J Pharmacol Sci **117**, 170 – 179 (2011)

Journal of Pharmacological Sciences

Full Paper

In Vitro and In Vivo Effects of Ketamine on Generation and Function of Dendritic Cells

Jingxian Zeng[1,*], Shuxuan Xia[1], Wa Zhong[2], Jie Li[1], and Liling Lin[1]

[1]*Department of Anesthesiology,* [2]*Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No. 107 Yanjiang Xi Road, Yuexiu District, 510210 Guangzhou, China*

Received June 26, 2011; Accepted September 26, 2011

Abstract. The question about how intravenous anesthetic reagents affect the development and function of dendritic cell subsets still has no comprehensive answers. Bone marrow cells differentiated with FMS-like tyrosine kinase 3 ligand in vitro represented the steady-state dendritic cell subsets. The effects of ketamine on the generation and function of dendritic cell subsets were investigated. We found that dendritic cell subsets responded to the anesthetic reagent ketamine in several aspects: 1) The in vitro and in vivo development of plasmacytoid dendritic cells were inhibited by ketamine at high concentrations; 2) The endocytosis of dendritic cells were not influenced by ketamine at concentrations from 50 – 200 μM; 3) The maturation markers of conventional dendritic cells were not changed by ketamine upon LPS or CpG stimulation, although the cytokines mRNA profiles were affected; 4) The allogenic-stimulatory activity of dendritic cells was suppressed by ketamine. In conclusion, ketamine hampered plasmacytoid dendritic cell subset development both in vivo and in vitro. The dendritic cells maturation and downstream responses towards different toll-like receptor stimuli were differently regulated by ketamine treatment.

Keywords: anesthesia, ketamine, dendritic cell, plasmacytoid dendritic cell, conventional dendritic cell

Introduction

Anesthetics and sedatives have been commonly used in stability management and safety control during surgical operation and clinical care. However, under circumstances of surgical trauma, pre-existing diseases, and possible infections, the immune system of patients may be easily vulnerable, so slight effects of anesthetics on the immune system may affect the immunological system, causing an undesirable aftermath (1, 2).

Among the intravenous anesthetic reagents, ketamine had relatively high clearance, which accounted for its short elimination half-life of less than 3 h in adults (3). However, this popular anesthetic chemical had effects on the immune system based on both in vivo and in vitro studies (4 – 10). Ketamine was supposed to be immunosuppressive due to its negative effects on the synthesis of inflammatory cytokines, cell adherence, and migration (11 – 13). In the mouse macrophage cell line, ketamine treatment significantly inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) mRNA synthesis (14).

Dendritic cells (DCs) are sparse although widely distributed in the body and recognized as the only professional antigen-presenting cell (APC) to stimulate naïve T cells. DCs are a mixture of heterogeneous cell subsets that can be divided into several categories with different functions and diverse distribution (15). DCs consist of plasmacytoid DCs (pDC); conventional DCs (cDC), which include migratory DCs and lymphoid tissue–resident DCs; and inflammatory DC that appear as a consequence of infection or inflammation and are not found in the steady state (15). When the body countered pathogens or cellular danger signals, the migratory immature DCs in peripheral tissues took up antigens and then migrated into lymph tissue to induce T cell immunity (16). The lymphoid tissue–resident DCs remained immature until they met antigens within lymphoid tissues and organs (17). Due to the elaborate procedure and low yield of

*Corresponding author. jingxian.zeng@yahoo.com
Published online in J-STAGE on October 29, 2011 (in advance)
doi: 10.1254/jphs.11113FP

isolating DC in vivo, the in vitro generation of DCs from bone marrow cells under the help of granulocyte macrophage colony stimulating factor (GM-CSF) or FMS-like tyrosine kinase 3 (Flt3) ligand, referred to as GM-DC and FL-DC, respectively, were frequently used in the functional study of DCs. Many studies supported the viewpoint that GM-DCs were similar to monocyte-derived inflammatory DCs, whereas FL-DCs were the equivalents of steady-state resident DC subsets in lymph organs and tissues (15, 18). GM-DCs and FL-DCs differed in several aspects, including origin, morphology, surface markers, cytokines secretion, migrating capacity, and stimulatory capacity (15, 19, 20). The in vitro cultured FL-DCs can be divided into pDC, equivalent $CD8^+$ cDC ($eCD8^+$ cDC), equivalent $CD8^-$ cDC ($eCD8^-$ cDC) subsets, which were equivalents to splenic pDC, $CD8^+$ cDC, $CD8^-$ cDC subsets (15). $CD8^-$ cDC resembled migratory DCs that were critical in antigen presentation, inflammatory cytokine synthesis, and T helper cell induction, whereas $CD8^+$ cDC played key roles in cross presentation, immune tolerance, and $CD8^+$ T cell activation (21 – 23). To trigger downstream activation and the maturation pathway, DCs expressed a set of toll-like receptors (TLRs) to recognize different pathogens and to mediate the downstream cascade (24). TLR expression patterns differed in DC subsets (25), in which pDC highly expressed TLR7 and TLR9 for type I interferon synthesis, while $CD8^+$ cDC expressed TLR3 and TLR9 for IL-12p40 expression (26, 27).

The direct effects of ketamine on DCs were rarely reported. In 2009, It was reported that ketamine modulated murine GM-DCs in cytokine production, maturation makers expression, and T helper 1 (Th1) cells polarization (5). Nevertheless, the effects of ketamine on lymphoid-resident DCs were not clear, and mouse FL-DCs developed from bone marrow cells offered a good platform to answer this question. Our study focused on the direct effects of ketamine on FL-DC subsets in vitro and in vivo, aiming to dissect the role of ketamine in DC development, endocytosis, TLRs-mediated maturation, and allogenic T cell stimulation.

Materials and Methods

Materials and animals

Ketamine hydrochloride solution was purchased from Gutian Pharmaceutical, Ltd. (Fujian Province, China). Phosphate-buffered saline (PBS) or saline buffer were used as the vehicle control for ketamine in this study. Specific pathogen free (SPF) C57BL/6 mice were purchased from the experimental animal center of Sunyatsen University (Guangzhou, China) and used in this study with the approval of the Animal Care Committee (ACC) of Sunyatsen University. Cell culture medium and other supplements were purchased from Invitrogen (Carlsbad, CA, USA). The Flt3 ligand was purchased from R&D systems (Minneapolis, MN, USA). Direct fluorescence-conjugated antibodies for flow cytometry were provided by eBioscience (San Diego, CA, USA) and BD Biosciences (San Jose, CA, USA). The RNA extraction kit was from QIAGEN (Hilden, German) and real-time reagents and kit was from TAKARA (Otsu).

