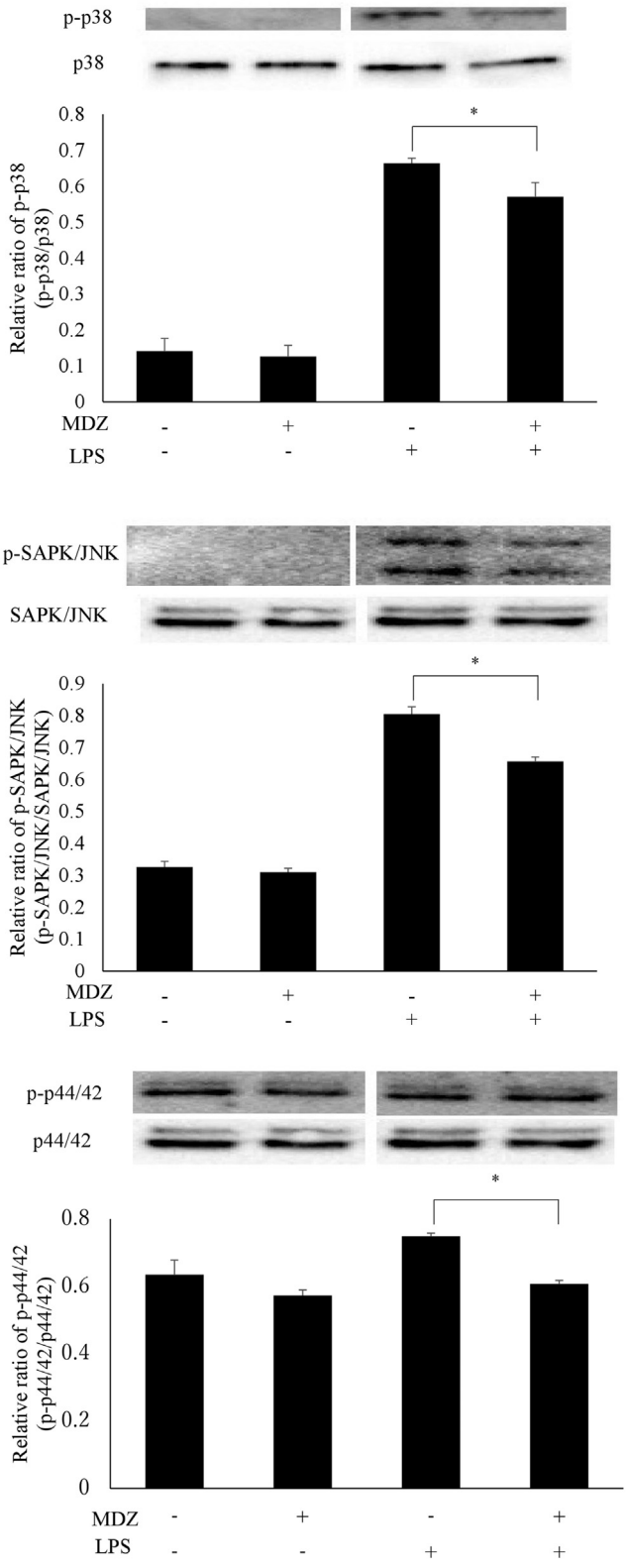

**Fig. 2.** The effects of midazolam on LPS-induced activation of NF-κB/AP-1 and proteolysis of IκBα.
(A) The effects of midazolam on the activation of the transcription factor NF-κB/AP-1 in THP-1 Xblue CD14 cells. Results are presented as mean ± SD ($N = 6$). THP-1 Xblue CD14 cells are seeded in 96-well plates at $10^6$ cells/mL. Pretreatment with midazolam for 24 h and subsequent stimulation with LPS for 24 h are performed. Promoter activity of NF-κB/AP-1 is measured by detecting accumulation of SEAP in the culture medium. The amount of SEAP in the culture supernatant is analyzed by 2 h exposure to Quanti-Blue alkaline phosphatase substrate and is measured at an optical density of 600 nm. Midazolam suppresses the activation of NF-κB/AP-1 in THP-1 Xblue CD14 cells. $*p < 0.05$, control vs. 7.5, 15, or 30 μM midazolam.
(B) The effects of midazolam on the proteolysis of IκBα in THP-1 cells. The expression of protein was analyzed by Western blot analysis. THP-1 cells are incubated with midazolam (15 μM) or vehicle control for 24 h and are subsequently stimulated with or without LPS for 30 min. Midazolam prevented the proteolysis of IκBα. Bars represent the relative protein quantification relative to controls. Results are presented as mean ± SD ($N = 6$). $*p < 0.05$, control versus midazolam.

### 3.4. Effect of midazolam on the expression of TSPO in THP-1 macrophages stimulated by LPS

We assessed the effects of the expression of *TSPO* gene in LPS-treated THP-1 macrophages and the effects of midazolam. The expression of *TSPO* mRNA in THP-1 macrophages did not change after stimulation with LPS. However, the expression of *TSPO* mRNA was decreased in LPS-stimulated THP-1 macrophages by the treatment with midazolam (Fig. 4).

### 3.5. Midazolam suppressed LPS-induced activation of PMDMs

We assessed the effects of midazolam on PMDMs. As observed in the macrophage cell line, LPS treatment in PMDMs upregulated the expression of CD80 and the release of IL-6 and NO (nitrates/nitrites), and




these responses were suppressed in a dose-dependent manner by midazolam pretreatment (Fig. 5). These results strongly suggest that midazolam can suppress the inflammatory activation of macrophages in vivo.

**Fig. 3.** The effects of midazolam on the activation of phosphorylation of MAPKs in THP-1 macrophages.
The effects of midazolam on the activation of phosphorylation of MAPKs in THP-1 cells. Western blot analysis demonstrates protein expression. THP-1 cells are incubated with midazolam (15 μM) or vehicle control for 24 h and are subsequently stimulated with or without LPS for 30 min. Midazolam inhibits phosphorylation of p38 (p-p38) MAPK, phosphorylation of SAPK/JNK (p-SAPK/JNK) and phosphorylation of p44/42 (p-p44/42) MAPK. Bars represent the relative protein quantification of p-p38/p38, p-SAPK/JNK/SAPK/JNK, and p-p44/42/p44/42. Results are presented as mean ± SD ($N = 6$). *$p < 0.05$, control vs. midazolam.

