Ketamine Affects *In Vitro* Differentiation of Monocyte into Immature Dendritic Cells

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ABSTRACT

Background: Monocytes (MOs) have the unique ability to differentiate into immature dendritic cells (iDCs) (MO→iDC) under the influence of interleukin-4 and granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF). In this study, the authors investigated the influence of ketamine on the process of MO→iDC.

Methods: iDCs were cultured from MO obtained from 36 subjects in the presence of IL-4 and GM-CSF and ketamine at 100, 10, and 1 µg/ml for 5 days. In some of the experiments, the authors used nonspecific *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801, NMDA, or a neutralizing antibody for transforming growth factor β (TGFβ). The expression of surface markers and functional assays were used to assess the effect of ketamine on IL-4&GM-CSF-stimulated MO. IL-4&GM-CSF-stimulated MO's supernatants were assessed for cytokine levels.

Results: Ketamine at 10 µg/ml, and higher concentrations, diminished the expression of CD1a on IL-4&GM-CSF-stimulated MO and retarded both their ability to process DQ ovalbumin and mixed lymphocyte reaction stimulation. The addition of ketamine to IL-4&GM-CSF-differentiated MO resulted in the persistent expression of CD14 and unchanged expression of CD86 and CD206. The phagocytic abilities of IL-4&GM-CSF-differentiated MO were not changed by ketamine. MK-801, a nonselective NMDA agonist, mimicked ketamine's effect on MO→iDC differentiation. Adding exogenous NMDA to IL-4&GM-CSF-stimulated MO in the presence of ketamine partially restored the level of CD1a^{+}. TGFβ was elevated in supernatants of IL-4&GM-CSF-stimulated MO in the presence of ketamine. Adding neutralizing TGFβ antibody or TGFβR1 blocker (SB431542) resulted in the full recovery of MO→iDC, despite the presence of ketamine.

Conclusions: Ketamine diminishes the process of MO→iDC *in vitro*. This is mediated *via* NMDA-dependent mechanisms and TGFβ. (ANESTHESIOLOGY 2015; 123:628-41)

KETAMINE is an agent with unique anesthetic, analgesic, and immune-modulatory properties.[1–8] Ketamine effects are immunoinhibitory by virtue of its depression of levels of cytokines in serum or cellular response to bacterial pathogens.[9–11] However, little is known about the effect of ketamine on cellular immunity. Increasing use of ketamine infusion for sedation, treatment of chronic pain and delirium, and aid to alleviate acute depression are new therapeutic applications with potentially significant consequences for the immune system.[12–17] Also, some experimental therapies call for the use of ketamine during cardiopulmonary bypass, septic shock, and for central nervous system repair.[2,4–6,11,18–20] Ketamine and similar chemical derivatives are also frequently abused, further increasing the number of individuals whose immune system is under the prolonged influence of ketamine.[21]

Peripheral monocytes (MOs) are pluripotent cells that can differentiate into dendritic cells (DCs). DCs are potent regulators of the immune system that are able to focus on

What We Already Know about This Topic

- Monocytes (MOs) have the unique ability to differentiate into immature dendritic cells (iDCs) (MO→iDC) under the influence of interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF). In this study, the authors investigated the influence of ketamine (an agent with unique anesthetic, analgesic, and immune-modulatory properties) on the process of MO→iDC.

What This Article Tells Us That Is New

- At 10 µg/ml and higher concentrations, ketamine diminished the expression of CD1a on interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF)-stimulated monocytes and retarded both their ability to process DQ ovalbumin and mixed lymphocyte reaction stimulation. The phagocytic abilities of IL-4&GM-CSF-differentiated monocytes were not changed by ketamine.

initially indiscriminate immunological activation into a more specific response.[22–24] Their emergence from peripheral MO depends on the local cytokine milieu, with some

cytokines being supportive (interleukin [IL]-4, IL-13, granulocyte–monocyte colony-stimulating factor [GM-CSF]) or inhibitory (M-CSF, GM-CSF alone, IL-10, transforming growth factor [TGF]-β, prostaglandin E2 [PGE$_2$]) to MO→DC.[23,25–31] This is a two-step process with initial differentiation of MO into immature DC (iDC) followed by its acquisition of the fully functional properties of mature DC (mDC). Failed differentiation of MO into iDC precludes the subsequent steps. In contrast to "classical" DC (such as Langerhans cells, plasmacytoid DC, *etc.*), MO-derived DC can emerge quickly.[30,32] Therefore, it is likely that they play a pivotal role in responses to critical care insults compared with "classical" DC. Thus, the effect of ketamine infusion on their differentiation may be substantial. This is important clinically because deficits of MO-derived DC have been reported in sepsis, trauma, and burns and is linked to unfavorable clinical outcomes.[33,34]

Data on the effect of ketamine on MO-derived DC are sparse. Ohta *et al.*[35] concluded that ketamine-treated bone marrow mice DC precursors have a lower propensity to become mDC. No precise mechanism of ketamine's effect on MO→iDC was examined. Although it is difficult to apply data from mice to human immunology, especially in the field of DC biology, several mechanisms can be elucidated.[22,24,36] For example, aberrations in the initial, and necessary, step of MO to iDC differentiation would explain Ohta's observation. Prior studies described ketamine as capable of inducing several cytokines inhibitory to the MO→iDC process.[2,7,8,19] So far no data have been presented indicating the effect of ketamine on the human MO/DC system.

Therefore, we decided to focus on the effects of ketamine on the initial stage of the MO→DC process—the emergence of iDC from precursor MO. We hypothesized that ketamine would interfere with this process by inducing production of immunoinhibitory cytokines or altering the expression of cytokine receptors that are critical for the differentiation fate of MO.[18,25,27–29,37–39] The study is intended as an *in vitro* project assessing whether ketamine has an effect on the MO→iDC process in human MO.

Materials and Methods

Generation of iDCs from Peripheral MO
Human MOs were procured from healthy individuals by the Human Immunology Core affiliated with the University of Pennsylvania, Philadelphia, Pennsylvania, under a permit issued by the Institutional Review Board (IRB) at the University of Pennsylvania. MOs obtained from 36 anonymous donors were used in this study.

To generate iDC, fresh MOs were incubated in X-VIVO 15 Media with gentamicin and phenol red (Lonza, USA) supplemented with human IL-4 (PeproTech, USA) at 500 IU/ml and human GM-CSF (PeproTech) at 1,000 IU/ml at 37°C 5% CO_2 in the dark. In some cultures, ketamine hydrochloride (Ket) (JHP Pharmaceuticals, USA) was

added to the final concentrations of 1, 10, and 100 μg/ml. Ten μg/ml of ketamine had been used before by Ohta *et al.* On day 3, 50% of the X-VIVO15/10 was replenished with fresh media along with 50% of the initial cytokine concentration. No additional ketamine was added at this step. Supernatants were collected at this time and immediately frozen for subsequent cytokine analysis. The cultures were terminated on day 5 when supernatants cells were collected. Some MOs were cultured with or without *N*-methyl-D-aspartate (NMDA) nonselective receptor antagonist MK-801 (Cayman Chemicals, USA) at 100 and 10 μM. In another series of experiments, we added NMDA (Tocris, United Kingdom) at 100 μM, incubated for 15 min, and followed with the addition of 100 μg/ml of ketamine. Alternatively, rabbit polyclonal neutralizing antibody for TGFβ (Abcam, United Kingdom) was used at a concentration of 5 μg/ml. An appropriate isotype immunoglobulin was used in control experiments. Blockade of the TGFβ receptor activation was achieved by incubating cells with SB431542 (Santa Cruz Biotechnology, USA) at 0.3 μM. Downstream activation of Erk1/2 *via* TGFβ was suppressed by addition of PD 98059 (Sigma, USA) at 1 μM. All the experiments were followed by 5 days of incubation with IL-4 and GM-CSF, as described (see paragraph 2, Materials and Methods), unless specified differently in the text.

The harvesting of the IL-4&GM-CSF-stimulated MO was done by a brief incubation in 1 ml of 10 mM EDTA buffered to pH of 7.4 (Gibco, USA) on ice, followed by the washout of all cells with phosphate buffered solution (PBS) without Ca^{2+} or Mg^{2+} (Gibco). Cells were counted with the Countess® Automated Cells Counter (LifeTechnologies, USA) as per the manufacturer's instructions. Cells were suspended at 10^7 live cells per milliliter in X-VIVO15 for further studies.