Mouse FL-DCs culture

FL-DCs were generated as described (28) with minor modifications. The bone marrow cells of C57BL/6 mice (6 – 8-week-old) were harvested by flushing femurs and tibias with PBS. Red cells were hypotonically lysed, and the residual cells were washed and kept on ice until used. Cells were cultured at a concentration of $1.5 – 2 \times 10^6$ cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U of penicillin, and 100 μg of streptomycin (osmolity adjusted to 310 mOsm/l). The murine Flt3 ligand was added at the final concentration of 100 ng/ml. The culture lasted for 9 – 11 days at 37°C in a 10%-CO_2 incubator. Fluorescence-labeled mAbs to CD11c, CD45RA, CD24, and CD11b were applied to identify DC subsets. Ketamine was added into the culture at different time-points as indicated.

TLRs-mediated DC maturation and cytokines expression

Bone marrow cells were cultured with Flt3 ligand for 9 – 11 days. Ketamine was included in the final 48 h, and then LPS (100 ng/ml) (Sigma, St. Louis, MO, USA) or CpG 2216 (100 nM) (InvivoGen, San Diego, CA, USA) was included in the final 16 – 24 h. At day 11, the cells were subjected to flow cytometry analysis for CD80, CD86, CD40, and major histocompatibility complex class II (MHC II) expression in $CD11c^{high}CD45RA^-$ cells.

For cytokines assay, FL-DCs with 24-h treatment of ketamine were sorted in the BD Aira instrument with fluorescent antibody for CD11c and CD45RA. The sorted cDCs and pDCs were cultured at the density of 2×10^5/well in a 96-well plate in the presence of ketamine and LPS (100 ng/ml) or CpG (100 nM) for 6 h. Total RNAs were isolated from the cells with the RNeasy Mini kit (QIAGEN) following the manufacturer's instructions. Each RNA sample was subjected to Dnase I digestion. The mRNA was reverse-transcribed to cDNA for quantitative real-time PCR. The measurement of mRNA level of cytokine was performed on a thermal cycler instrument (ABI 7500). Sense and antisense primers were synthesized for housekeeping gene glyceralde-

hyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-TGAAGCAGGCATCTGAGGG-3′ and reverse, 5′-CGAAGGTGGAAGAGTGGGA-3′) and for target genes: interferon-beta 1 (IFN-β1) (forward, 5′-CCCTATGGAGATGACGGAGA-3′ and reverse, 5′-CTGTCTGCTGGTGGAGTTCA-3′); interferon-alpha 1 (IFN-α1) (forward, 5′-GAAGGACTTTGGATTCCCGC-3′ and reverse, 5′-GTCAGGGGAAATTCCTGCAC-3′); interferon-alpha 4 (IFN-α4) (forward, 5′-AGAAGGCTCAAGCCATCCTT-3′ and reverse, 5′-CTGCATCACACAGGCTTTGA-3′); TNF-α (forward, 5′-CGTCAGCCGATTTGCTATCT-3′ and reverse, 5′-CGGACTCCGCAAAGTCTAAG-3′); IL-6 (forward, 5′-AGTTGCCTTCTTGGGACTGA-3′ and reverse, 5′-TCCACGATTTCCCAGAGAAC-3′); interleukin-12 subunit p40 (IL-12p40) (forward, 5′-AGGTCACACTGGACCAAAGG-3′ and reverse, 5′-CTGGTTTGATGATGTCCCTG-3′).

DC endocytosis assay

At day 9, ketamine was added into FL-DC culture for 48 h and then collected for endocytosis assay. Briefly, 50 μl of FL-DC (2×10^5 cells) in 2% FBS-PBS were added into triplicate wells of 96-well U-bottom plates before adding fluorescein isothiocyanate (FITC)-dextran (40 kDa, Sigma) for a final concentration of 1 mg/ml. One plate was incubated at 37°C and the second one was incubated at 4°C for 30 min. Both plates were gently tapped every 5 – 10 min to ensure adequate mixing. After incubation, plates were washed with 2% FBS-PBS twice to remove excessive FITC-dextran, followed by cell surface markers staining and flow cytometry analysis. The geometric mean fluorescent intensity difference between 37°C and 4°C was considered as the result of antigen uptake.

Allogenic T cell response in mixed lymphocyte reaction (MLR) test

Allogenic $CD4^+$ T cells were purified from spleens of BALB/c mice using $CD4^+$-positive microbeads (#130-049-201; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. FL-DCs were treated with ketamine (100 μM) from day 9 to 11 and LPS (100 ng/ml) was added for the final 18 h. DCs were then treated with mytomycin C (25 μg/ml, 1 h) and adequate washing. The ketamine-treated or control DCs (1×10^5 cells) were co-cultured with 5×10^5 T cells for about 72 h with ^{3}H-thymidine incorporation for the final 18 h. The radioactivity was determined by a beta scintillation counter (Pharmacia, Uppsala, Sweden). TdR incorporation assay was performed in 96-well plates. For the cytokines release assay, a mouse Th1/Th2-plex kit (BMS820FF; Bender Systems, Novato, CA, USA) was used to measure cytokines in the supernatant of the DC–T-cell co-culture.

The effect of ketamine on plasmacytoid DC development in vivo

Four-week-old C57BL/6 mice (6 mice per group) received ketamine (8 mg/kg) or saline buffer injections intraperitoneally once a week for 4 weeks. At week 8, the mice were sacrificed and the splenic DCs were isolated by using a nycodenz gradient (29). Briefly, the spleen was harvested and digested with collagenase and Dnase I. Splenic DCs were enriched with Nycodenz gradient centrifugation (1.077 g/cm^3) and then stained for DC populations with CD11c, PDCA-1, CD8, CD4 fluorescent antitbodies.

Statistics

Statistics data for samples were calculated by using Student's *t*-test. When $P < 0.05$, the difference was considered statistically significant. All statistical analysis was performed by Prism software.