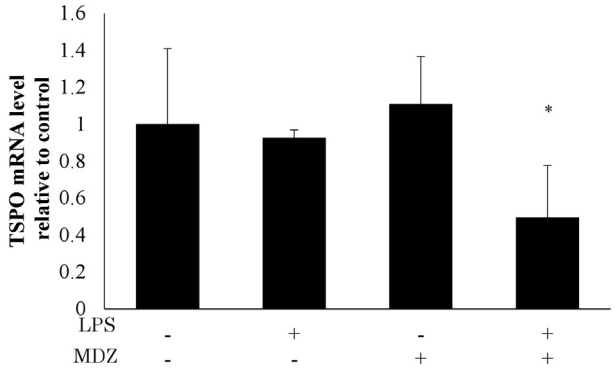



**Fig. 4.** The effect of midazolam on expression level of TSPO mRNA in THP-1 macrophages treated by LPS.
The effect of midazolam on expression level of *TSPO* mRNA in THP-1 macrophages treated by LPS. THP-1 cells were differentiated into macrophages and were treated with or without midazolam. After 24 h, THP-1 macrophages are stimulated with 1000 ng/mL LPS or not. Results are presented as mean ± SD ($N = 6$). The expression of *TSPO*-mRNA is compared with the negative control in THP-1 macrophages using qRT-PCR analysis. Midazolam inhibited the expression of *TSPO* mRNA level in LPS treated THP-1 macrophages. *$p < 0.05$, control versus midazolam.

### 3.6. *TSPO ligands, but not GABA$_A$ receptor ligands, suppressed LPS-induced pro-inflammatory changes in THP-1 cells and PMDMs*

We examined whether benzodiazepine receptors contribute to the suppressive effects of midazolam on macrophage activation by pretreating THP-1 cells and PMDMs with various TSPO ligands (Ro5-4864, etifoxine, and FGIN1-27) and GABA$_A$ receptor ligands (vigabatrin and muscimol) prior to LPS stimulation. Similar to the findings for midazolam, TSPO ligands suppressed LPS-induced upregulation of CD80 (Fig. 6A) and LPS-enhanced release of IL-6 (Fig. 6B). In contrast, GABA$_A$ receptor ligands did not suppress either LPS-induced CD80 upregulation or IL-6 release. TSPO ligands, but not GABA$_A$ receptor ligands, reduced LPS-induced NF-κB/AP-1 activation in THP-1 Xblue CD14 cells (Fig. 6C). These results suggest that midazolam reduces the expression of pro-inflammatory factors by activating TSPO signaling, which in turn blocks transactivation of associated pro-inflammatory genes by NF-κB/AP-1.

TSPO ligands and GABA$_A$ ligands made similar effects on the expression of CD80 and IL-6 in PMDMs in similar manners with THP-1 cells. TSPO ligands suppressed LPS-induced upregulation of CD80 and LPS-enhanced release of IL-6, but GABA$_A$ receptor ligands did not suppress either LPS-induced CD80 upregulation or IL-6 release (Fig. 6D, E).

### 3.7. *Immunosuppressive effects of midazolam were dependent on TSPO*

To further examine whether the suppressive effects of midazolam on LPS-treated THP-1 cells are dependent on TSPO signaling, we compared the dose dependence of midazolam between WT THP-1 cells and THP-1 cells stably overexpressing TSPO cDNA through lentiviral vector

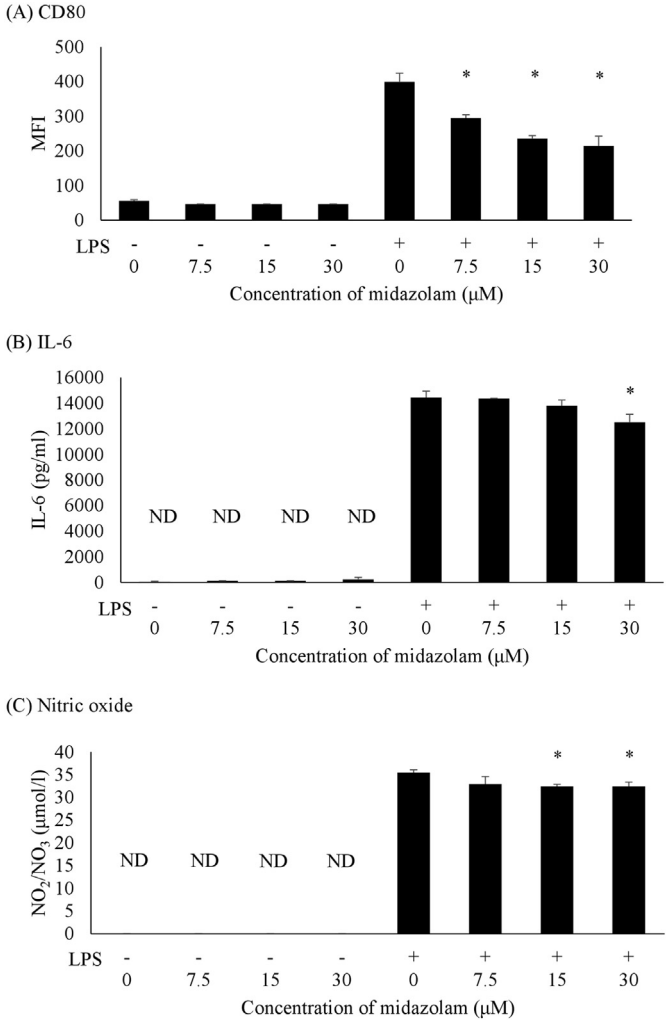



**Fig. 5.** The effects of midazolam on the LPS-induced pro-inflammatory responses of PMDMs.

The effects of midazolam on the expressions of (A) CD80, (B) IL-6, and (C) NO in PMDMs. PMDMs are incubated with midazolam or vehicle control for 24 h and are subsequently stimulated with or without LPS for 24 h. After stimulation with LPS, phenotypic changes are analyzed. All results are presented as mean ± SD ($N = 6$). PMDMs treated with midazolam show lower expression of CD80 and less secretion of IL-6 and NO.