Randomization was not used to assign cells to treatments. Study personnel were kept unaware what sample they were running through the use of simple numerical values for experimental designs/stimulation. Only after completion of the experiments, all the data were combined and analyzed.

Flow Cytometry
A total of 1×10^5 cells were incubated with fluorescence activated cell sorting (FACS) media (PBS without Ca^{2+} or Mg^{2+} with 0.01% sodium azide and 1% fetal bovine serum) enriched with human TrueStainFcX (BioLegend, USA) for 15 min at 4°C in the dark. Next, antibodies were added at a predetermined concentration. Incubation took place for 30 min at 4°C in the dark with additional mixing of the cells at 15 min. Cells were washed twice in FACS media and centrifuged at 500*g* 4°C for 5 min. Excess FACS media was evacuated, and cells were resuspended in 100 μl of FACS media and 100 μl of the 1% Flow Fix (Polysciences, USA). The following antibodies were used: CD1a (clone HI149; BioLegend), CD14 (clone Tuk4; Invitrogen, USA), CD83 (clone HB15e; BD, USA), CD206 (clone 15-2; BioLegend),

CD116 (clone 4H1; BioLegend), CD115 (clone AFS98; BioLegend), CD124 (clone G077F6; BioLegend), CD126 (clone UV4; BioLegend), and CD210 (clone 3F9; BioLegend).

DQ ovalbumin (OVA DQ) (LifeTechnologies) is a self-quenched dye that exhibits green fluorescence upon proteolytic digestion and red fluorescence when it accumulates at organelles at high concentration.[40] This mimics the process of antigen uptake and processing by antigen-processing cells. An OVA DQ assay was done by dissolving lyophilized OVA DQ in PBS without Ca^{2+} or Mg^{2+} for a final concentration of 1 mg/ml. Four microliter of such solution was added to 200 μl of cell suspension at 10^6 cells per milliliter and incubated for 60 min at 37°C 5% CO_2. The negative control was incubated at 4°C 5% CO_2. After 1 h, 3 ml of FACS media was added. Collected cells were spun down, resuspended in 200 μl of FACS media, and 5 μl of Tryptan Blue (LifeTechnologies) to quench any nonspecific binding of OVA DQ to the cell surface. Data for antigen processing were analyzed on the BD FACSCalibur at FL1 (505 nm) and FL3 (630 nm) for assessment of antigen uptake and antigen processing, respectively. Data are presented as mean fluorescent intensity.

Zymosan A *Saccharomyces cerevisiae* BioParticles®*FITC (Lifetechnologies) were used to study phagocytic properties of the cells. In brief, zymosan was reconstituted at 20 mg/ml in PBS without Ca^{2+} or Mg^2 and opsonized as per manufacturer's recommendation. A total of 10^5 cells were incubated at 0.5 μg/ml with zymosan particles for 30 min at 37°C 5% CO_2. The negative control was incubated at 4°C 5% CO_2. After incubation, cells were washed three times and subjected to flow cytometry.

Cells were analyzed with an LSR (BD) or an FACSCalibur (BD). Both flow cytometers are maintained by the University of Pennsylvania Flow Cytometry Core. Appropriate compensation settings were established using antigen captured by AbC Anti-Mouse Bead (Invitrogen). At least 10^4 cells were collected for each assessment. Cells of interest were gated based on their forward and side scatters to remove duplets or dead cells.

Measurement of Cell Toxicity and Apoptosis

Cells were harvested after 24 h and suspended in X-VIVO15 media. A 10-mM stock solution of Mitotracker Red® dye (LifeTechnologies) was prepared by dissolving it in a culture-grade dimethyl sulfoxide (Fisher Scientific, USA). The working solution of the Mitotracker Red was prepared by diluting the stock solution in X-VIVO15 to a concentration of 10 μM. For the assay, 4 μl of working solution of the Mitotracker Red was added to 1 ml of solution containing 5×10^5 cells and incubated for 30 min at 37°C 5% CO_2. Cells were washed and resuspended in the annexin-binding buffer (10 mM HEPES, 2.5 mM $CaCl_2$, 140 mM NaCl; pH = 7.4) and incubated with 5 μl of Annexin V*FITC solution (LifeTechnologies). Cells were incubated at room temperature in the dark for 15 min. After that the annexin incubation buffer was added and cells were kept on ice and immediately analyzed through flow cytometry. Cell fluorescence emission was measured at 530 and 585 nm, and these results were plotted against each other. Live cells were located in the upper left quadrant, whereas early apoptotic cells exhibited a right downward shift.

Five microliter of 1 mg/ml of 7-aminoactinomycin D (7AAD) solution (Sigma-Aldrich, USA) in PBS was added to 10^5 cells suspended in 100 μl of FACS media. Cells were incubated for 30 min at 4°C in the dark. Cells were immediately analyzed on a flow cytometer by plotting the cells on the dot plot with forward scatter *versus* 670-nm bandpass filter. Apoptotic cells showed a right shift. A positive control for the experiment was obtained by incubating THP-1 cells line in 5 μM camptothecin (Tocris) for 6 h at 37°C 5% CO_2 to induce apoptosis. Cells were then washed and processed for the aforementioned apoptosis tests.

Finally, we assessed cell count and viability using Tryptan Blue. In brief, 200×10^3 cells suspended in 20 μl of PBS were mixed with an equivalent volume of 0.04% Tryptan Blue. Fifteen microliter of cell suspension was loaded onto hemocytometer cassette. Cell count and viability were measured using an automatic counting machine using a Countess® Automated Cells Counter (LifeTechnologies).

Mixed Lymphocyte Reaction

A one-way mixed lymphocyte reaction (MLR) was used in this study; 2×10^4 of harvested cells (IL-4&GM-CSF-stimulated MO; stimulators) were added to a 96-well plate. Responder allogeneic T cells were obtained from the Human Immunology Core at the University of Pennsylvania from an anonymous donor, and 2×10^5 T cells were added to the antigen-presenting cells and incubated for 1 h. Twenty microliter of Alamar Blue (LifeTechnologies) was subsequently added. Three sets of controls consisted of stimulator cells, T cells alone, and media mixed with Alamar Blue alone. After 18 h of incubation, the level of absorbance was measured using the Opsys MR (Thermo Laboratories, USA) with Revelation software (Thermo Laboratories, USA). The absorbance at 570 nm (with a reference filter at 630 nm) was measured. The degree of Alamar Blue reduction was calculated per manufacturer's specification (Invitrogen). It has been shown previously that the degree of reduction correlates well with T-cell proliferation.[41]

High-performance Liquid Chromatography Assessment of Ketamine Concentrations

Measurement of supernatant levels of ketamine was done through the modification of an existing protocol.[42] The chromatographic system consisted of a variable wavelength ultraviolet detector operated at 205 nm, a Type U6K injector equipped 500-μl sample loop and ultrasphere reverse-phase column (4.6 mm × 250 mm) (Beckman Coulter, USA). All high-performance liquid chromatography were operated by the 32 Karat 7.0 System (Beckman Coulter). They were carried out at ambient temperature at a flow rate 1.0 ml/min with a mobile phase of a mixture of acetonitrile 46% and 20 μM NaH_2PO_4 54% containing 10 μM sodium lauryl sulfate.

Cytokine Measurements

Supernatant level of cytokines (TNFα, IL-6, and IL-10) was measured using a magnetic multiplex kit (Millipore, USA) specifically customized to our needs. Cytokines were prepared following the manufacturer's protocol and analyzed on the Bio-Rad platform (USA).

Enzyme-linked immunosorbent assays were performed for M-CSF (R&D, USA), PGE$_2$ (Cayman Chemical), and TGFβ (eBioscience, USA) following their manufactures' protocols. Enzyme-linked immunosorbent assays were read using the Opsys MR (Thermo Laboratories) with Revelation software.

IRB Approval

Because all the cells were procured from the Human Immunology Core at the University of Pennsylvania, no separate consent was needed for this study. Human Immunology Core affiliated with the University of Pennsylvania is operating under a permit issued by the IRB at the University of Pennsylvania.