Results

The effects of ketamine on the early and late differentiation of FL-DCs in vitro

To investigate whether ketamine played a certain role in the generation of DC. Ketamine at different concentrations was added into the FL-DC culture at the early differentiation stage (day 2 – 6) and the very late differentiation stage (day 9 – 11). Since the half-life of ketamine is short (< 3 h) (30) and usual infusion time is no more than 48 h, we incubated FL-DCs with ketamine for no more than 96 h. As shown in Fig. 1A, bone marrow cell developed into $CD11c^+$ DCs in the presence of Flt3 ligand. The pDC subset ($CD45RA^{high}CD11c^+$) and cDC subset ($CD45RA^-CD11c^{high}$) became dominant (total > 90%) in FL-DC culture after 11 days. Ketamine did not affect the in vitro differentiation of both pDC and cDC subsets at concentrations lower than 200 μM; however, ketamine significantly suppressed the development of pDC at concentrations higher than 200 μM (Fig. 1B). This result was not due to the cytotoxicity effect since dead cells were excluded by propidium iodide (P.I.) staining. Although the differentiation of total cDC was not affected by ketamine, the subpopulations were diversely regulated. The surface markers for discriminating different DC subsets were adopted from a previous study (26). Ketamine negatively regulated the differentiation of $eCD8^+$ cDC ($CD45RA^-CD11c^{high}CD24^{high}CD11b^-$) population while promoted the development of $eCD8^-$ cDC ($CD45RA^-CD11c^{high}CD24^{lo}CD11b^+$) at concentrations higher than 100 μM (Fig. 1B). Another point worthy to mention was that ketamine hampered FL-DC survival at

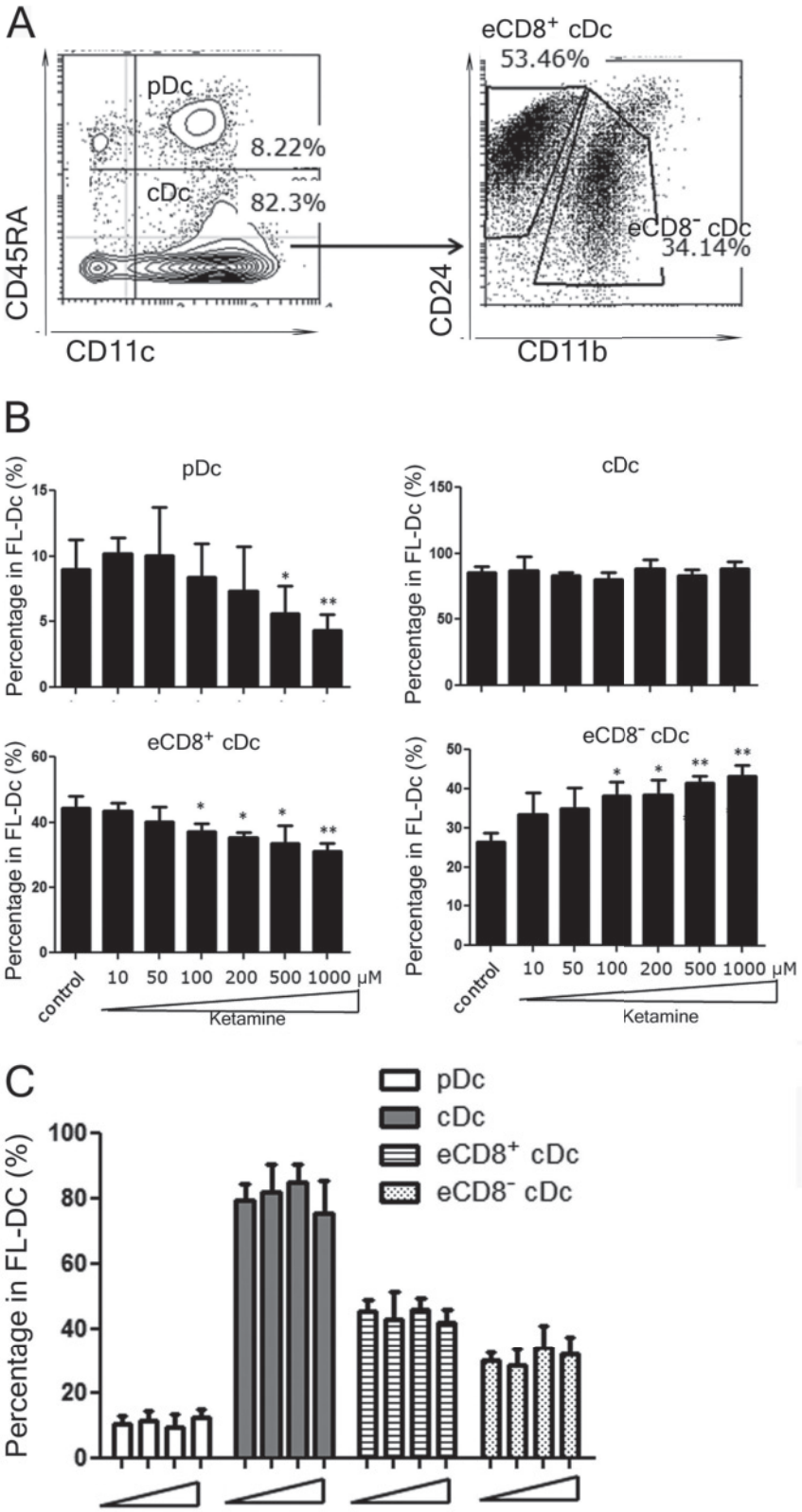

Fig. 1. The effect of ketamine on the in vitro development of FL-DCs. A) The FL-DCs was generated from mouse bone marrow cells using Flt3 ligand for 9 – 11 days in vitro. The DC subpopulations were analyzed by flow cytometry with surface markers CD45RA, CD11c, CD24, and CD11b to distinguish FL-DC subpopulations (pDC, cDC, eCD8$^+$ cDC, eCD8$^-$ cDC) in P.I. negative cells. B) The effect of ketamine on FL-DC subpopulations at the early differentiation stage is shown. Ketamine was included in the culture system from day 2 to 6 and then replaced with fresh culture medium. Data was analyzed at day 11. C) The effect of ketamine on FL-DC subpopulations at the very late differentiation stage is shown. Ketamine was included in the culture system from day 9 to 11 and FL-DC subsets were characterized by flow cytometry. The increaing concentrations (0, 50, 100, 200 μM) of ketamine was indicated (triangle symbol). *$P < 0.05$, **$P < 0.01$, compared to control. The data are representative of three independent experiments.

the concentration of 1000 μM, as indicated by P.I. staining at day 11 (data not shown).