(A) The effects of midazolam on CD80 expression are analyzed in CD11b-gated cell samples of PMDMs using flow cytometry. *$p < 0.05$, control vs. 7.5, 15, or 30 μM midazolam. MFI = mean fluorescence intensity. (B) The effects of midazolam on IL-6 production are analyzed in the culture supernatant of PMDMs using cytokine-specific enzyme-linked immunosorbent assay. *$p < 0.05$, control vs. 30 μM midazolam. (C) The effects of midazolam on NO expression are measured in the culture supernatant of PMDMs using colorimetric assay. *$p < 0.05$, control vs. 15 or 30 μM midazolam.

transfection. Expression of the *TSPO* gene was 71% higher in transfected cells than in the other cells (Fig. 7A). Overexpression of TSPO did not affect the viability or growth rate of THP-1 cells (data not shown), but it did suppress basal expression of CD80 and IL-6. Moreover, the suppressive effects of midazolam on LPS-induced CD80 and IL-6 upregulation were enhanced (i.e., midazolam sensitivity was higher) in THP-1 cells overexpressing TSPO when compared with WT THP-1 cells (Fig. 7B, C).

We established THP-1 cells in which the expression of TSPO was knocked down by transfection of siRNA against TSPO, and we analyzed the effects of midazolam on inflammatory response in WT THP-1 cells and THP-1 cells lacking TSPO expression. Expression of the *TSPO* gene was significantly lower in TSPO knockdown THP-1 cells than in the

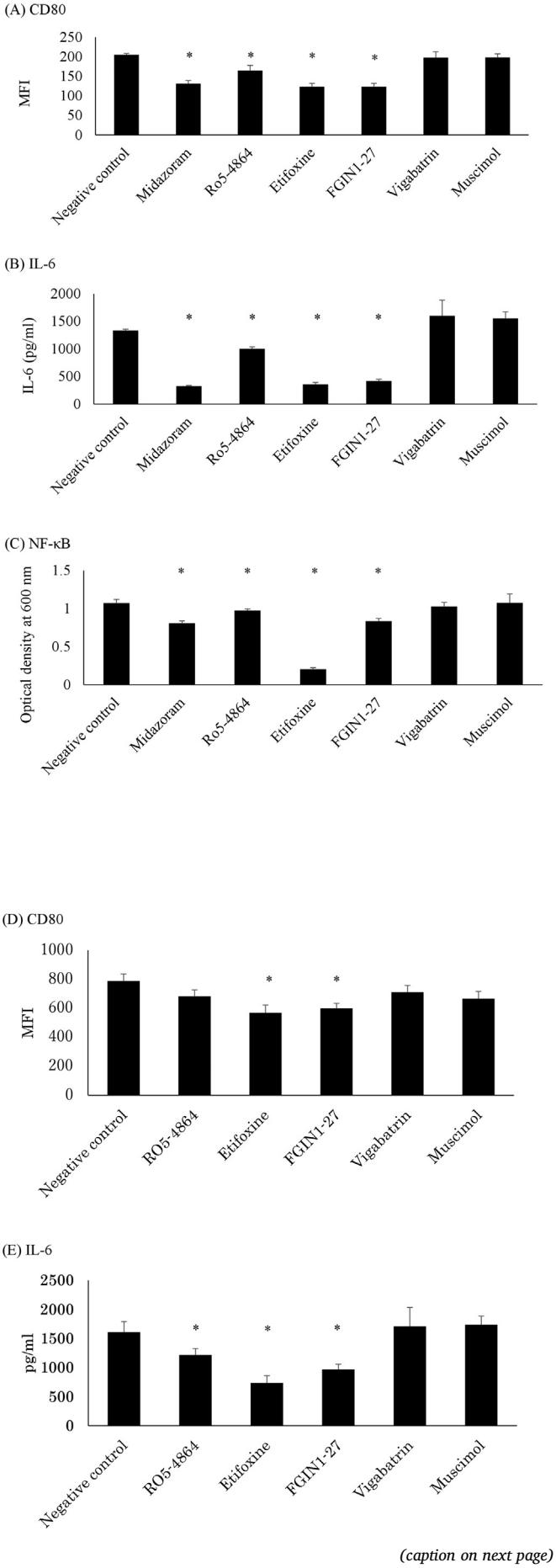



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**Fig. 6.** The effects of benzodiazepine receptor ligands on the LPS-induced pro-inflammatory responses of THP-1 macrophages and PMDMs.
The various effects of benzodiazepine receptor ligands on the expressions of CD80 (A) and IL-6 (B) in THP-1 macrophages and on the activation of the transcription factor NF-κB in THP-1 Xblue CD14 cells (C) are assessed. THP-1 macrophages are exposed or not exposed to benzodiazepine receptor ligands (midazolam, Ro5-4864, etifoxine, FGIN1-27, vigabatrin, and muscimol). The concentration of each ligand is 15 μM. After incubation with each ligand for 24 h, THP-1 macrophages (A, B) and THP-1 Xblue CD14 cells (C) are subsequently stimulated with 1000 ng/mL LPS. All results are presented as mean ± SD ($N = 6$). THP-1 cells treated with midazolam, Ro5-4864, etifoxine, and FGIN1-27 show less expression of CD80, IL-6, and nitrite/nitrate, and those treated with vigabatrin and muscimol show no change. THP-1 Xblue CD14 cells treated with midazolam, Ro5-4864, etifoxine, and FGIN1-27 show less activation of NF-κB, and those treated with vigabatrin and muscimol show no change. (A) After stimulation with LPS, the expression of CD80 is analyzed using flow cytometry in CD11b-gated cell samples of THP-1 macrophages. $^*p < 0.05$, control vs. midazolam, Ro5-4864, etifoxine, or FGIN1-27. MFI = mean fluorescence intensity. (B) The secretion of IL-6 is measured in THP-1 cell cultures using cytokine-specific enzyme-linked immunosorbent assay. $^*p < 0.05$, control vs. midazolam, Ro5-4864, etifoxine, or FGIN1-27. (C) The amount of SEAP in the culture supernatant is analyzed by 2 h exposure to Quanti-Blue alkaline phosphatase substrate and is measured at an optical density of 600 nm. After stimulation with LPS, THP-1 Xblue cells are exposed to QUANTI-Blue™ phosphatase substrate for 2 h. The accumulation of SEAP is measured at an optical density of 600 nm. $^*p < 0.05$, control vs. midazolam, Ro5-4864, etifoxine, or FGIN1-27.
Then the effects of benzodiazepine receptor ligands on the expressions of CD80 (D) and IL-6 (E) in PMDMs are assessed. PMDMs are exposed or not exposed to benzodiazepine receptor ligands (Ro5-4864, etifoxine, FGIN1-27, vigabatrin, and muscimol). The concentration of each ligand is 15 μM. After incubation with each ligand for 24 h, PMDMs are subsequently stimulated with 100 ng/mL LPS. All results are presented as mean ± SD ($N = 6$). PMDMs treated with etifoxine and FGIN1-27 show less expression of CD80, and treated with Ro5-4864, etifoxine, and FGIN1-27 show less expression of IL-6. Those treated with vigabatrin and muscimol show no change. (D) After stimulation with LPS, the expression of CD80 is analyzed using flow cytometry in CD11b-gated cell samples of PMDMs. $^*p < 0.05$, control versus etifoxine or FGIN1-27. MFI = mean fluorescence intensity. (E) The secretion of IL-6 is measured in PMDMs cultures using cytokine-specific enzyme-linked immunosorbent assay. $^*p < 0.05$, control versus Ro5-4864, etifoxine, or FGIN1-27.