Statistical Analysis

No *a priori* statistical power calculations were conducted. The initial sample sizes were based on previous experience. Additional experiments and samples were added by request during the peer-review process. Each experiment was done at least six times using cells obtained from six different donors.

Initially, a descriptive analysis of the data was performed. Parametric data are presented as mean and SD (X ± SD), whereas nonparametric data are presented as median and interquartile ranges (M$_e$ ± IQ). Lavene and Shapiro–Wilk tests were performed to evaluate the parametric characteristics of the data. The two-group comparison *t* test and Wilcoxon matched pair test were conducted for data with parametric and nonparametric characteristics, respectively. ANOVA was conducted for multiple group comparisons. Bonferroni test was used for *post hoc* analysis. The data were flagged as significant if two-tailed hypothesis was significant at P value less than 0.05, unless otherwise specified in the article. Statistica v8.0 (Statistica, USA) was used for all analyses.

Results

Ketamine Causes Significant Dose-dependent Diminution in the Differentiation of IL-4&GM-CSF-stimulated MO into iDCs

In our first set of experiments, we added ketamine at varied concentrations to IL-4&GM-CSF-stimulated MO for 5 days. Our observations strongly suggested that ketamine has an inhibitory effect on the differentiation of MO into DC under the influence of IL-4&GM-CSF in a dose-depended manner (fig. 1). We noticed that ketamine concentrations of 10 μg/ml, or higher, added at the beginning of stimulation of MO with IL-4&GM-CSF significantly suppressed the expression of CD1a, an early marker for iDC (fig. 1, A and B).[22,26,36] There was some interindividual variability among the subjects, especially at a dose of 10 μg/ml (fig. 1B). A dose of 1 μg/ml of ketamine had no effect on MO→iDC.

In the next step, we assessed the functional features of IL-4&GM-CSF MO costimulated with 100 and 10 μg/ml of ketamine with respect to their DC-like functions.[23,26] First, we tested their ability to capture, and to process, an antigen.[22,26,36] To this end, we used the DQ ovalbumin assay. It is a synthetic protein that antigen-presenting cells can uptake and digest similarly to a pathogen being processed by DCs. As the DQ ovalbumin is digested by DC, its fluorochrome is unquenched and assembled, causing increased signal in FL1 (uptake and initial processing) and FL3 (late processing and postuptake modification) channels, respectively.[36] Accordingly, we found that ketamine costimulated with IL-4&GM-CSF MO has a significantly diminished ability to perform both antigen uptake and processing functions at both effective concentrations (fig. 1, C and D). In another experiment, we mixed IL-4&GM-CSF MO with allogeneic T cells, performing a classical MLR. The degree to which Alamar Blue is reduced is directly proportional to the ability of antigen-presenting cells to stimulate T cells. Here, we found that adding at least 10 μg/ml of ketamine reduces the ability of IL-4&GM-CSF-treated MO to reduce Alamar Blue by more than 50%, more than those observed in IL-4&GM-CSF MO alone (M$_e$ [Reduction of Alamar Blue]; MO$_{IL-4\&GM-CSF}$ = 31.6%, MO$_{IL-4\&GM-CSF+ket\,100}$ = 15.6%, MO$_{IL-4\&GM-CSF+ket\,10}$ = 21.6%, MO$_{IL-4\&GM-CSF+ket\,1}$ = 26.7%; $P < 0.05$).

Culturing IL-4&GM-CSF-treated MO with Ketamine Induces the Emergence of Atypical MO while Retarding Differentiation of iDC or Macrophage-like Cells

It is possible that observed ketamine-induced blockade of MO to iDC differentiation is related to an accelerated differentiation of MO into mDC (mDC) because mDC exhibit diminished expression of CD1a.[23,32,34,36] Therefore, we analyzed the expression of CD83, a selective marker for mDC. This marker was diminished as well after 5 days of culture (%CD83$^+$ MO$_{IL-4\&GM-CSF}$ = 48.7 ± 27.92% *vs.* MO$_{IL-4\&GM-CSF+ket100}$ = 29.6 ± 24.93% *vs.* MO$_{IL-4\&GM-CSF+ket10}$ = 33.6 ± 12.31%; $P < 0.036$).

Next, we investigated whether ketamine induces macrophage-like characteristics in IL-4&GM-CSF-treated MO. The expression of MO-specific marker CD14 was statistically elevated only in the MO treated with 100 μg/ml of ketamine (fig. 2). The persistence of CD14 on IL-4&GM-CSF+Ket100-stimulated MO suggests that adding ketamine at the highest dose preserves CD14. Because CD14 is present on the surface of macrophages/MO, we further investigated the flow cytometric properties of 4&GM-CSF+Ket100-stimulated MO.[21,24,41] However, the surface density of CD86 was not changed on the surface of IL-4&GM-CSF MO in the presence of ketamine (mean fluorescent intensity CD86
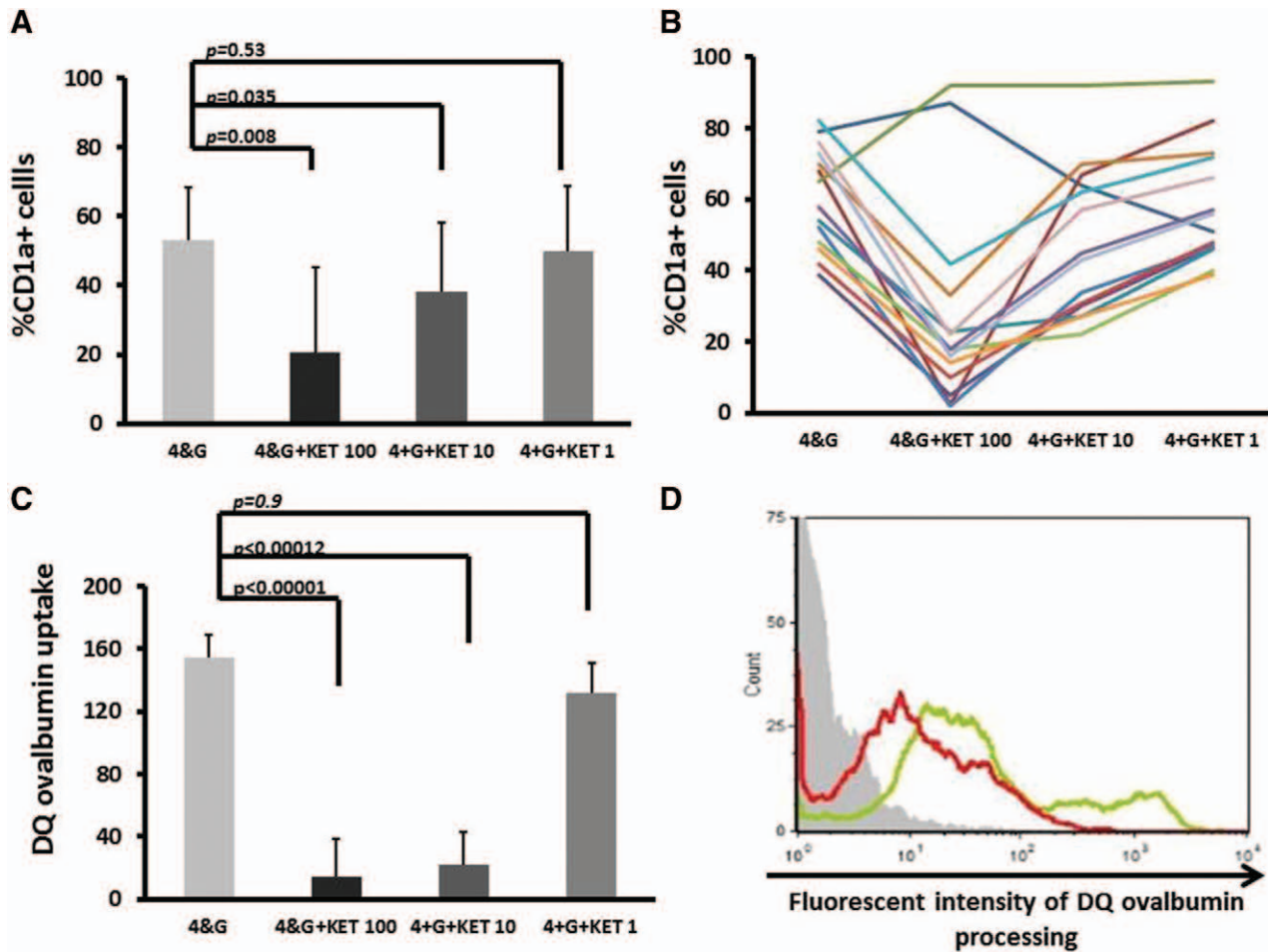
Fig. 1. Addition of ketamine reduces the percentage of CD1a-positive cells in interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF)-cultured monocytes (MOs) after 5 days of incubation, suggesting diminished rate of differentiation of monocytes into immature dendritic cells (*A* and *B*). (*A*) Shows the aggregated data from all experiments (n = 133). (*B*) Shows the data in which all four experimental groups were set up at the same time (n = 14). Concomitantly, ability of IL-4&GM-CSF-differentiated MO to uptake (*C*) and process (*D*) antigen is severely affected if ketamine was present during their differentiation process, confirming that ketamine prevents acquisition of typical feature for dendritic cells by IL-4&GM-CSF-stimulated MO (*green line* = IL-4&GM-CSF alone; *red line* = IL-4&GM-CSF + ketamine; *gray histogram* = negative control). 4&G = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 µg/ml stimulated; 4&G+Ket10 = interleukin-4 and granulocyte macroph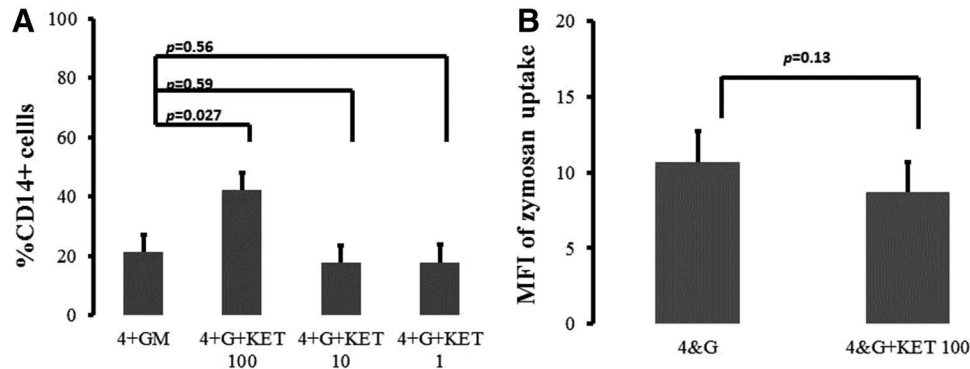age colony-stimulating factor + ketamine 10 µg/ml stimulated; 4&G+Ket1 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 1 µg/ml stimulated.