At the very late differentiation stage (day 9 – 11), ketamine ($\leq$ 200 μM) has no significant effects on the maintenance of different DC subsets. At day 11, the percentages of pDC, cDC, eCD8$^+$ cDC, and eCD8$^-$ cDC

subsets in ketamine groups (50 – 200 μM) were similar to the respective levels in the control groups (Fig. 1C).

The effects of ketamine on LPS- or CpG-mediated DC maturation

TLRs activation had long been acknowledged as a trigger of DC maturation, so herein we used LPS (TLR4 ligand) and CpG 2216 (TLR9 ligand) to examine the maturation of DCs when exposed to different doses of ketamine. Ketamine was incubated with FL-DC culture from day 9 to 11 and then LPS/CpG was added for the final 24 h. At day 11, the expression of maturation markers CD80, CD86, CD40, and MHC II in the cDCs ($CD11c^{high}CD45RA^{-}$) was detected in flow cytometry analysis. The vast majority of FL-DCs had an immature phenotype before TLR ligands stimulation. Both LPS (Fig. 2A) and CpG (Fig. 2B) successfully boosted the surface expression of CD86, CD40, and CD80, as compared with the un-stimulated group (gray background). The expression of MHC II was positive in unstimulated FL-DCs and was not dramatically enhanced by LPS/CpG stimulation. However, the additional ketamine treatment (50 – 200 μM) had no evident impacts on the expressions of mature molecules on DCs after LPS/CpG stimulation. As in Fig. 2, in the cDC subsets from FL-DC culture, the expression patterns of CD80, CD86, CD40, and MHC II in the ketamine-treated groups (color lines) were very similar to those in CpG/LPS groups alone (black line).

Because cytokine synthesis is another aspect of DC maturation, we investigated the expression of inflammatory cytokines and type I interferons, respectively, in LPS-treated and CpG 2216–treated FL-DCs, by real-time PCR. LPS rendered rapid expression of inflammatory cytokines within 6 h; however, co-treatment of LPS and ketamine (100 μM) led to a small but significant decrease in IL-6 (40%) and IL-12p40 (42%) mRNA levels. Meanwhile, pDC possessed less IL-6 (39%) and IL-12p40 (24%) mRNA level after ketamine (100 μM) treatment upon LPS stimulation (Fig. 3A). For TLR9 signaling, cDC is a weak responder and low mRNA levels of type I interferons were detected upon CpG stimulation (Fig. 3B). pDC strongly responded towards CpG treatment (6 h) and demonstrated a dramatic increase in IFN-β1, IFN-α1, and IFN-α4 mRNA level as compared with un-stimulated FL-DC. Co-treatment of CpG and ketamine (100 μM) further enhanced the expression levels of IFN-β1, IFN-α1, and IFN-α4 to 1.44-, 1.60-, and 1.64-fold, respectively (Fig. 3B). Both $CD8^{+}$ cDC and monocyte-derived DC contributed to the production of IL-12p70 upon bacteria infection (27). Consistently,

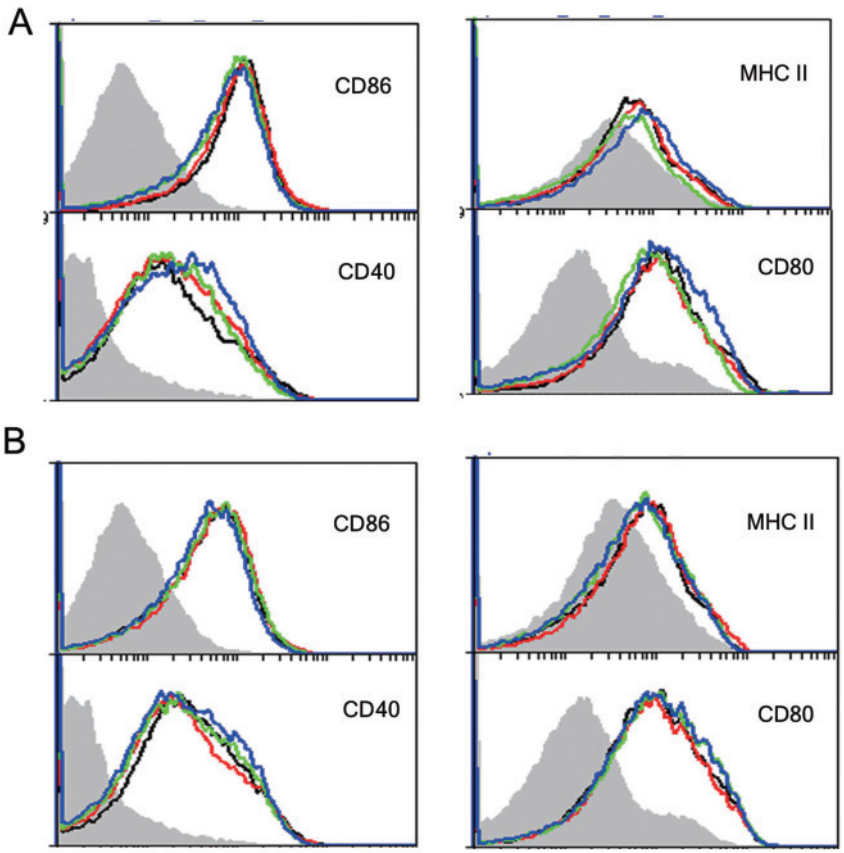

Fig. 2. The effect of ketamine on surface expression of maturation marker. A) The surface expression of CD80, CD86, MHC II, and CD40 in $CD11c^{high}CD45RA^{-}$ FL-DC induced by LPS treatment and the effect of ketamine are presented. B) The surface expression of CD80, CD86, MHC II, CD40 in $CD11c^{high}CD45RA^{-}$ FL-DC stimulated by CpG treatment and the effect of ketamine are presented. Untreated FL-DCs served as the background (gray area); LPS (100 ng/ml) or CpG (100 nM) treatment of 16 h is indicated by the black curve. Ketamine was pre-incubated with FL-DC in different doses (50 μM indicated by the red curve, 100 μM indicated by the green curve, and 200 μM indicated by the blue curve), followed by LPS/CpG treatment for another 16 h. The assay was analyzed in P.I. negative cells and the data are representative of three independent experiments.