other cells (Fig. 8A). Knockdown of the *TSPO* gene did not affect the viability or growth rate of THP-1 cells (data not shown). The suppressive effects of midazolam on LPS-induced upregulation of CD80 and IL-6 were attenuated in TSPO knockdown THP-1 cells when compared with WT THP-1 cells (Fig. 8B, C).

## 4. Discussion

Midazolam inhibited the activation of human macrophages (a human macrophage cell line and PMDMs) as evidenced by reduced expression of costimulatory CD80 and lower secretion of the inflammatory mediators IL-6, TNF-α, and NO (nitrates/nitrites). In addition, the secretion of IL-10, which down-regulates inflammatory immune responses, was inhibited by midazolam, [15,16]. LPS signaling has been shown to independently activate NF-κB, AP-1, and MAPKs, which are master regulators of inflammatory mediators, such as IL-6, TNF-α, and NO [14,17–24]. Considering the involvement of MAPKs in the activation of the LPS signaling pathway, we analyzed the association of MAPKs in the suppressive effects of midazolam on LPS-induced inflammatory changes [3]. The inhibitory effects of midazolam were identified by analyzing the phosphorylation of MAPKs. We found that midazolam inhibited the activation of both NF-κB/AP-1 and MAPK pathways. All assessed TSPO ligands showed the same effects as midazolam on the expression of pro-inflammatory mediators and NF-κB/AP-1. The suppressive response to midazolam was enhanced by TSPO overexpression and attenuated by TSPO deficiency.

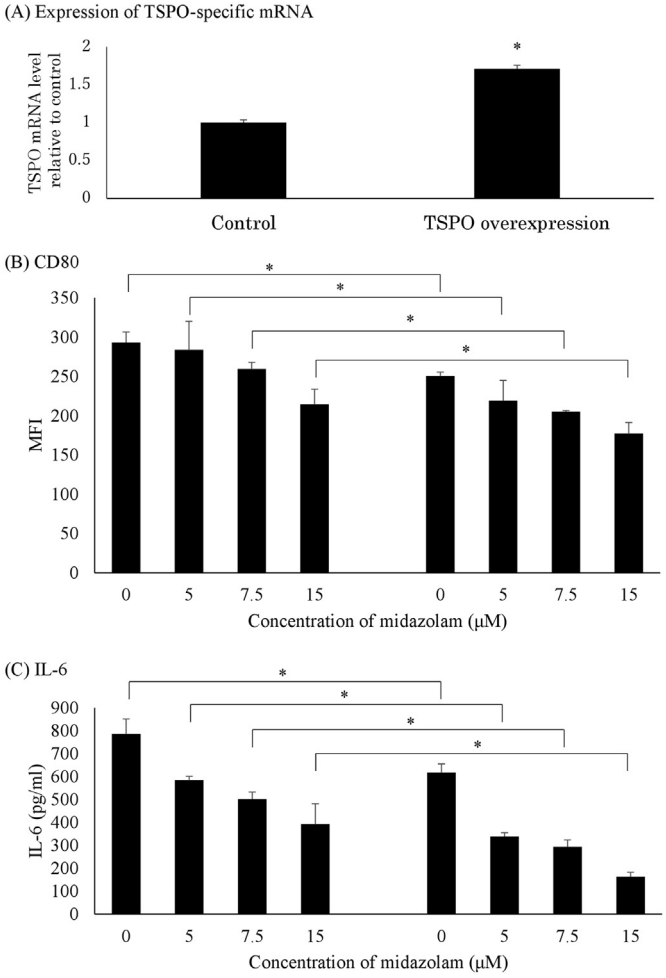


**Fig. 7.** The effects of midazolam on the LPS-induced pro-inflammatory responses of TSPO-overexpressed THP-1 macrophages.
TSPO overexpression suppresses the expressions of CD80 and IL-6 in THP-1 macrophages. THP-1 cells are transduced with a TSPO expression vector, and control cells are exposed or not exposed to midazolam as indicated after differentiation into macrophages. After 24 h, THP-1 macrophages are stimulated with 1000 ng/mL LPS. Results are presented as mean ± SD ($N = 6$). THP-1 cells stably overexpressing TSPO cDNA suppress basal expressions of CD80 and IL-6. The suppressive effects of midazolam on LPS-induced CD80 and IL-6 upregulation are enhanced in THP-1 cells overexpressing TSPO when compared with WT THP-1 cells. (A) The expression of TSPO-mRNA is compared with the negative control in THP-1 cells using qRT-PCR analysis. (B) The effects of midazolam on CD80 expression is analyzed in CD11b-gated cell samples of THP-1 cells using flow cytometry. MFI = mean fluorescence intensity. (C) The effects of midazolam on IL-6 production are analyzed in the culture supernatant of THP-1 cells using cytokine-specific enzyme-linked immunosorbent assay. There is a significant difference in the expressions of CD80 and IL-6 between control and THP-1 macrophages with TSPO overexpression ($p < 0.05$). $^*p < 0.05$, control vs. TSPO overexpression at 0, 7.5, 15, or 30 μM midazolam.