$MO_{IL\text{-}4\&GM\text{-}CSF}$ = 191.7±64.52%, $MO_{IL\text{-}4\&GM\text{-}CSF+ket100}$ = 211.5±96.64; P = 0.54). Because macrophages are unique cells with phagocytic activity, we measured how ketamine can affect IL-4&GM-CSF-stimulated MO with respect to phagocytosis.[24,26,43,44] To study the phagocytosis, we incubated cells with fluorescently marked zymosan particles. Again, ketamine addition to IL-4&GM-CSF-stimulated MO resulted in decreased uptake of zymosan particles at both concentrations (data not shown). Finally, we measured the expression of CD206, a specific marker expressed on the surface of alternatively activated type 2 macrophages.[26,43] The expression of this marker was not elevated when ketamine was added to the cultures (%CD206 $MO_{IL\text{-}4\&GM\text{-}CSF}$ = 9.7±8.19%, $MO_{IL\text{-}4\&GM\text{-}CSF+ket100}$ = 9.5±10.56; P < 0.43).

This suggests a similarity between ketamine-exposed IL-4&GM-CSF-stimulated MO and atypical MO.[23,26,43]

These data, taken together, suggest that ketamine retards the ability of MO to differentiate into immature or mature DC. At the same time, ketamine-costimulated IL-4&GM-CSF MO do not resemble "classical" or alternatively activated macrophages.[13,19,24,26,43]

Impaired Differentiation of MO to DC in the Presence of Ketamine Is Not an Effect of Increased Apoptosis and/or Diminished Numbers of Precursor MO

Ketamine can activate apoptosis *via* NMDA activation.[11,18,45] This can significantly hamper the ability of MO to differentiate into DC by eliminating the pool of precursors.[23,25,26]

Fig. 2. Addition of ketamine to interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF)-cultured mono-cytes results in perseverance of monocyte marker—CD14 (*A*). However, phagocytic activity of IL-4&GM-CSF-cultured mono-cytes stimulated with ketamine during differentiation process remains unchanged (*B*). 4&GM = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 µg/ml stimulated; 4&G+Ket10 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 10 µg/ml stimulated; 4&G+Ket1 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 1 µg/ml stimulated; MFI = mean fluorescent intensity.

Also, ingestion of apoptotic bodies facilitates the emergence of macrophages, which in turn can retard the MO→iDC process.[25,26,43] Thus, we measured the level of apoptosis by comparing early (Mitotracker), middle (Annexin V), and late (7AAD) markers of inducible programmed death.[46,47] However, we did not observe that ketamine induces an early or late apoptosis process in 24-h windows as visualized by combined staining with Mitotracker and Annexin V (fig. 3, A and B) and 7AAD (fig. 3C), respectively.

Furthermore, we measured the frequency of precursor CD11c-positive MO after 24 h of stimulation of MO with IL-4 and GM-CSF. The frequencies were comparable across all studied groups and at different concentrations of ketamine (%CD11c$^+$$_{IL-4\&GM-CSF}$ = 92+4; CD11c$^+$$_{IL-4\&GM-CSF\&ketamine\ 100}$ = 89+4; *P* = 0.34).

In addition, we analyzed cell recovery and viability at 24 h, 3 days, and 5 days between different concentrations of ketamine with the reference point of cells stimulated with IL-4&GM-CSF alone. No differences in the percentage of recovered cells (table 1), or cells stained with Tryptan blue (table 2), were seen at any time point or with any stimulant. These data suggest overwhelmingly that ketamine does not induce apoptotic and/or necrotic processes when added to IL-4&GM-CSF-costimulated MO.

NMDA Plays a Role in Ketamine's Effect on MO Differentiation into DC

There are two main mechanisms by which ketamine exerts the bulk of its biological effect. One of the most prominent is an NMDA antagonism.[11] Ketamine is also a weak agonist toward µ- and σ-opioid receptors.[9–11] Stimulation of these receptors can potentially affect MO activation and differentiation.[23,26,48,49]

In the first round of experiments, we showed that addition of NMDA antagonist MK-801 mimics the effect of ketamine.[18,50] It was previously reported that MK-801 at 100

µM had an effect on osteoclast formation, a remote offspring of MO.[51] In our experiments, when MK-801 was added at a concentration of 100 or 10 µM (data not shown), a significant suppression of CD1a expression was seen (fig. 4). However, the addition of NMDA (100 µM) only partially prevented ketamine from interfering with the emergence of iDC from MO (fig. 4). This suggests that NMDA antagonism is the mechanism implicated in the effect of ketamine on MO differentiation.

Ketamine is also a weak opioid receptor agonist. To test the effect on µ- and σ-receptors, we added fentanyl to our colonies. Fentanyl addition did not diminish the expression of CD1a after differentiating MO with IL-4 and GM-CSF (data not shown).