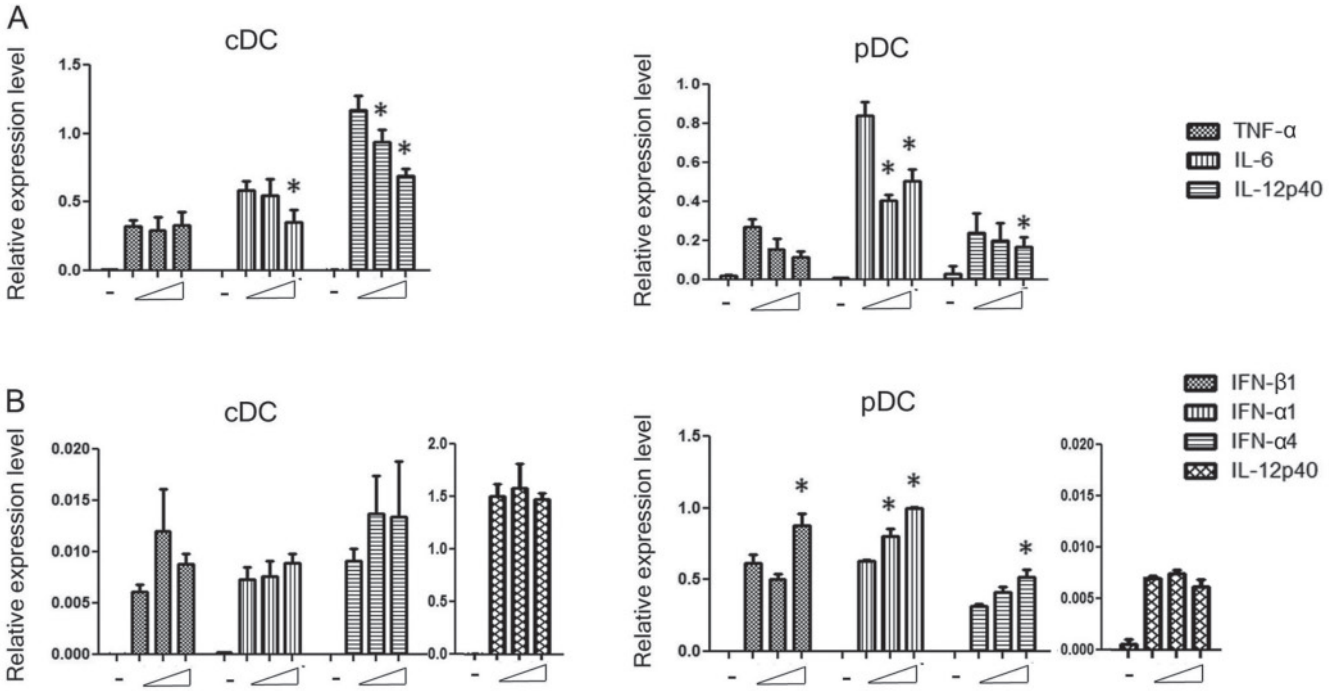

Fig. 3. The impact of ketamine on mRNA synthesis of cytokines after TLRs stimulation by real-time PCR. A) Inflammatory cytokines mRNA level in cDC and pDC after LPS (100 ng/ml) treatment for 6 h and the effect of ketamine are shown. B) Type I interferons and inflammatory cytokines mRNA level in cDC and pDC after CpG (100 nM) treatment for 6 h and the effect of ketamine are shown. "–" column represents the unstimulated control; the other columns show LPS/CpG treatment plus increasing concentrations (0, 50, 100 μM) of ketamine as indicated the triangle symbol. *$P < 0.05$, compared to LPS/CpG control.

IL-12p40 was majorly expressed by cDC but not pDC upon CpG stimulation in our study. Unlike LPS stimulation, ketamine played a negligible role in the expression of IL-12p40 upon CpG stimulation (Fig. 3B).

The effects of ketamine on the endocytosis capability of FL-DC

The capacity of FL-DCs to uptake extracellular antigens was assessed in a FITC-dextran uptake assay. Both $eCD8^+$ cDC and $eCD8^-$ cDC from FL-DC culture were reported to be capable of endocytosing foreign antigens, although being less active than their splenic counterparts (31). From our result, an evident amount of FITC-dextran was efficiently taken up by $CD11c^+$ FL-DC. However, ketamine in the range of 50 – 200 μM did not have significant impact on the FITC-dextran uptake by FL-DCs (Fig. 4).

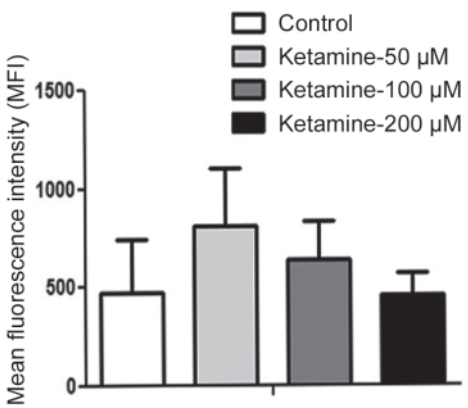

Fig. 4. The endocytosis of FITC-dextran by FL-DC and the effect of ketamine. Ketamine was pre-incubated with FL-DC for 48 h before endocytosis analysis. The FITC fluoresence intensity was analyzed in P.I. negative $CD11c^+$ cells by flow cytometry. The result is shown as the solution of mean fluorescence intensity (MFI) at 37°C subtracting MFI at 4°C. The data are representative of three independent experiments, with similar results.

