Midazolam Suppresses Maturation of Murine Dendritic Cells and Priming of Lipopolysaccharide-induced T Helper 1-type Immune Response

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ABSTRACT

Background: Dendritic cells (DCs), as antigen-presenting cells, play a key role in the induction and regulation of adaptive immune response. Midazolam is reported to have immunomodulatory properties that affect immune cells. However, the effect of midazolam on DCs has not been characterized. We examined the immunomodulatory properties of midazolam on DC-mediated immune response.

Methods: After allowing murine bone marrow–derived DCs induced by granulocyte macrophage colony stimulating factor to mature, we analyzed their expression of costimulatory molecules (CD80 and CD86), major histocompatibility complex class II molecules, and the secretion of interleukin-12 p40. In vitro, we evaluated the effect of midazolam on maturing DCs in mixed cell cultures containing DCs and T cells. In vivo, we investigated the contact-hypersensitivity response.

Results: Midazolam suppressed the expression of CD80, CD86, and major histocompatibility complex class II molecules from murine DCs. Treated with midazolam, DCs also secreted less interleukin-12 p40. In mixed cell cultures with CD3-positive T cells, midazolam-treated DCs showed less propensity to stimulate the proliferation of CD3-positive T cells and the secretion of interferon-γ from CD4-positive T cells. Midazolam-treated DCs impaired the induction of contact-hypersensitivity response. Treatment with ligands for peripheral benzodiazepine receptor inhibited the up-regulation of CD80 during DC maturation.

Conclusion: Midazolam inhibits the functional maturation of murine DCs and interferes with the lipopolysaccharide-induced T helper 1-type immune response in the mouse through the activation of peripheral benzodiazepine receptors.

What We Already Know about This Topic

- Dendritic cells are potent antigen-presenting cells and play a key role in the regulation of the immune response.

What This Article Tells Us That Is New

- Midazolam inhibits the maturation of dendritic cells, interfering with the lipopolysaccharide-induced T helper 1-type immune response in the mouse through the activation of peripheral benzodiazepine receptors.
Midazolam Inhibits Dendritic Cell Functions

Materials and Methods

Animals
Female C57BL/6 and Balb/c mice (aged 4–6 weeks) were purchased for cell samples (Japan CLEA, Tokyo, Japan). Animals were housed with food and water available ad libitum in the specific pathogen-free central animal facility of Osaka University Medical School (Suita, Japan). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Osaka University Medical School (Suita, Japan) and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents and Antibodies
Recombinant mouse granulocyte macrophage colony stimulating factor was purchased from R&D Systems (Minneapolis, MN). Fluorescein isothiocyanate-conjugated dextran (FITC-dextran [40,000 molecular mass]) and lipopolysaccharide from Escherichia coli 055:B5 were obtained (Sigma-Aldrich, St Louis, MO). FITC- or phycoerythrin-conjugated monoclonal antibodies (mAbs) were used to detect the expression of CD11c (HL3), MHC class II (I-Ab), (AF6-120.1), CD80 (16-10A1), and CD86 (GL1) were purchased from BD Biosciences (San Diego, CA). For intracellular cytokine detection, we used mAbs for tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin 4 (11B11, MP6-XT22, and XMG1.2; BD Biosciences). Ligands Ro5-4864 and PK11195, specific to PBR, and clonazepam, specific to CBR, were purchased from Sigma-Aldrich. Ligands were dissolved separately in dimethyl sulfoxide, reserved, and diluted with culture medium immediately before use.

Isolation and Culture of DC
Using a previously described method with minor modifications,5,10 DCs were generated from murine bone marrow cells. In brief, murine bone marrow was flushed from the tibiae and femurs of C57BL/6 mice and then depleted of red cells with ammonium chloride. Bone marrow cells were suspended in complete media (RPMI-1640 supplemented with 10% fetal bovine serum albumin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells for each mouse were plated on 10-cm plates at 5 × 10⁵/ml and cultured in the presence of 20 ng/ml recombinant mouse granulocyte macrophage colony stimulating factor at 37°C in 5% CO₂. On days 3 and 5 of the culture, floating cells were gently removed and fresh medium supplemented with recombinant mouse granulocyte macrophage colony stimulating factor was added. On day 7, nonadherent cells and loosely adherent proliferating DCs were harvested and, by centrifugation at 600g for 20 min at room temperature, purified. Samples of DCs were cultured for another 2 days with and without midazolam and then stimulated with 100 ng/ml lipopolysaccharide for 24 h.

The viability of cultured cells was assessed by trypan blue exclusion testing. Cell viability of more than 90% was observed in all experimental cultures used in this study.