The Change in the Expression Pattern of Cytokine Receptors Is Not Responsible for the Inhibitory Effects of Ketamine on MO to DC Conversion

A potential explanation for this finding is that ketamine affects the expression of critical receptors, or cytokines, involved in the differentiation fate of MO working *via* an NMDA-dependent mechanism.[10,11,18,22–24,26,36,45] We measured the expression of CD124 (IL-4R), CD116 (GM-CSFR), CD126 (IL-6R), CD115 (M-CSFR), and CD210 (IL-10R) after 24 h of cultures. Only the surface density of CD116 was up-regulated by the ketamine at a dose of 100 µg/ml (*P* = 0.021) (table 3). However, the expression of the receptor for IL-4 (IL-4R; CD124), which is pivotal for the emergence of DC, was unchanged in the terms of receptor density and percentage of positive cells.[22–24,26,36,52] Because overexpression of GM-CSFR may interfere with the ability of IL-4R to induce differentiation of MO into DC, we measured the expression of CD209, a surface marker that is triggered by the influence of IL-4.[24,26,52] Therefore, CD209 is a convenient measure of the biological effect of IL-4 on the cells.[52] Expression of CD209 was
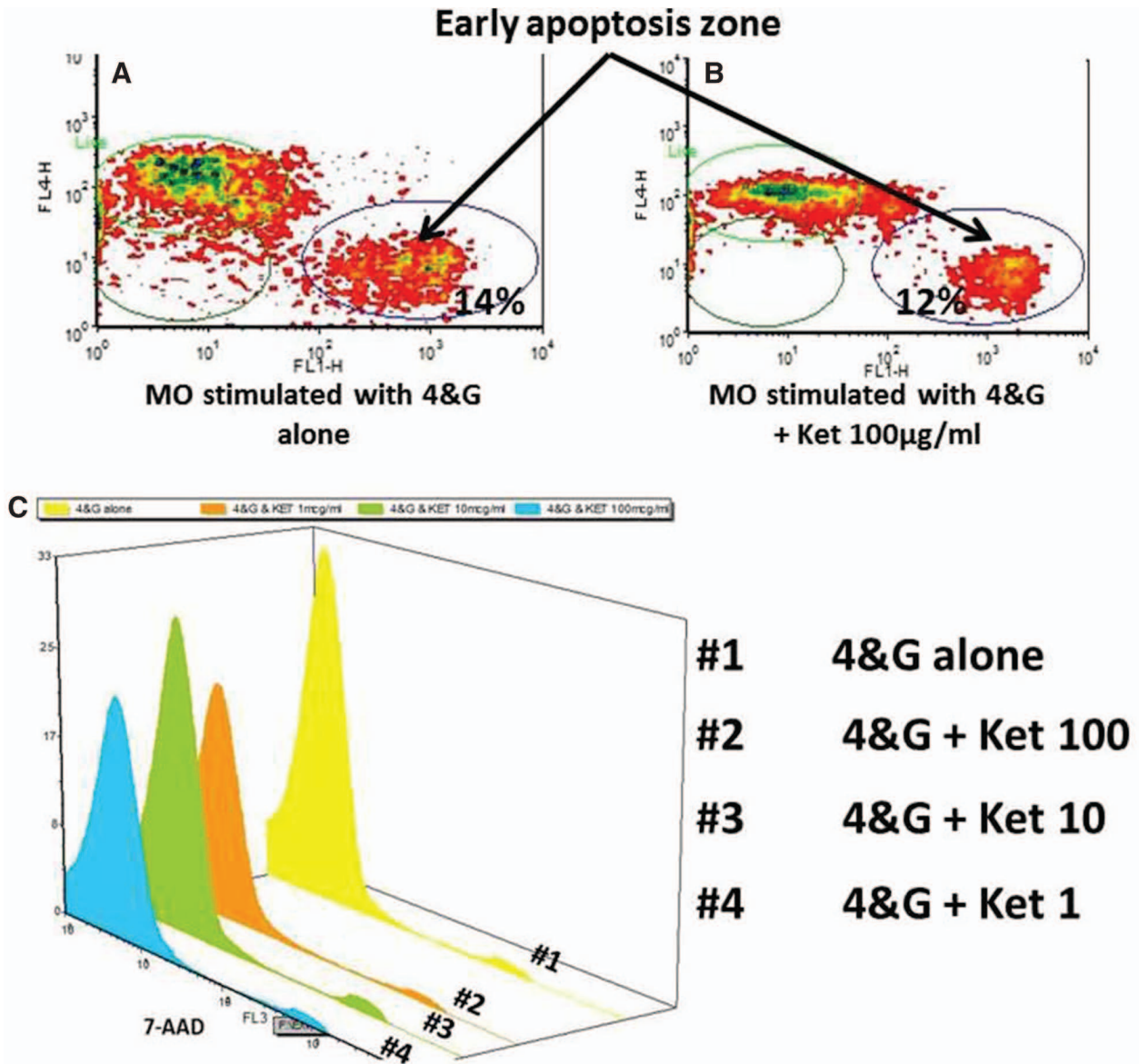
Fig. 3. Approximately 14% of cells had early marker of apoptosis showed by decreased fluorescence of the Mitotracker dye (FL4) and elevated fluorescence of Annexin V (*A*). Addition of ketamine did not induce significant increase in percentage of cells in the early apoptosis zone (*B*). Similarly, no increased percentage of 7-aminoactinomycin D (7AAD) fluorescence was seen in interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF)-cocultured monocytes (MO) regardless the concentration of ketamine (*C*). 4&G = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 µg/ml stimulated; 4&G+Ket10 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 10 µg/ml stimulated; 4&G+Ket1 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 1 µg/ml stimulated.

Table 1. Cells Recovery (Defined as a Percentage of Original Number) at 3 Days of IL-4&GM-CSF Cultures

	24 h (%)	3 Days (%)	5 Days (%)
4&G	91 ± 15.26	72.4 ± 12.82	81.1 ± 13.39
4&G + Ket 100	78.7 ± 11.6	83.3 ± 25	81 ± 12.93
4&G + Ket 10	81.8 ± 13.32	80.4.6 ± 14.52	84.1 ± 17.69
4&G + Ket 1	80.1 ± 7.84	83.8 ± 12.74	88.1 ± 20.37

Values are average ± SDs of percentage of cells remained after initial seeding of the cells.

4 = interleukin-4; G = granulocyte–monocyte colony-stimulating factor; IL-4&GM-CSF = interleukin-4 granulocyte–monocyte colony-stimulating factor; ket 100 = ketamine at 100 µg/ml; ket 10 = ketamine at 10 µg/ml; ket 1 = ketamine at 1 µg/ml.

Table 2. Cell Viability at 3 Days of IL-4&GM-CSF Cultures

	24 h (%)	3 Days (%)	5 Days (%)
4&G	90 ± 4.58	86 ± 3.24	89.9 ± 5.18
4&G + Ket 100	78.6 ± 11.6	88.7 ± 2.01	87.2 ± 5.67
4&G + Ket 10	81.9 ± 13.3	85.6 ± 3.36	80.4 ± 14.52
4&G + Ket 1	80.1 ± 7.84	84.5 ± 5.69	83.8 ± 12.74

Values are average ± SDs of percentage of cells remained after initial seeding of the cells.
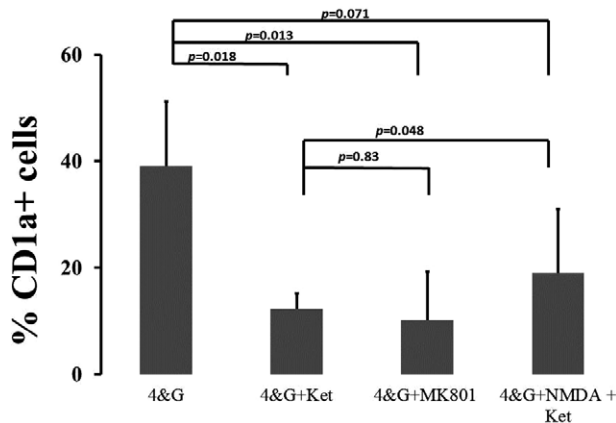
4 = interleukin-4; G = granulocyte–monocyte colony-stimulating factor; IL-4&GM-CSF = interleukin 4 granulocyte–monocyte colony-stimulating factor; ket 100 = ketamine at 100 µg/ml; ket 10 = ketamine at 10 µg/ml; ket 1 = ketamine at 1 µg/ml.