The stimulatory effects of ketamine on allogenic T cell response

Although FL-DCs exhibited weaker allo-stimulating activity as compared with GM-DCs, their stimulatory capacity for allogenic T cells was significantly enhanced after IL-2 or LPS treatment (32, 33). In our observation, FL-DCs at day 10 were approximately 79% – 91% positive for CD11c and contained less than 2% of T cells (data not shown). In our case, FL-DCs demonstrated enhanced allo-stimulatory capacity after LPS treatment (approximately 10-fold) (Fig. 5A). However, 100 μM

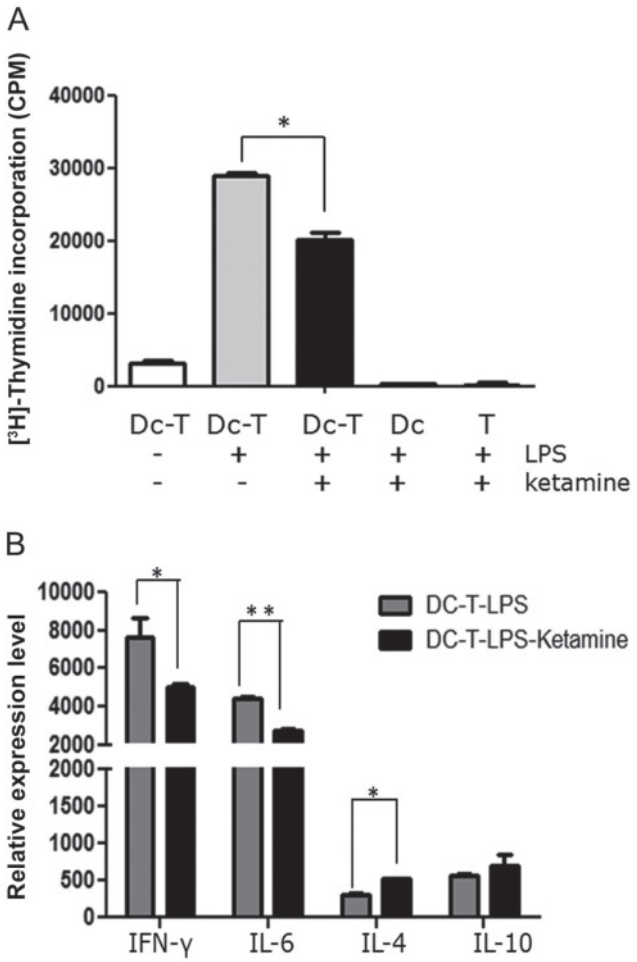

Fig. 5. Ketamine impeded the proliferation and inflammatory cytokine release of allogeneic T cells. FL-DCs were incubated with or without ketamine (100 μM) from day 9 to 11 with additional LPS treament for the final 18 h. The DCs were washed, inactivated, and cocultured with $CD4^+$ T cells derived from Balb/c mice. A) Mixed cell culture was incubated for 3 days and subjected to $[^3H]$-TdR incorporation for the final 18 h. Cell proliferation was estimated based on uptake of $[^3H]$ radioactivity using a liquid scintillation counter. B) Mixed cell culture lasted for 3 days and supernatant was collected and subjected to the cytokines profile assay. $^*P < 0.05$, $^{**}P < 0.01$.

ketamine repressed the proliferation of allogenic T cell compared with LPS treatment alone (20172 ± 1096 cpm vs. 29089 ± 514 cpm), which indicated ketamine was a potent inhibitor for the allogenic T cell response. FL-DCs or allogenic T cells alone cannot proliferate efficiently (Fig. 5A). We also analyzed the cytokines in the supernatant of DC–T-cell co-culture. As shown in Fig. 5B, ketamine-treated FL-DCs led to a decreased secretion of IFN-γ in DC–T-cell co-culture as compared with the control group (5033 ± 133 vs. 7627.27 ± 1060 pg/ml, $P < 0.05$). IL-6 secretion showed the same inhibition in the ketamine group (2721 ± 96 vs. 4387 ± 12 pg/ml, $P < 0.01$), while IL-4 secretion exhibited an increasing tendency (512 ± 16 vs. 311 ± 29 pg/ml, $P < 0.05$).

Ketamine affected pDC development in vivo

The intraperitoneal injection of ketamine had been used to study the effect of ketamine on cytokine production in mice (34) and rats (35). Four-week-old mice were subjected to ketamine treatment once per week, lasting for 4 weeks. The surface markers for identifying splenic pDC ($CD11c^{mid}PDCA\text{-}1^+$), $CD8^+$ cDC ($CD11c^{high}PDCA\text{-}1^-CD8^+CD4^-$), $CD8^-$ cDC ($CD11c^{high}PDCA\text{-}1^-CD8^-CD4^+$) have been characterized previously (36 – 38). The pDC of ketamine-treated mice diminished as compared with the control group ($3.21 \times 10^5 \pm 4.34 \times 10^4$ cells/per mice vs. $5.46 \times 10^5 \pm 7.32 \times 10^4$ cells/per mice, $P < 0.05$). However, $CD8^+$ cDC ($CD11c^{high}PDCA\text{-}1^-CD8^+CD4^-$) and $CD8^-$ cDc ($CD11c^{high}PDCA\text{-}1^-CD8^-CD4^+$) did not show any significant change (Fig. 6). DC isolated from treated mice did not show enhanced P.I. staining, which indicated the blockage of pDC development was not due to cell death.

Discussion

It had been reported that the plasma concentration of ketamine was about 3 μg/ml (about 11 μM) during anesthetic surgery and even 100 μM falls within the range of clinical relevance (39 – 42). Ketamine has no toxic action on DC survival at the concentration of 100 μM in vitro (5, 14), although exposure of macrophages to 1000 μM of ketamine resulted in an increase of cell death and a decrease in cell migration (14). In our study, ketamine also resulted in cytotoxicity at a concentration of 1000 μM. The dose range of ketamine in our study was between 50 – 200 μM in most of the experiments without obvious cytotoxicity (data not shown). Moreover, most of the cellular analysis was based on the exclusion of P.I. positive cells to avoid the interference of dead cells. A new finding in our study is that ketamine hampered the development of pDC both in vivo and in vitro. Since pDC was a vital component in type I interferon synthesis, the repressed pDC development may impair the defensive response towards pathogens, as well as B cell and NK cell activation (43, 44).