Collectively, these results strongly suggest that midazolam exerts anti-inflammatory effects on LPS-stimulated macrophages through TSPO-mediated suppression of NF-κB/AP-1 and MAPK activation, which in turn reduces the activation of pro-inflammatory genes, such as those encoding CD80, IL-6, TNF-α, and NO. This interference with macrophage activation may contribute to the deleterious side effects observed following midazolam anesthesia in some critically ill patients [1,2].

These results confirm previous findings that the production of inflammatory mediators by macrophages and neutrophils, including IL-6 and NO, is suppressed by midazolam [3]. In addition, we identified

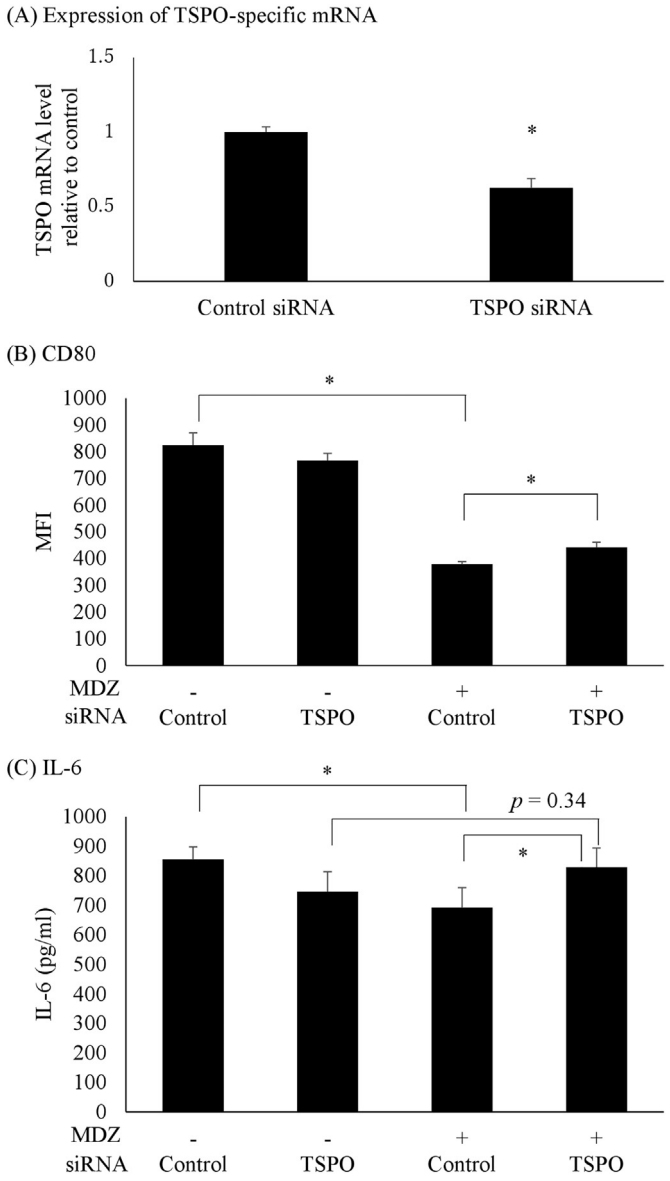


**Fig. 8.** The effects of midazolam on the LPS-induced pro-inflammatory responses of TSPO-knockdowned THP-1 macrophages.
THP-1 cells transfected with siRNA against TSPO and control cells are exposed or not exposed to midazolam and are stimulated with or without LPS. Results are presented as mean ± SD ($N = 6$). The suppressive effects of midazolam on LPS-induced CD80 and IL-6 upregulation are attenuated in TSPO knockdown THP-1 cells when compared with WT THP-1 cells. (A) The expression of TSPO mRNA is compared with the negative control in THP-1 cells using qRT-PCR analysis. (B) The effects of midazolam on CD80 expression are analyzed in CD11b-gated cell samples of THP-1 cells using flow cytometry. MFI = mean fluorescence intensity. (C) The effects of midazolam on IL-6 production are analyzed in the culture supernatant of THP-1 cells using cytokine-specific enzyme-linked immunosorbent assay. There is a significant difference in the effects of midazolam on the expressions of CD80 and IL-6 between control and TSPO knockdown THP-1 macrophages ($p < 0.05$).

TSPO-mediated inhibition of the activation of LPS-TLR4-NF-κB/MAPKs pathways. All assessed TSPO ligands recapitulated the effects of midazolam, which is consistent with previous reports implicating TSPO signaling in the anti-inflammatory response to benzodiazepines [11,25,26]. Further, TSPO may act in a critical physiological control mechanism for limiting macrophage-dependent inflammation. In mouse retina, LPS was found to increase TSPO protein expression by microglia, and both a synthetic TSPO ligand (PK11195) and an endogenous benzodiazepine ligand (diazepam binding inhibitor) were found to decrease inflammatory reactions, such as elevated NO activity and TNF-α secretion [11]. In the current study, overexpression of TSPO in macrophages decreased basal CD80 expression and augmented the inhibitory effects of midazolam on LPS-induced activation. Conversely, siRNA-mediated knockdown of TSPO has been reported to enhance LPS-stimulated production of inflammatory cytokines [11,27]. These findings strongly suggest that TSPO acts as an endogenous negative regulator of inflammatory mediators in macrophages.

Thus, in most cells, NF-κB is present as a latent, inactive, IκBα-bound complex in the cytoplasm. LPS-TLR4 signaling induces the phosphorylation of IκBα protein and the subsequent degradation of IκBα by proteasome. Release of NF-KB from IκBα leads to the translocation of NF-κB into nucleus and the transcription of pro inflammatory mediators. We showed that the administration of midazolam or TSPO ligands inhibited the degradation of IκBα and subsequent activation of NF-κB induced by LPS-TLR4 signaling. This is the first report that TSPO ligands can make effects on the phosphorylation and the degradation of IκBα.