Fig. 4. An addition of nonspecific *N*-methyl-D-aspartate (NMDA) antagonist mimics the effect of ketamine on interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF)-stimulated monocytes (MOs). Coculturing the MO in the presence of ketamine induces partial reversal of the ketamine-mediated block of MO to immature dendritic cells differentiation as compared with IL-4&GM-GM-CSF alone. Partial reversal by NMDA was statistically significant if one one-tail hypothesize was formulated during comparison to 4&G+Ket 100. 4&GM = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine stimulated; 4&G+MK-801 = interleukin-4 and granulocyte macrophage colony-stimulating factor + MK-801 stimulated; 4&G+NMDA+Ket = interleukin-4 and granulocyte macrophage colony-stimulating factor + *N*-methyl-D-aspartate receptor + ketamine stimulated.

the same across all groups when the data were analyzed at day 1 or 5 (data not shown), suggesting that IL-4 influence is not opposed by GM-CSFR's effect on MO to DC differentiation.

Induction of TGFβ Production Is the Mechanism for Ketamine-mediated Interference in IL-4&GM-CSF-mediated MO Differentiation into DC

In the next step, we hypothesize that ketamine induces cytokines that interfere with the process of MO differentiation into DC. However, the levels of IL-6 and TNFα were not significantly elevated by ketamine culture supernatants (table 4).[25,27,28] In the second step, we analyzed the expression of immunoinhibitory cytokines that interfere with the MO to iDC process.[43] Although the supernatant levels of PGE_2 and IL-10 were not elevated, we noticed a marked and dose-dependent effect of ketamine on TGFβ (table 4). We hypothesize that this was a dose-dependent effect given the increase in level of this cytokine that was seen between 10 and 100 µg/ml. We also measured the levels of TGFβ in the 5-day supernatants after stimulating MO with different NMDA antagonist and agonist. We found that addition of an NMDA antagonist (MK-801) to IL-4&GM-CSF-cultured MO resulted in a statistically significant dose-dependent increase in supernatant TGFβ, whereas addition of NMDA resulted in a moderated increase in this cytokine ($TGFβ_{\text{IL-4\&GM-CSF alone}}$ = 17.2 + 6.55 pg/ml; $TGFβ_{\text{IL-4\&GM-CSF+ket 100}}$ = 84.7 + 21.1 pg/ml; $TGFβ_{\text{IL-4\&GM-CSF+MK-801 [100 µM]}}$ = 78.2 + 6.7 pg/ml; $TGFβ_{\text{IL-4\&GM-CSF+MK-801 [10 µM]}}$ = 38.4 + 17.87 pg/ml; $TGFβ_{\text{IL-4\&GM-CSF+NMDA}}$ = 21.9 + 4.07 pg/ml). The increase of TGFβ was confirmed by flow cytometry assessment of expression of latency associated peptide (LAP)/TGFβ on the surface of the IL-4&GM-CSF-costimulated MO after 5 days (fig. 5, A and B).[53] MK-801 triggered an increased in the surface density of LAP/TGFβ as well, even at a concentration of 10 µM (fig. 5, A and C).

Because TGFβ has a profoundly inhibitory effect on the emergence of DC from MO, we conducted an experiment with the addition of neutralizing antibody to TGFβ (FN TGFβ).[43,54] Addition of FN TGFβ resulted in total restoration of the ability of IL-4&GM-CSF to induce differentiation of MO into iDC. This was shown by reestablishment of %CD1a^{+} level in IL-4&GM-CSF-stimulated MO (fig. 6A). Also, the functional properties of DC were restored, as shown by MLR and OVA DQ assays (fig. 6, B and C). Isotype control antibody had no effect on ketamine-induced deterioration in IL-4&GM-CSF-induced MO differentiation. Supernatant TGFβ was low in the IL-4&GM-CSF cultures if FN TGFβ was present ($TGFβ_{\text{IL-4\&GM-CSF alone}}$ = 17.2 + 6.55; $TGFβ_{\text{IL-4\&GM-CSF+ket 100}}$ = 84.7 + 21.1; $TGFβ_{\text{IL-4\&GM-CSF+ FN TGFβ + ket 100}}$ = 13.7 + 5.53; $P < 0.005$).

Binding of TGFβ to its receptors triggers activation of Smads, p38, and Erk followed by translocation of nuclear factor κ-light-chain-enhancer of activated B cells.[53] In contrast to other kinases, Smad are specifically activated by engagement of TGFβ by its ligand. Therefore, we decided to use a specific inhibitor of Smad binding to TGFβ—SB431542.[53,54] If this compound was added to the supernatants before ketamine, it almost completely abrogated the effects of ketamine on differentiation of MO into iDC. In the cultures with SB431542, there was almost complete recovery of CD1a, ability to stimulate T cells, and antigen processing (fig. 7). In contrast, Erk ½ kinase inhibitor (PD35901) did not interfere with the MO→iDC process (data not shown).

Table 3. Expression of Critical Receptors for Monocytes Differentiation in Terms of Positive Cells and Receptor Density

	IL-4&GM-CSF Alone	IL-4&GM-CSF + ket 100 µg/ml	IL-4&GM-CSF + ket 10 µg/ml	IL-4&GM-CSF + ket 1 µg/ml	Significance
% CD124	65±34.2	72±25.2	66±25.8	72±30.2	ns
% CD116	86±16.0	89±12.4	86±14.5	88±16	ns
% CD126	90±17.4	89±14.8	89±15.7	90±14.9	ns
% CD115	82±21.3	80±17.7	77±24.5	77±26	ns
% CD210	72±37.4	77±34.8	62±35.7	61±34.3	ns
MFI CD124	68±33	75±24.8	69±24.6	74±28.9	ns
MFI CD116	*51 ± 18*	*64 ± 20.9*	47±19.4	40±15.9	0.01
MFI CD126	56±21.2	47±18.3	51±20.9	62 ± 31.3	ns
MFI CD115	72±42.4	69±38.9	67±33.8	78±48.4	ns
MFI CD210	42±19.9	43±19.9	38±21.2	50±30.8	ns

Values are average ± SDs of percentage of cells remained after initial seeding of the cells. Entries in italics are statistically significant at $P = 0.048$.
CD = luster of differentiation; CD115 = M-CSF receptor; CD116 = GM-CSF receptor; CD124 = IL-4 receptor; CD126 = IL-6 receptor; CD210 = IL-10 receptor; GM-CSF = granulocyte–monocyte colony-stimulating factor; IL-4 = interleukin-4; ket = ketamine; MFI = mean fluorescent intensity; % = percent positive cells.

Table 4. Only the Concentration of the TGFβ Was Elevated in a Dose-dependent Manner in Supernatant of IL-4&GM-CSF-stimulated Monocytes if Ketamine Was Added

Part B	IL-4&GM-CSF Alone	IL-4&GM-CSF + ket 100 µg/ml	IL-4&GM-CSF + ket 10 µg/ml	IL-4&GM-CSF + ket 1 µg/ml	Significance
IL-6	33±36.4	35±35.3	24±38.5	24±23.3	ns
TNFα	115±80.1	76±42	73±24.6	36±22.7	ns
IL-10	25±29.6	21±26.3	10±14.1	6±5.9	ns
PGE₂	10±10.6	11±14	10±14.1	11±20.6	ns
TGFβ	15±6.31	100.9±21.1**	49.5±19.2*	10.8±1.94	0.0001

The supernatant levels of other cytokines were not elevated in statistically significant manner. Values are average ± SDs of percentage of cells remained after initial seeding of the cells.
*$P < 0.05$; **$P < 0.01$.
GM-CSF = granulocyte–monocyte colony-stimulating factor; IL = interleukin-4; ket = ketamine; PGE₂ = prostaglandin E2; TGFβ = transforming growth factor-β; TNFα = tumor necrosis factor-α.