Comparatively, GM-DCs were larger and more granular than FL-DCs in phenotype; GM-DCs secreted more inflammatory cytokines while FL-DCs were more potent in type I interferon synthesis; the allo-stimulatory capability of FL-DC was weaker than that of GM-DCs (19, 32). The study on the GM-DCs showed that 100 μM ketamine inhibited the expression of IL-12p40 during the maturation induced by LPS, as well as the maturation markers (CD80, CD86, CD40, MHC II) and the allogenic

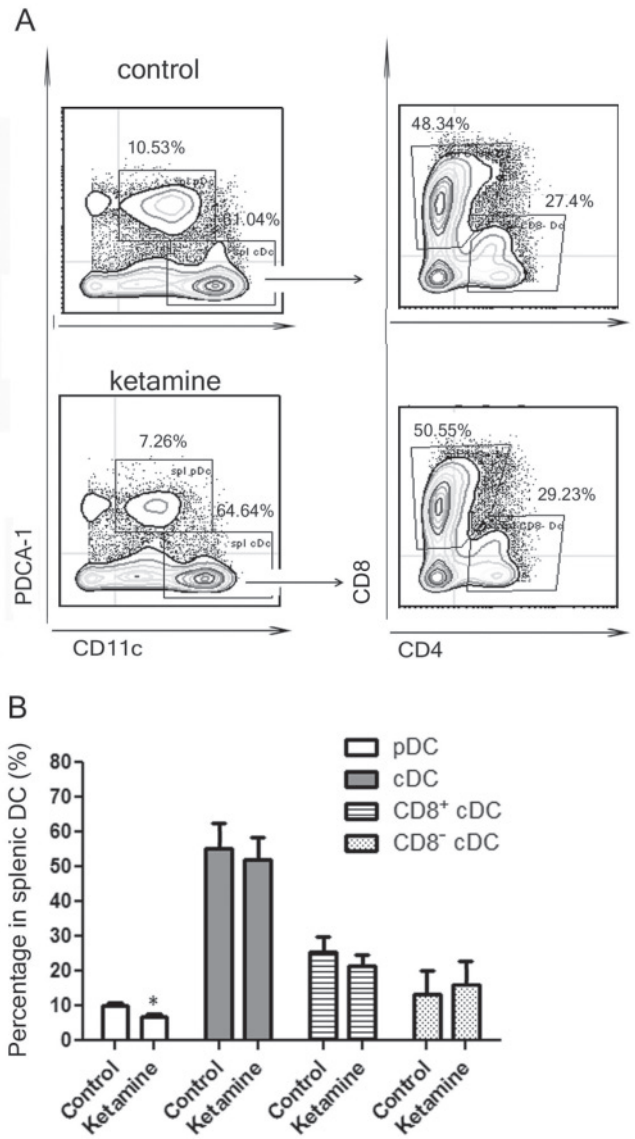

Fig. 6. The development of pDC was inhibited by ketamine in vivo. Four-week-old mice were subjected to ketamine (8 mg/kg) injection intraperitoneally once per week for 4 weeks. A) Mouse splenic DCs were enriched and analyzed with different DC markers (CD11c, PDCA-1, CD8, CD4) to indicate different DC subpopulatios (pDC, cDC, $CD8^+$ cDC, $CD8^-$ cDC) in P.I. negative cells. B) The effects of ketamine on DC subsets development in vivo are shown. The data are representative of four independent experiments. $*P < 0.05$, compared to control.

T-cell response (5). In our case, LPS-induced inflammatory cytokines (IL-6 and IL-12) mRNA synthesis was attenuated by ketamine in both cDCs and pDCs subpopulations. The allogenic stimulatory capacity of FL-DCs was also down-regulated by ketamine, as demonstrated by the allogenic T-cell proliferation and inflammatory cytokines profile. However, ketamine cannot induce significant changes in maturation marker expression upon either LPS or CpG stimulation. The endocytic activity was repressed by ketamine in macrophages and GM-DCs (5, 14), which was not observed in our FL-DCs study. These results indicated the immunomodulation of ketamine in GM-DCs and FL-DCs shared some similar mechanism, yet some variance existed. Another point was that although ketamine exerted a negative effect on TLR4 stimulation as indicated in our observation and other studies, it worked in a quite different way towards TLR9 stimulation. CpG 2216 was a strong inducer for type I interferon in DCs and we found that ketamine further enhanced the mRNA levels of IFN-β1, IFN-α1, and IFN-α4 in pDC. These data strongly suggested that ketamine rendered FL-DCs with different responses to different TLRs stimuli.

The interferon regulatory factor (IRF) family governed many key steps in DC developmental process and functional activity (23, 45, 46). GM-CSF favored $CD8^-$ cDC development via IRF4, while Flt3 ligand supported pDC and $CD8^+$ cDC development via IFR8 (47). IRF7 and IRF8 controlled type I interferon production in DC, but inflammatory cytokines were produced in an IRF5-dependent manner (23, 45, 48). Based on the fact that ketamine only interfered with particular DC subsets generation and played different roles in different TLR signaling pathways, we presumed that ketamine may act directly or indirectly on specific IRFs and thus affect different DC subsets development and different TLRs signaling pathways. Besides IRFs, different downstream signaling molecules recruited by TLR4 and TLR9 may partially explain the discrepancy between the down-regulated cytokines mRNA level in LPS-stimulated pDCs and the up-regulated type I interferon mRNA level in CpG-stimulated pDCs (23, 49). TLR4 can use both the myeloid differentiation factor 88 (MyD88)-dependent signaling pathway and the Toll/interleukin-1 receptor domain-containing adaptor-inducing beta interferon (TRIF)-dependent signaling pathway to mediate the TLR4 activation; while TLR9 only employed the MyD88-dependent signaling pathway (49). Therefore, ketamine may differentially affect these two pathways to modulate the immune capacity of DCs.

In conclusion, we have characterized a variety of effects exerted by ketamine on FL-DCs. Ketamine modulated the in vitro and in vivo development of DC subsets, TLR signaling pathways, as well as the downstream T-cell response. Therefore, ketamine provided an alternative way of modulating the immunostimulatory capacity of DCs and it should be noted during sedation and analgesia management in critical care.