Midazolam suppressed the expression of *TSPO* gene at the stimulation of macrophages with LPS. We speculate that signaling from TSPO ligands can negatively regulate the expression of *TSPO* mRNA. The mechanism of this phenomenon occurred only at the time of LPS administration is unknown from current experiment.

It is controversial whether benzodiazepines can modulate $GABA_A$ receptor activity in immune cells. Several reports have indicated that $GABA_A$ receptor activation can suppress immune cells, including antigen-presenting cells [28–31]. In contrast, GABA signaling reportedly had no effects on lymphocyte proliferation and cytokine production [28]. In the current study, none of the assessed $GABA_A$ receptor ligands altered LPS-induced activation of macrophages. However, it is possible that the functions of various immune cells besides macrophages are differentially regulated by benzodiazepine receptor subtypes.

The secretions of IL-6 and NO were less susceptible to midazolam in PMDMs than in THP-1 cells. This can be partly explained by the cellular heterogeneity of PMDMs. THP-1 cells were part of a cell line and represented a somewhat homogenous cell population, and the influence of midazolam was clearly detected. On the other hand, the less remarkable effects of midazolam on in vivo macrophages than on the cell line suggest that benzodiazepines alone cannot cause dangerous suppressive effects in the entire body. In addition, the suppressive effects of midazolam on the secretion of IL-6 were more remarkable than the effects on the activation of NF-kB/AP-1 in THP-1 cells. One possible explanation for this phenomenon is that midazolam has effects independent of NF-kB/AP-1. The production of IL-6 from cells can be regulated at multiple steps, including transcription, stabilization of the transcript, translation, and secretion from cells [32–35]. Benzodiazepines have been reported to affect other functions of cytokine production [36,37].

Consistent with the important physiological role of TSPO in immune regulation, it has been reported that microglial TSPO expression is upregulated in inflammatory central nervous system disorders, such as Alzheimer's disease, Parkinson's disease, and intracerebral hemorrhage [38–40]. In general, TSPO expression appears to increase during nerve degeneration and returns to normal when the regeneration process terminates [41], which is also consistent with involvement in a negative feedback loop that controls inflammatory responses. Indeed, upregulation of TSPO and endogenous benzodiazepine receptor ligands was recently reported as a potential compensatory mechanism against excessive inflammation in sepsis [42]. Exogenous benzodiazepines administered as sedative agents, such as in the case of midazolam, may augment this endogenous negative feedback loop, resulting in amplified immunosuppression.

The present study has some limitations. First, the physiological functions of TSPO in vivo remain unclear. TSPO knockout (KO) mice (lacking TSPO throughout the entire body) were found to be viable and

showed no apparent abnormalities, including in steroidogenesis, at least when healthy [43,44]. Thus, TSPO does not appear necessary for life or basic physiological functions, although the reactions of these animals to disease, injury, and other triggers for immune activation were not studied in detail. Furthermore, differences in immune responses between KO and WT mice have not yet been examined.

Second, there may be some concern about the concentrations of midazolam used in the current study. We showed dose-dependent changes (0–30 μM) in the effects of midazolam on the expression of inflammatory mediators from THP-1 cells and PMDMs (Figs. 1 and 4). Clinically relevant plasma concentrations of midazolam in the induction of anesthesia are thought to range between 0.12 and 2.25 μM (0.04 and 0.85 μg/mL), and these levels are lower than the levels used in vitro in this study. We selected the concentration (15 μM) of midazolam as a proof of principle that was supported by the analysis of CD80 at a lower concentration (2.25 μM), which is more clinically relevant. It has been reported that midazolam binds to plasma proteins, and the free-drug fraction was found to be 4–6% in vivo [45,46], and it decreased by infusion of lipoproteins [47]. We administered midazolam in an in vitro system. We used complete medium with 10% fetal bovine serum to maintain macrophages in current experiments, and there was a possible effect on the unbound medium concentration of midazolam. The concentration of midazolam used in our study has been used previously in various experiments analyzing the in vitro effects of benzodiazepine on immune cells [3,5]. Furthermore, the molecular mechanisms of midazolam associated with TSPO were supported by the findings of the genetic analysis using THP-1 cells overexpressing TSPO and THP-1 cells deficient in TSPO expression.

It has been reported that prolonged midazolam infusion causes an increase in the plasma concentration because of progressively reduced clearance. To achieve deep sedation, such as that associated with a Ramsay Sedation Scale score higher than 6, 6.75 μM (2.2 μg/mL) of midazolam is required [48]. Furthermore, hypothermia treatment in patients with brain injury can depress cytochrome P450 function; therefore, the plasma midazolam concentration may exceed 30 μM (10 μg/mL) [49]. Thus, in critical care settings requiring deep sedation, in vivo concentrations may reach levels sufficient to suppress macrophage-mediated immune responses.

In conclusion, the use of midazolam for sedation and analgesia in critical care may suppress macrophage-mediated immune responses. These effects likely occur via TSPO-induced suppression of NF-κB transcriptional activation for genes encoding pro-inflammatory mediators, such as CD80, IL-6, TNF-α, and NO.

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## Declaration of interest

None.

## Author contribution

Yu Horiguchi designed and conducted the study, analyzed the data, performed statistical analysis, and wrote the manuscript. Noriyuki Ohta designed and conducted the study, analyzed the data, and wrote the manuscript. Syunsuke Yamamoto designed and conducted the study and analyzed the data. Moe Koide designed and conducted the study and analyzed the data. Yuji Fujino designed the study and analyzed the data.