Discussion

Our study shows the ability of ketamine to disrupt the differentiation process of MO→iDC *in vitro*. This was a dose-dependent effect because different concentrations of ketamine had varying effects on the degree of MO→iDC differentiation. Prior work by Ohta *et al.*[35] focused on the effects of ketamine on the mDC in mouse bone marrow progenitors. Similar to our work, they stimulated DC progenitors with 10.4 µg/ml (40 µM) ketamine and showed diminished frequency of mDC if ketamine was present. In contrast, our work focused on the early stages of human MO differentiation into iDCs. If that step is disrupted, maturation of DC cannot take place.[22–24,26,36] Therefore, our study provides a mechanism for their finding. Ohta *et al.* suggested that maturation block is due to the induction of the high endocytosis state at the level of iDC.[29,32,49] Our finding suggests that the blockade in the differentiation fate of MO is at the level of the earliest stages of MO→DC. The blockade of MO→iDC represents a cumulative effect of ketamine because the process of iDC emergence is time consuming. This blockade is not related to the emergence of macrophages, as evidenced by frequency of surface markers (CD206). In addition, phagocytic activity tends to be lower when ketamine was present, further suggesting that the ketamine-stimulated MO are not macrophages.[49]

One of the most important aspects of our study is its clinical applicability. The concentration of 10 µ/mg is similar to peak concentrations observed during anesthesia or continuous infusion of ketamine in certain clinical situations. Bolze *et al.*[42] shown that a bolus of 150 mg followed by short-term infusion of ketamine in intensive care unit patients can produce sustained levels of ketamine only slightly lower than the one used in our study. Others reported concentrations of serum ketamine slightly below our concentration.[55–57] No study investigated the serum concentration of ketamine after prolonged infusion. We believe that our concentration of ketamine is reasonable and clinically relevant. Furthermore, *in vivo* even lower concentrations of ketamine can exert their action on MO differentiation. Sepsis and surgery are accompanied by activation of the immune system.[9,24,26,30,33,34,39] In these clinical conditions, serum inflammatory products prime peripheral MO to become MO while prohibiting the emergence of DC. This may render MO to be even more sensitive to the inhibitory effects of ketamine. It is also worth mentioning that the concentration of ketamine used in this study has been used before for *in vitro* studies.[35,44]
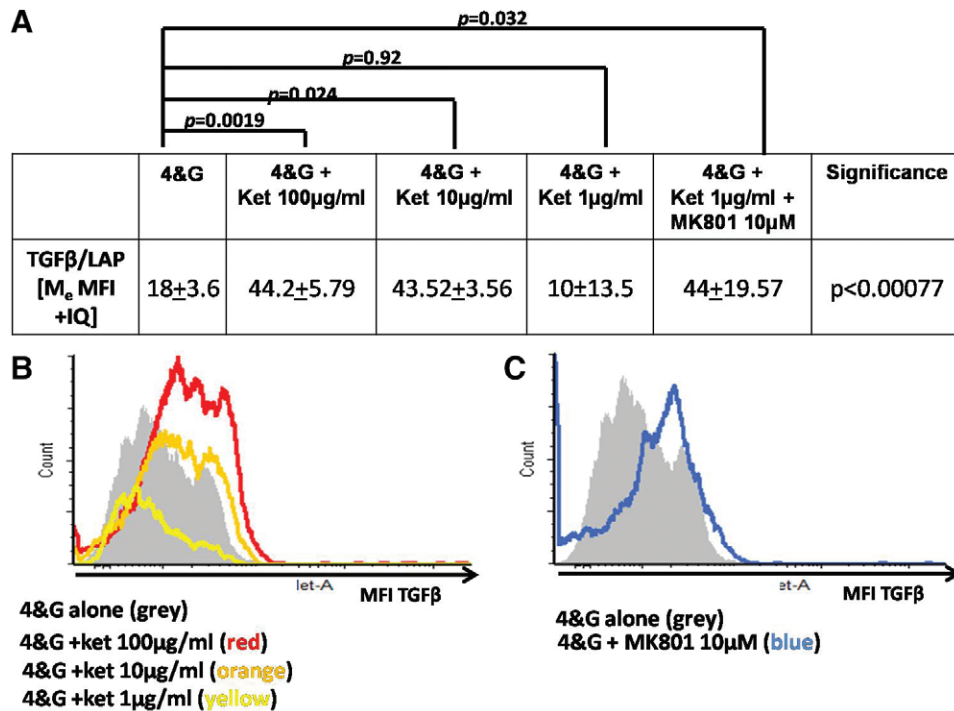
Fig. 5. An addition of ketamine causes dose-dependent increase in surface density of the latency associated peptide (LAP)/transforming growth factor (TGF)-β (*A* and *B*). Also MK-801, noncompetitive *N*-methyl-D-aspartate inhibitor, significantly increased LAP/TGFβ (*A* and *C*). IQ = interquartile; MFI = mean fluorescent intensity; 4&G alone = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 μg/ml stimulated; 4&G+Ket10 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 10 μg/ml stimulated; 4&G+Ket1 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 1 μg/ml stimulated; 4&G+MK-801 = interleukin-4 and granulocyte macrophage colony-stimulating factor + MK-801 stimulated.

The important aspect of clinical relevance of our finding is whether ketamine needs to affect MO for prolonged period of time to diminish the process of MO→iDC. Alternatively, ketamine could "prime" MO over short period of time and thus preventing them from becoming iDC even if the ideal cytokine environment is present. Similarly, sustained influence of both IL-4 and GM-CSF is critical while even a brief exposure to IL-6 or M-CSF interrupt MO differentiation into iDC by triggering acquisition of macrophage characteristic. If ketamine can affect MO differentiation over brief period of time, then it is possible that such effect can be exerted by giving ketamine during routine induction of anesthesia. However, in our study, we deliberately presented that our study is more relevant to clinical scenarios with sustained level of ketamine.

The mechanism of action for ketamine-induced MO→iDC inhibition *in vitro* is related to NMDA antagonism. Ketamine is a potent NMDA receptor antagonist.[2,11] Ketamine's effect on MO→iDC differentiation *in vitro* was mimicked by MK-801, a nonspecific NMDA receptor antagonist. Previously, ketamine was shown to interfere with the emergence of some peripheral macrophages.[51] Thus, NMDA antagonism seems to arrest MO development into peripheral offspring in general. L-NMDA, an NMDA receptor agonist, was able to reverse partial ketamine-induced

MO→iDC differentiation blockade, further supporting the pivotal role of NMDA receptors. The partial efficacy of NMDA may stem from the much shorter half life of NMDA *versus* ketamine or MK-801.