Flow-cytometric Analysis of Surface Molecules
The expression of surface molecules on DCs was analyzed under flow cytometry. At each step of staining, to prevent nonspecific binding of antibodies, 1–2 × 10⁵ cells were incubated for 15 min on ice in staining buffer containing anti-CD16/CD32 mAbs. Cells were stained with specific antibodies. After mAb staining, 7-amino actinomycin D viability staining solution (eBioscience, Inc., San Diego, CA) was applied to stain dead cells. Samples were analyzed using BD FACSCalibur and BD CellQuest Pro (BD Biosciences). We used FITC- or phycoerythrin-labeled mAbs to stain for MHC class II, CD80, CD86, and CD11c. Using flow cytometry, dead cells were gated out by staining with 7-amino actinomycin D and only live cells were phenotypically assessed.

Interleukin-12 p40 Enzyme-linked Immunosorbent Assay
DCs were cultured in the presence versus absence of midazolam, followed by stimulation with lipopolysaccharide (100...
ng/ml) for 12 h. Following manufacturer instructions, we analyzed culture supernatants using IL-12 p40 enzyme-linked immunosorbent assay kits (R&D Systems).

**Intracelluar Cytokine Assay**
For intracellular cytokine assay, T cells were restimulated with ionomycin and phorbol myristate acetate in the presence of BD GolgiStop (BD Biosciences). Following manufacturer instructions, intracellular cytokines were detected using standard Cytofix/Cytoperm Plus Kits (BD Biosciences). We used phycoerythrin-labeled mAbs to stain TNF-α, IFN-γ, and interleukin 4, and FITC-labeled mAbs for staining CD4.

**Quantification of Antigen Uptake**
As described by Sallusto et al., endocytosis was quantified. In brief, 2 x 10⁵ cells were equilibrated at 37°C or 4°C for 10 min and then pulsed with 1 mg/ml FITC-dextran. Cold staining buffer was added to stop the reaction. Cells were washed three times and stained with phycoerythrin-conjugated anti-CD11c antibodies before analysis with BD FACSCalibur. Nonspecific binding of FITC-dextran to DCs was assessed by evaluating FITC-dextran uptake at 4°C. Mean fluorescence intensity at 37°C — mean fluorescence intensity at 4°C was used as the measure of antigen uptake.

**Reverse Transcription–Polymerase Chain Reaction Analysis of PBR-specific Messenger RNA (mRNA)**
After 7-day culture, BM-DCs were purified by magnetic sorting using magnetic-activated column sorting column and beads coated with anti-CD11c antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA from BM-DCs was prepared with RNeasy mini kit (QUIAGEN, Hilden, Germany) and complementary DNA was synthesized (Superscript III; Invitrogen Life Technologies, Tokyo, Japan). To amplify PBR complementary DNA, the following primers were used: 5′-AGTGTCTTCTACGGAA-CAAC-3′ and 5′-AACTGTGTCTCGGAGACT-3′. After heating at 95°C for 10 min, complementary DNA was amplified for 35 cycles, each cycle consisting of 95°C for 15 s then 60°C for 60 s. Amplified products were separated by electrophoresis on 1.8% agarose gel and stained with 1 μg/ml ethidium bromide.

**Allogeneic Mixed Cell Culture Reaction**
DCs induced from C57BL/6 mice (haplotype I-Ab) were incubated in the presence and absence of midazolam. Subsequently, DCs were stimulated by 100 ng/ml lipopolysaccharide for 12 h and treated with 50 μg/ml mitomycin C. To enrich CD3-positive T cells from splenocytes, splenocytes isolated from 6-week-old female Balb/c mice (haplotype I-Ad) were purified in magnetic-activated column sorting. Enriched CD3-positive T cells were cocultured with mitomycin C–treated DCs. In 96-well round-bottom plates, mixed samples were cultured for 4 days in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Cell proliferation was estimated based on uptake of [3H]thymidine. For this purpose, cells were pulsed with [3H]thymidine for the final 18 h of mixed cell culture. Then, radioactivity was measured using a liquid scintillation counter (PerkinElmer, Waltham, MA). Intracellular cytokines were detected, following manufacturer instructions, using standard Cytofix/Cytoperm Plus Kits, as described.

**Contact Hypersensitivity Model and Model with Adoptive Transfer of DCs**
In positive control groups for the original contact hypersensitivity (CHS) model, 2,4-dinitro-1-fluorobenzene (DNFB) was used as a contact-sensitizing agent, and DNFB, 0.5%, was dissolved in 4:1 aceton/olive oil was applied to the shaved abdomen on day 0. On day 5, mice were challenged by epicutaneous application of DNFB on the right ear. After 24 h, using a spring-loaded micrometer (Mitutoyo Corporation, Kawasaki, Japan), we measured right (challenged) and left (unchallenged) ear thickness. Increased ear thickness was evaluated by simple subtraction: thickness of challenged ear — thickness of unchallenged ear.