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## References

[1] E. Obiora, R. Hubbard, R.D. Sanders, P.R. Myles, The impact of benzodiazepines on occurrence of pneumonia and mortality from pneumonia: a nested case-control and survival analysis in a population-based cohort, Thorax 68 (2013) 163–170.
[2] R.R. Riker, Y. Shehabi, P.M. Bokesch, D. Ceraso, W. Wisemandle, F. Koura, P. Whitten, B.D. Margolis, D.W. Byrne, E.W. Ely, M.G. Rocha, SEDCOM (Safety and Efficacy of Dexmedetomidine Compared With Midazolam) Study Group, Dexmedetomidine vs midazolam for sedation of critically ill patients: a randomized trial, JAMA 301 (2009) 489–499.
[3] S.C. Son, S.M. Lee, C.S. Kim, D.G. Yoo, S.K. Lee, G.M. Hur, J.B. Park, B.H. Jeon, Midazolam inhibits proinflammatory mediators in the lipopolysaccharide-activated macrophage, Anesthesiology 105 (2006) 105–110.
[4] E.B. Lasunskaia, M.N. Campos, M.R. de Andrade, R.A. Damatta, T.L. Kipnis, M. Einicker-Lamas, W.D. Da Silva, Mycobacteria directly induce cytoskeletal rearrangements for macrophage spreading and polarization through TLR2-dependent PI3K signaling, J. Leukoc. Biol. 80 (2006) 1480–1490.
[5] K. Nishina, H. Akamatsu, K. Mikawa, M. Shiga, N. Maekawa, H. Obara, Y. Niwa, The inhibitory effects of thiopental, midazolam, and ketamine on human neutrophil functions, Anesth. Analg. 86 (1998) 159–165.
[6] J.F. Tallman, S.M. Paul, P. Skolnick, D.W. Gallager, Receptors for the age of anxiety: pharmacology of the benzodiazepines, Science 207 (1980) 274–281.
[7] C. Braestrup, R.F. Squires, Specific benzodiazepine receptors in rat brain characterized by high-affinity (3H)diazepam binding, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 3805–3809.
[8] F. Zavala, Benzodiazepines, anxiety and immunity, Pharmacol. Ther. 75 (1997) 199–216.
[9] V. Selvaraj, D.M. Stocco, The changing landscape in translocator protein (TSPO) function, Trends Endocrinol. Metab. 26 (2015) 341–348.
[10] H. Wilms, J. Claasen, C. Röhl, J. Sievers, G. Deuschl, R. Lucius, Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells in vitro, Neurobiol. Dis. 14 (2003) 417–424.
[11] M. Wang, X. Wang, L. Zhao, W. Ma, I.R. Rodriguez, R.N. Fariss, W.T. Wong, Macroglia-microglia interactions via TSPO signaling regulates microglial activation in the mouse retina, J. Neurosci. 34 (2014) 3793–3806.
[12] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.
[13] T.A. Hamilton, J.A. Major, D. Armstrong, J.M. Tebo, Oxidized LDL modulates activation of NFkappaB in mononuclear phagocytes by altering the degradation if IkappaBs, J. Leukoc. Biol. 64 (1998) 667–674.
[14] T. Kawai, S. Akira, TLR signaling, Cell Death Differ. 13 (2006) 816–825.
[15] G.H. Giambartolomei, V.A. Dennis, B.L. Lasater, P.K. Murthy, M.T. Philipp, Autocrine and exocrine regulation of interleukin-10 production in THP-1 cells stimulated with Borrelia burgdorferi lipoproteins, Infect. Immun. 70 (2002) 1881–1888.
[16] P.K. Murthy, V.A. Dennis, B.L. Lasater, M.T. Philipp, Interleukin-10 modulates proinflammatory cytokines in the human monocytic cell line THP-1 stimulated with Borrelia burgdorferi lipoproteins, Infect. Immun. 68 (2000) 6663–6669.
[17] D. Hwang, B.C. Jang, G. Yu, M. Boudreau, Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappaB signaling pathways in macrophages, Biochem. Pharmacol. 54 (1997) 87–96.
[18] Z. Guan, S.Y. Buckman, B.W. Miller, L.D. Springer, A.R. Morrison, Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells, J. Biol. Chem. 273 (1998) 28670–28676.
[19] C.J. Tsi, Y. Chao, C.W. Chen, W.W. Lin, Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IkappaB kinase, extracellular signal regulated kinase, and p38 mitogen-activated protein kinase inhibition, Mol. Pharmacol. 62 (2002) 90–101.
[20] C. Marie, P.R. Philippe, Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases, Microbiol. Mol. Biol. Rev. 75 (2012) 50–83.
[21] A. Neininger, D. Kontoyiannis, A. Kotlyarov, R. Winzen, R. Eckert, H.D. Volk, H. Holtmann, G. Kollias, M. Gaestel, MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels, J. Biol. Chem. 277 (2002) 3065–3068.
[22] S. Sato, H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, S. Akira, Essential function for the kinase TAK1 in innate and adaptive immune responses, Nat. Immunol. 6 (2005) 1087–1095.
[23] P.A. Baeuerle, T. Henkel, Function and activation of NF-kappa B in the immune system, Annu. Rev. Immunol. 12 (1994) 141–179.
[24] M.S. Lee, Y.J. Kim, Signaling pathways downstream of pattern-recognition receptors and their cross talk, Annu. Rev. Biochem. 76 (2007) 447–480.
[25] A. Fafalios, A. Akhavan, A.V. Parwani, R.R. Bies, K.J. McHugh, B.R. Pflug, Translocator protein blockade reduces prostate tumor growth, Clin. Cancer Res. 15 (2009) 6177–6184.
[26] N. Ohta, Y. Ohashi, C. Takayama, T. Mashimo, Y. Fujino, Midazolam suppresses