Initially, we speculated that the mechanism of ketamine action on MO to iDC differentiation will be an elevated secretion of the immunoinhibitory cytokines.[8,19,44,58] Indirect effect of ketamine on cytokines receptors, which are critical for the MO differentiation process, would be another plausible mechanism.[2,6,7,9,10,18,59,60] None of these hypotheses appears to be correct. However, an increase in TGFβ supernatant levels was observed when ketamine was added to the IL-4&GM-CSF-stimulated MO. Moreover, we showed that surface expression of LAP, an inactivated TGFβ attached to the cells, was elevated. This TGFβ can be released over time under certain conditions.[53] In addition, the LAP molecule itself has complex modulatory properties.[53] An increase in TGFβ influence is consistent with the functional and phenotypical characteristics of the ketamine-costimulated IL-4&GM-CSF MO observed in this study.[53] These cells do not express typical features of iDCs and have poor antigen-processing capability.[54] Also, their phagocytic abilities are not enhanced, suggesting that these cells are not macrophages. These findings
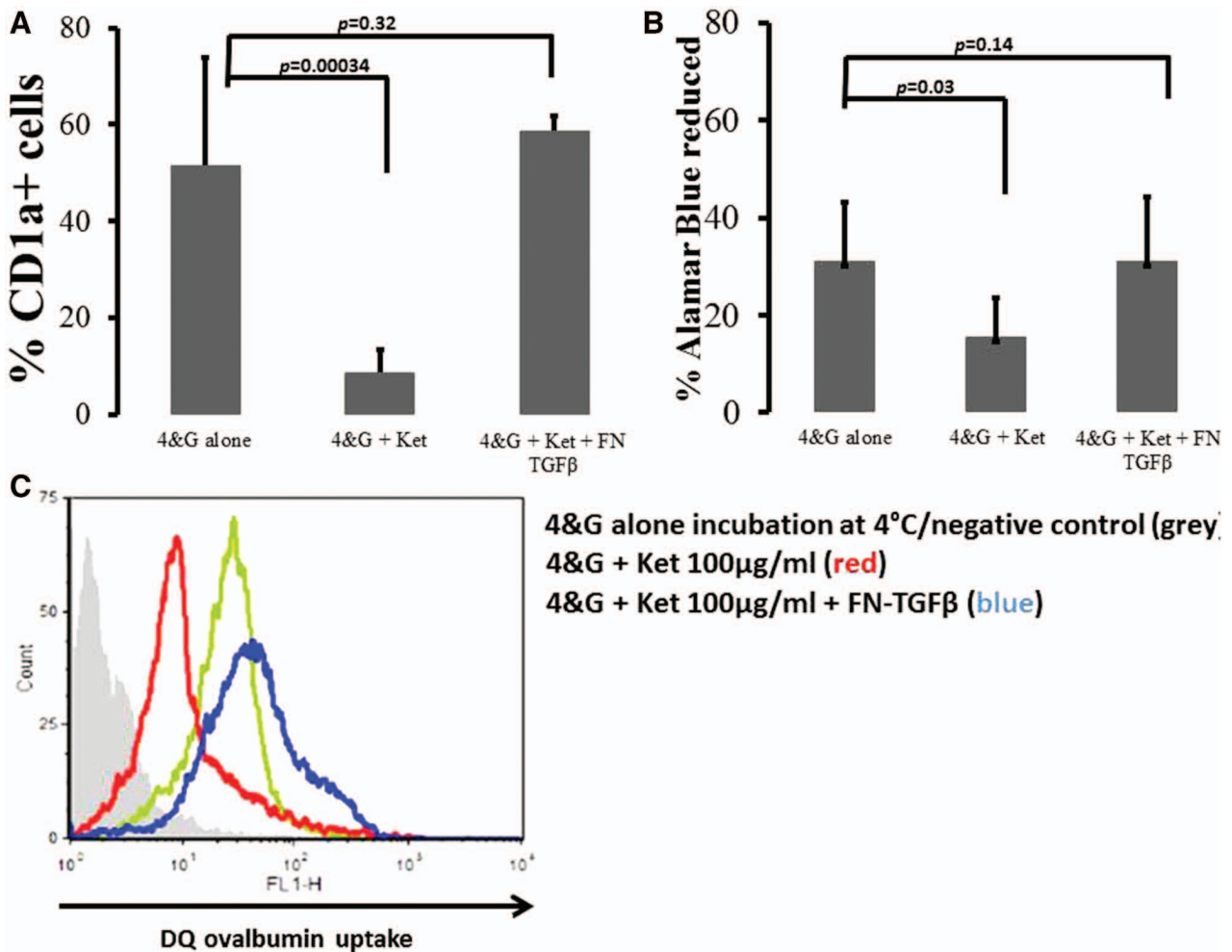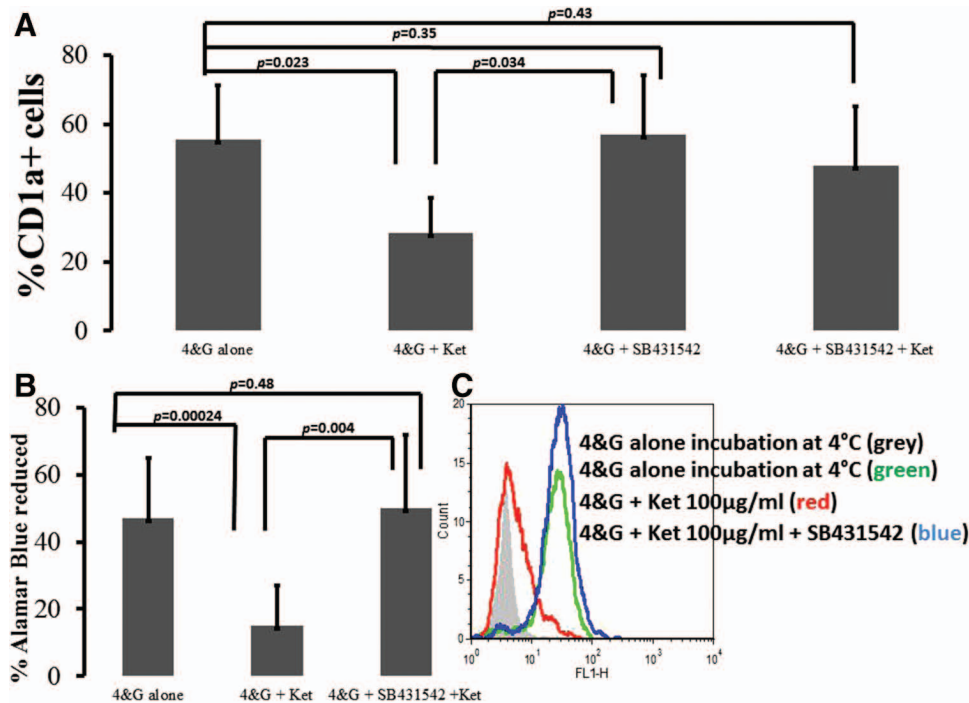
Fig. 6. An addition of neutralizing antibody for transforming growth factor-β (FN TGFβ) resulted in recovery of CD1a-positive marker in ketamine-costimulated monocytes (MOs) differentiated with interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF) (*A*). Also, FN TGFβ resulted in recovery of ability of MO differentiated with IL-4&GM-CSF to stimulate allogeneic T cells (*B*) and process antigen (*C*) (*green line* = IL-4&GM-CSF alone; *red line* = IL-4&GM-CSF + ketamine; *blue line* = IL-4&GM-CSF + FN TGFβ + ketamine, *gray histogram* = negative control). 4&G alone = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 μg/ml stimulated; 4&G+Ket100 + FN-TGFβ = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 μg/ml + neutralizing antibody for TGFβ stimulated.

are consistent with deactivated MO.[13,19,24,61] TGFβ was shown to deactivate MO or diminish their ability to stimulate T cells.[31,37,53,61] Ketamine did not induce nonspecific MO deactivation because TGFβ secretion was elevated.

Our study suggests that ketamine specifically triggers TGFβ secretion *via* a nonapoptotic, NMDA-related mechanism. Neutralizing ketamine-induced elevation in TGFβ alleviated the inhibitory influences of ketamine on the MO differentiation process. Ketamine triggers phosphorylation of the TGFβ-related Smad molecules.[53] Blocking the downstream effect of TGFβR/Smad with SB431511 abrogated the effect of ketamine.[53] It is unknown how the NMDA antagonism translated into increased secretion of TGFβ blocking the MO→iDC process. Some studies suggested that ketamine may induce cell death.[18,45]

This would be a very plausible explanation of our finding because ketamine-induced cell death and the subsequent ingestion of cell bodies by surviving cells can result in enhanced TGFβ production.[18,45,62] However, using several different tests, we showed that ketamine was not toxic at any concentration.

In summary, our study showed that ketamine disrupts the process of human MO differentiation into DC *in vitro via* induction of TGFβ. This study is the first demonstration of ketamine regulation of DC emergence in human subjects. We also showed for the first time the ability of MO to secrete TGFβ upon ketamine stimulation. Our study suggests that inducing immunomodulation *via* interference with the emergence of DC could contribute to the lack of long-term benefits of ketamine use in clinical situations.

Fig. 7. An addition of Smad inhibitor SB431542 resulted in recovery of CD1a-positive marker in ketamine-costimulated monocyte (MO) differentiated with interleukin-4 granulocyte–monocyte colony-stimulating factor (IL-4&GM-CSF) (*A*). Also, we observed recovery of ability of MO differentiated with IL-4&GM-CSF to stimulate allogeneic T cells (*B*) and process antigen (*C*) if SB431542 was added (*green line* = IL-4&GM-CSF alone, *red line* = IL-4&GM-CSF + ketamine; *blue line* = IL-4&GM-CSF + SB431542 + ketamine, *gray histogram* = negative control). 4&G alone = interleukin-4 and granulocyte macrophage colony-stimulating factor stimulated; 4&G+Ket100 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 μg/ml stimulated; 4&G+Ket100 + SB431542 = interleukin-4 and granulocyte macrophage colony-stimulating factor + ketamine 100 μg/ml + transforming growth factor-βR1 blocker (SB431542) stimulated.

Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Laudanski: Department of Anesthesiology and Critical Care, University of Pennsylvania, Dullas 780A, 3400 Spruce Street, Philadelphia, Pennsylvania 19104. krzysztof.laudanski@uphs.upenn.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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