A CHS model involving adoptive transfer of BM-DCs pulsed with peptide-antigen was designed as described in a previous report and used. DCs were prepared as described in isolation and culture of DC. After culture in the presence or absence of midazolam, 5 x 10⁵ DCs (100 μl saline) were pulsed with 100 μg/ml 2,4-dinitrobenzene sulfonic acid (DNBS: a water-soluble analog of DNFB) and injected subcutaneously on day 0. After 5 days, mice were challenged on both sides of the right ear by the application of 10 μl DNFB, 0.2%, in 4:1 aceton/olive oil solution. At the time of initial immunization, negative control animals were injected with 100 μl saline and exposed to DNFB 5 days later. After 24 h, using a spring-loaded micrometer, ear thickness was evaluated as described above.

**Statistical Analysis**
In a preliminary study, power analysis showed a group size of eight (N = 8) as sufficient to detect a 50% reduction of surface-molecule expression by midazolam with a power of 0.8 at an α level of 0.05. Normality of distribution was tested using the Kolmogorov-Smirnov test. Data are expressed as mean ± SD or median (25–75% interquartile range), as appropriate. Comparison of means was performed using the Student t test or ANOVA and the post hoc Tukey test. Comparison of medians was performed using the Mann–Whitney U test. All analyses were performed using two-tailed tests. A P value of less than 0.05 was considered statistically significant. Statistical analysis was conducted using JMP version 8.0 (SAS Institute, Cary, NC) and STATISTICA 06 software (StatSoft, Inc., Tulsa, OK).
Midazolam Inhibits Maturation of Murine DCs

At first, we examined whether midazolam influences the maturation of murine DCs. Our preliminary investigations of DC culture showed that midazolam has no effect on DC viability in concentrations up to 200 μM (data not shown).

To examine how midazolam influences the maturation of DCs, we cultured immature DCs in the absence versus presence of 15 μM midazolam. We measured the expression of costimulatory molecules and MHC class II molecules after 24-h lipopolysaccharide stimulation. As DCs matured, we observed higher expression of MHC class II, CD80, and CD86 molecules (fig. 1A). Furthermore, midazolam suppressed the expression of CD80 molecules in a dose-dependent manner (fig. 1B).

Midazolam Inhibits Secretion of Interleukin-12 p40

Mature DCs are important for the synthesis and secretion of cytokines that affect T-cell differentiation, being responsible for the quality of immune response. DCs produce proinflammatory cytokines. In particular, IL-12 production is a marker of DC maturation and can be used as a method of selecting the Th1-dominant adjuvant. Here, we tested the expression of IL-12 p40 from lipopolysaccharide-stimulated DCs. As figure 2A shows, midazolam inhibited IL-12 p40 secretion.

Midazolam Induces Immature DCs with High Endocytotic Capacity

Evaluation of surface molecule and IL-12 p40 expression indicated that exposure to midazolam significantly suppresses phenotypical and functional maturation of DCs generated in vitro. These results did not, however, exclude the possibility that midazolam causes a general inhibition of DC function.

In addition, we investigated whether midazolam exposure alters the ability of DCs to capture antigen through the uptake of FITC-dextran. As figure 2B shows, DCs exposed to midazolam for 7 days and then left in the presence of lipopolysaccharide for 24 h displayed increased endocytotic capacity for FITC-dextran. All our findings—including those for surface molecule and IL-12 p40 expression as well as those for endocytotic activity—strongly suggest that midazolam prevents DC maturation.

Midazolam Inhibits T-cell Proliferation and Th1-type Immune Response

To clarify how midazolam-mediated alteration of DC function might affect DC-induced immune response, we investigated
how this medication affected mixed cell culture reaction in DC lymphocytes. In mixed cell culture reaction, DCs from C57BL/6 mice (haplotype I-Ab) cultured with allogeneic T cells from Balb/c mice (haplotype I-Ad) induced Balb/c T cells to proliferate and differentiate into Th1-secreting IFN-\(\gamma\) and TNF-\(\alpha\). In mixed cell culture reaction, DCs matured with lipopolysaccharide in the presence or absence of midazolam were tested for their capacity to stimulate allogeneic T cells. Coculturing DCs without midazolam effectively enhanced proliferative responses assessed by increase of \(\text{[3H]thymidine uptake by T cells. By contrast, with DCs that had been treated with midazolam, there was less [3H]thymidine uptake by allogeneic T cells (fig. 3A).}

Another determinant of DC potency for priming T cells is how well they are able to adhere to and form clusters on T cells (fig. 3B). Compared with control, we found that midazolam-treated DCs formed fewer clusters on T cells.