maturation of murine dendritic cells and priming of lipopolysaccharide-induced T helper 1-type immune response, Anesthesiology 114 (2011) 355–362.
[27] K.R. Bae, H.J. Shim, D. Balu, S.R. Kim, S.W. Yu, Translocator protein 18 kDa negatively regulates inflammation in microglia, J. NeuroImmune Pharmacol. 9 (2014) 424–437.
[28] R. Bhat, R. Axtell, A. Mitra, M. Miranda, C. Lock, R.W. Tsien, L. Steinman, Inhibitory role for GABA in autoimmune inflammation, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2580–2585.
[29] Y. Wang, D. Feng, G. Liu, Q. Luo, Y. Xu, S. Lin, J. Fei, L. Xu, Gamma-aminobutyric acid transporter 1 negatively regulates T cell-mediated immune responses and ameliorates autoimmune inflammation in the CNS, J. Immunol. 181 (2008) 8226–8236.
[30] S.K. Mendu, L. Akesson, Z. Jin, A. Edlund, C. Cilio, A. Lernmark, B. Birnir, Increased GABA(A) channel subunits expression in CD8(+) but not in CD4(+) T cells in BB rats developing diabetes compared to their congenic littermates, Mol. Immunol. 48 (2011) 399–407.
[31] H. Bjurström, J. Wang, I. Ericsson, M. Bengtsson, Y. Liu, S. Kumar-Mendu, S. Issazadeh-Navikas, B. Birnir, GABA, a natural immunomodulator of T lymphocytes, J. Neuroimmunol. 205 (2008) 44–50.
[32] C.A. Hunter, S.A. Jones, IL-6 as a keystone cytokine in health and disease, Nat. Immunol. 16 (2015) 448–457.
[33] K. Masuda, B. Ripley, R. Nishimura, T. Mino, O. Takeuchi, G. Shioi, H. Kiyonari, T. Kishimoto, Arid5a controls IL-6 mRNA stability, which contributes to elevation of IL-6 level in vivo, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 9409–9414.
[34] S.R. Viswanathan, J.T. Powers, W. Einhorn, Y. Hoshida, T.L. Ng, S. Toffanin, M. O'Sullivan, J. Lu, L.A. Phillips, V.L. Lockhart, S.P. Shah, P.S. Tanwar, C.H. Mermel, R. Beroukhim, M. Azam, J. Teixeira, M. Meyerson, T.P. Hughes, J.M. Llovet, J. Radich, C.G. Mullighan, T.R. Golub, P.H. Sorensen, G.Q. Daley, Lin28 promotes transformation and is associated with advanced human malignancies, Nat. Genet. 41 (2009) 843–848.
[35] A. Waage, P. Brandtzaeg, A. Halstensen, P. Kierulf, T. Espevik, The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome, J. Exp. Med. 169 (1989) 333–338.
[36] V. Taupin, P. Jayais, B. Descamps-Latscha, J.B. Cazalaa, G. Barrier, J.F. Bach, F. Zavala, Benzodiazepine anesthesia in humans modulates the interleukin-1 beta, tumor necrosis factor-alpha and interleukin-6 responses of blood monocytes, J. Neuroimmunol. 35 (1991) 13–19.
[37] V. Taupin, J. Gogusev, B. Descamps-Latscha, F. Zavala, Modulation of tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6, interleukin-8, and granulocyte/macrophage colony-stimulating factor expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetraneuropeptide: evidence for a role of prostaglandins, Mol. Pharmacol. 43 (1993) 64–69.
[38] G.J. Liu, R.J. Middleton, C.R. Hatty, W.W. Kam, R. Chan, T. Pham, M. Harrison-Brown, E. Dodson, K. Veale, R.B. Banati, The 18 kDa translocator protein, microglia and neuroinflammation, Brain Pathol. 24 (2014) 631–653.
[39] A. Batarseh, V. Papadopoulos, Regulation of translocator protein 18 kDa (TSPO) expression in health and disease states, Mol. Cell. Endocrinol. 327 (2010) 1–12.
[40] F. Bonsack, C.H. Alleyne, S. Sukumari-Ramesh, Augmented expression of TSPO after intracerebral hemorrhage: a role in inflammation? J. Neuroinflammation 13 (2016) 151.
[41] M.K. Chen, T.R. Guilarte, Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair, Pharmacol. Ther. 118 (2008) 1–17.
[42] T. Clavier, M.C. Tonon, A. Foutel, E. Besnier, A. Lefevre-Scelles, F. Morin, P. Gandolfo, J.J. Tuech, M. Quillard, B. Veber, B. Dureuil, Increased plasma levels of endozepines, endogenous ligands of benzodiazepine receptors, during systemic inflammation: a prospective observational study, Crit. Care 18 (2014) 633.
[43] H. Wang, K. Zhai, Y. Xue, J. Yang, Q. Yang, Y. Fu, Y. Hu, F. Liu, W. Wang, L. Cui, H. Chen, J. Zhang, W. He, Global deletion of TSPO does not affect the viability and gene expression profile, PLoS One 11 (2016) e0167307.
[44] L.N. Tu, K. Morohaku, P.R. Manna, S.H. Pelton, W.R. Butler, D.M. Stocco, V. Selvaraj, Peripheral benzodiazepine receptor/translocator protein global knockout mice are viable with no effects on steroid hormone biosynthesis, J. Biol. Chem. 289 (2014) 27444–27454.
[45] J.X. de Vries, J. Rudi, I. Walter-Sack, R. Conradi, The determination of total and unbound midazolam in human plasma. A comparison of high performance liquid chromatography, gas chromatography and gas chromatography/mass spectrometry, Biomed. Chromatogr. 4 (1990) 28–33.
[46] H. Allonen, G. Ziegler, U. Klotz, Midazolam kinetics, Clin. Pharmacol. Ther. 30 (1981) 653–661.
[47] I. Walter-Sack, J.X. de Vries, J. Rudi, R. Conradi, M. Kohlmeier, B. Kohl, E. Weber, Lack of accumulation of midazolam in plasma and lipoprotein fractions during intravenous lipid infusions in patients on artificial respiration, Eur. J. Clin. Pharmacol. 42 (1992) 71–75.
[48] J. Barr, G.L. Fraser, K. Puntillo, E.W. Ely, C. Gélinas, J.F. Dasta, J.E. Davidson, J.W. Devlin, J.P. Kress, A.M. Joffe, D.B. Coursin, D.L. Herr, A. Tung, B.R. Robinson, D.K. Fontaine, M.A. Ramsay, R.R. Riker, C.N. Sessler, B. Pun, Y. Skrobik, R. Jaeschke, American College of Critical Care Medicine, Clinical practice guidelines for the management of pain, agitation, and delirium in adult patients in the intensive care unit, Crit. Care Med. 41 (2013) 263–306.
[49] N. Fukuoka, M. Aibiki, T. Tsukamoto, K. Seki, S. Morita, Biphasic concentration change during continuous midazolam administration in brain-injured patients undergoing therapeutic moderate hypothermia, Resuscitation 60 (2004) 225–230.