In mixed cell culture reaction, DCs matured by lipopolysaccharide also induced the cocultured CD4-positive T cells to differentiate into IFN-\(\gamma\)-secreting Th1 T cells. We evaluated whether midazolam had any effect on this function. Intracellular cytokine analysis revealed a lower density of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing CD4-positive cells stimulated by midazolam-treated BM-DCs (figs. 4A and B).

**Midazolam-treated DCs Failed to Elicit CHS that Would Normally Have Been Induced by Th-1 Type Immune Response**

Induced mainly by Th1-type T cells, including CD4-positive and CD8-positive T cells, CHS is a prototypical \textit{in vivo} model of cell-mediated immune response. In the original model, CHS was induced by epicutaneous immunization with DNBS into flanks and subsequent epicutaneous challenge with hapten, such as DNFB, to ears, followed by swelling of the challenged ears. CHS can be also induced by a single subcutaneous injection (day 0) of \(5 \times 10^5\) DCs that cells to differentiate into IFN-\(\gamma\)-secreting Th1 T cells.
have been pulsed with DNBS, followed, on day 5, by epicutaneous challenge with DNFB. We used this CHS model with adoptive transfer of DCs to assess the in vivo effect of midazolam on DC-induced Th1-type immune response in the whole animal. Immunization with midazolam-treated DCs elicited less CHS response than immunization with vehicle-treated DCs (fig. 5).

Effect on Expression of CD80 during DC Maturation of Ligands Specific to Peripheral and Central Benzodiazepine Receptors

Next, we investigated how benzodiazepine receptors are involved in the suppressive effect of midazolam by testing with ligands Ro5-4864 and PK11195, specific to PBR, and clonazepam, specific to CBR. The effect of these ligands on DC maturation was examined by evaluating surface-expression of CD80. Although no CD80 suppression was observed in the presence of clonazepam (within the range of concentration of PBR ligands), the dose-dependent suppression of these molecules was detected in the presence of Ro5-4864 and PK11195 (fig. 6A).

Identification of mRNA Specific for PBR

We next assessed the existence of PBR-specific mRNA in BM-DCs. From total RNA isolated from BM-DCs, complementary DNA was synthesized. After analysis for PBR-specific mRNA by reverse transcription–polymerase chain reaction, mRNA specific for PBR was identified (fig. 6B).

Discussion

In the current study, we demonstrated that midazolam inhibits the phenotypic maturation of DCs and suppresses the DC-induced induction of the Th1-type immune response in
flammation can be sometimes beneficial, but during the im-
sepsis, suppressive activity to reduce or avoid excessive in-
midazolam’s suppressive activity remain controversial. In
invading pathogens. In critical care, the beneficial effects of
zolam might compromise the immune response against in-
type immune response observed in the current study, mida-
alam on DC-induced Th1-response, suggest a candidate
whole animal.
mediated induction of Th1-type immune response in the
DCs cultured with midazolam, CHS was reduced. This re-
We found that, when mice were similarly exposed to DNBS-
the whole mouse. Our findings suggest that the suppressive
activity of midazolam on DCs is mediated by PBR signaling.
Previous investigators1–3 have suggested that development
toward Th1 cells is regulated by DC-derived cytokines, such
as IL-12. In addition, our study showed that lipopolysaccha-ide-stimulated DCs differentiate naive T cells into IFN-γ-
producing Th1 T cells. Our current finding, that midazolam
inhibits DC production of IL-12, shows that midazolam
affects the action of DCs on the differentiation of T cells to
Th1 T cells.
We conjectured that, in the whole animal, midazolam
probably impairs the ability of DCs to initiate immune re-
sponse mediated mainly by Th1 T cells. To test this hypo-
thesis, we used CHS, a well-established model for testing the
delayed hypersensitivity that is induced by Th1-type im-
une response. After confirming that subcutaneous im-
unization with DNBS-DC made mice susceptible to CHS, we
designed a CHS model for the adoptive transfer of DCs.12
We found that, when mice were similarly exposed to DNBS-
DCs cultured with midazolam, CHS was reduced. This re-
result provides evidence that midazolam suppresses the DC-
mediated induction of Th1-type immune response in the
whole animal.
In mice, repeated administration of diazepam has been
reported to impair cell-mediated immunity regulated by the
Th1-type immune response13,14. We speculate, in view of the
central role of DCs in the induction of immune response,
that the suppressive effects of benzodiazepine on cell-medi-
ated immunity is probably mediated by the effect of benzo-
diazepine on DCs.
With practical development in prospect for controlled
Th1-response induction, such as ex vivo manipulation of
DCs for DC therapy to treat malignant tumors or infectious
disease,15–17 our findings, which show the effect of midazo-
lon on DC-induced Th1-response, suggest a candidate
means to counter-regulate the immune response induced by
DC therapy. Considering the effects on DCs and the Th1-
type immune response observed in the current study, mida-
zolam might compromise the immune response against in-
vading pathogens. In critical care, the beneficial effects of
midazolam’s suppressive activity remain controversial. In
sepsis, suppressive activity to reduce or avoid excessive in-
flammation can be sometimes beneficial, but during the im-
munosuppressive conditions that are often complicated in
the course of sepsis, the suppressive effects of midazolam may
compromise the eradication of infection.18 However, as a
clinically administered drug, midazolam may be a good can-
didate in safely controlling DC-mediated immune response.
Pharmacologic experiments using ligands specific to
PBRs and CBRs showed that, although clonazepam did not
suppress surface-molecule expression on DCs, PBR ligands
did—in a manner similar to midazolam—suppress the up-
regulation of CD80 during DC maturation. The existence of
PBR-specific mRNA and our results using PBR- and CBR-
specific ligands, strongly suggest the involvement of PBRs in
the suppressive effect of midazolam on DCs. It has been
reported19,20 that PBR ligands are involved in autoimmune
arthritis in MRL/lpr mouse. In another instance, treatment
with PBR ligands was shown to suppress arthritic lesions in
MRL/lpr mice.1,2,3 Because DCs play a central role in the
induction and regulation of immune response, such evidence
may suggest that action on DC-PBR ligands mediates the
suppression of the immune responses observed in autoim-
mune arthritis.
This study has several limitations. First, our results are
based on experiments using mice; no human tissue was
tested. The BM-DC culture system used in most of the ex-
periments reported here has become a well-established
system as a widely used means for investigating DC bio-
ology.21–23 Culturing DCs induced from murine bone mar-
row with recombinant mouse granulocyte macrophage col-
ony stimulating factor has generated many results that are
comparable with standard human DC culture derived from
blood monocytes.3 It is possible that the results reported here
would differ in human cells. Our results need to be con-
formed using human cultured DCs derived from blood
monocytes.
In addition, the findings of our in vitro studies are of
questionable relevance to in vivo conditions found in whole
animals, where conventional DCs, plasmacytoid DCs, and
other DCs are present.3 Although the BM-DCs generated in
our study resemble commonly encountered monocyte-de-
ived DCs in conventional DC subsets, BM-DCs have no
counterpart in conventional DC subsets of lymphoid-organ-
residence DCs or plasmacytoid DCs.23 Further investigation of
vitro subsets of DCs is required to clarify the effect of
midazolam on in vivo DCs.
The effects of midazolam on DCs in this study were eval-
uated after exposure to 15 μM midazolam. In addition, we
tested for dose-dependent responses by exposing DCs to
0–50 μM midazolam and measuring the expression of sur-
face molecules. Changes in surface-molecule expression at 5
μM midazolam (actual clinical concentration) show the
same tendencies observed with 15 μM midazolam. We chose
15 μM midazolam as a proof of principle that was supported
by the response of CD80 expression at the lower concentra-
tion (5 μM), which is more clinically relevant.
In a pharmacokinetic-modeling study of midazolam-
based sedation, EC50 concentration for deep sedation (e.g.,


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Ramsay Sedation Scale score higher than 6) is reported to be 6.75 μM (2,200 ng/ml). When midazolam is administered to patients who have immunosuppression induced by therapeutic intervention or by diseases such as sepsis, the immunosuppressive activity of midazolam on DCs may increase immunosuppression. Thus, even if DCs are exposed to concentrations of midazolam that are lower than those investigated in the current study—such as those that occur in actual patients during critical care—there may be a similar inhibitory effect on DC-mediated immune responses. Furthermore, in clinical situations where very deep sedation is necessary (e.g., therapeutic hypothermia), midazolam concentrations exceeding 30 μM (10,000 ng/ml) have been reported. Thus, it is possible in critical care settings that clinically administered midazolam may suppress DC-mediated immune response.

In summary, midazolam can modify the immune response induced by DCs. Administration of midazolam may provide a nontoxic means of modulating the immunostimulatory capacity of DCs. During sedation and analgesia in critical care, the use of midazolam may suppress DC-mediated immune responses.

References
27. Ohta et al.