References

1 Chappell WA, White IW, Burman AL. Anaesthesia and the elderly. S Afr Med J. 1982;62:399–402.
2 Hogan BV, Peter MB, Shenoy HG, Horgan K, Hughes TA. Surgery induced immunosuppression. Surgeon. 2011;9:38–43.
3 Clements JA, Nimmo WS. Pharmacokinetics and analgesic effect of ketamine in man. Br J Anaesth. 1981;53:27–30.
4 Beilin B, Rusabrov Y, Shapira Y, Roytblat L, Greemberg L, Yardeni IZ, et al. Low-dose ketamine affects immune responses in humans during the early postoperative period. Br J Anaesth. 2007;99:522–527.
5 Ohta N, Ohashi Y, Fujino Y. Ketamine inhibits maturation of bone marrow-derived dendritic cells and priming of the Th1-type immune response. Anesth Analg. 2009;109:793–800.
6 Welters ID, Hafer G, Menzebach A, Muhling J, Neuhauser C, Browning P, et al. Ketamine inhibits transcription factors activator protein 1 and nuclear factor-kappaB, interleukin-8 production, as well as CD11b and CD16 expression: studies in human leukocytes and leukocytic cell lines. Anesth Analg. 2010;110:934–941.
7 Lankveld DP, Bull S, Van Dijk P, Fink-Gremmels J, Hellebrekers LJ. Ketamine inhibits LPS-induced tumour necrosis factor-alpha and interleukin-6 in an equine macrophage cell line. Vet Res. 2005;36:257–262.
8 Takenaka I, Ogata M, Koga K, Matsumoto T, Shigematsu A. Ketamine suppresses endotoxin-induced tumor necrosis factor alpha production in mice. Anesthesiology. 1994;80:402–408.
9 Takahashi T, Kinoshita M, Shono S, Habu Y, Ogura T, Seki S, et al. The effect of ketamine anesthesia on the immune function of mice with postoperative septicemia. Anesth Analg. 2010;111:1051–1058.
10 Wu GJ, Chen TL, Ueng YF, Chen RM. Ketamine inhibits tumor necrosis factor-alpha and interleukin-6 gene expressions in lipopolysaccharide-stimulated macrophages through suppression of toll-like receptor 4-mediated c-Jun N-terminal kinase phosphorylation and activator protein-1 activation. Toxicol Appl Pharmacol. 2008;228:105–113.
11 Larsen B, Hoff G, Wilhelm W, Buchinger H, Wanner GA, Bauer M. Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood. Anesthesiology. 1998;89:1218–1227.
12 Kawasaki C, Kawasaki T, Ogata M, Nandate K, Shigematsu A. Ketamine isomers suppress superantigen-induced proinflammatory cytokine production in human whole blood. Can J Anaesth. 2001;48:819–823.
13 Melamed R, Bar-Yosef S, Shakhar G, Shakhar K, Ben-Eliyahu S. Suppression of natural killer cell activity and promotion of tumor metastasis by ketamine, thiopental, and halothane, but not by propofol: mediating mechanisms and prophylactic measures. Anesth Analg. 2003;97:1331–1339.
14 Chang Y, Chen TL, Sheu JR, Chen RM. Suppressive effects of ketamine on macrophage functions. Toxicol Appl Pharmacol. 2005;204:27–35.
15 Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol. 2007;7:19–30.
16 Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev. 2002;82:97–130.
17 Wilson NS, El-Sukkari D, Belz GT, Smith CM, Steptoe RJ, Heath WR, et al. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. Blood. 2003;102:2187–2194.
18 Naik SH, O'Keeffe M, Proietto A, Shortman HH, Wu L. CD8+, CD8-, and plasmacytoid dendritic cell generation in vitro using flt3 ligand. Methods Mol Biol. 2010;595:167–176.
19 Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol. 2007;179:7577–7584.
20 Daro E, Pulendran B, Brasel K, Teepe M, Pettit D, Lynch DH, et al. Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but not CD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. J Immunol. 2000;165:49–58.
21 Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev. 2010;234:18–31.
22 Lehtonen A, Veckman V, Nikula T, Lahesmaa R, Kinnunen L, Matikainen S, et al. Differential expression of IFN regulatory factor 4 gene in human monocyte-derived dendritic cells and macrophages. J Immunol. 2005;175:6570–6579.
23 Tailor P, Tamura T, Ozato K. IRF family proteins and type I interferon induction in dendritic cells. Cell Res. 2006;16:134–140.
24 Hemmi H, Akira S. TLR signalling and the function of dendritic cells. Chem Immunol Allergy. 2005;86:120–135.
25 Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, et al. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol. 2003;33:827–833.
26 Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, et al. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol. 2005;174:6592–6597.
27 Zhan Y, Xu Y, Seah S, Brady JL, Carrington EM, Cheers C, et al. Resident and monocyte-derived dendritic cells become dominant IL-12 producers under different conditions and signaling pathways. J Immunol. 2010;185:2125–2133.
28 Bjorck P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. Blood. 2001;98:3520–3526.
29 Vremec D, Shortman K. The isolation and identification of murine dendritic cell populations from lymphoid tissues and their production in culture. Methods Mol Biol. 2008;415:163–178.
30 Clements JA, Nimmo WS, Grant IS. Bioavailability, pharmacokinetics, and analgesic activity of ketamine in humans. J Pharm Sci. 1982;71:539–542.
31 Kamphorst AO, Guermonprez P, Dudziak D, Nussenzweig MC. Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. J Immunol. 2010;185:3426–3435.
32 Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood. 2000;96:3029–3039.
33 Taieb A, Breitinger JJ, Unadkat JV, Shufesky WJ, Morelli AE, Thomson AW, et al. Intrinsic ability of GM+IL-4 but not Flt3L-induced rat dendritic cells to promote allogeneic T cell hyporesponsiveness. Clin Immunol. 2007;123:176–189.
34 Behrens MM, Ali SS, Dugan LL. Interleukin-6 mediates the increase in NADPH-oxidase in the ketamine model of schizophrenia. J Neurosci. 2008;28:13957–13966.

35 Sun J, Li F, Chen J, Xu J. Effect of ketamine on NF-kappa B activity and TNF-alpha production in endotoxin-treated rats. Ann Clin Lab Sci. 2004;34:181–186.
36 Wilson NS, Villadangos JA. Lymphoid organ dendritic cells: beyond the Langerhans cells paradigm. Immunol Cell Biol. 2004; 82:91–98.
37 Huang Y, Parker M, Xia C, Peng R, Wasserfall C, Clarke T, et al. Rabbit polyclonal mouse antithymocyte globulin administration alters dendritic cell profile and function in NOD mice to suppress diabetogenic responses. J Immunol. 2009;182:4608–4615.
38 Hochrein H, O'Keeffe M, Wagner H. Human and mouse plasmacytoid dendritic cells. Hum Immunol. 2002;63:1103–1110.
39 Dallimore D, Herd DW, Short T, Anderson BJ. Dosing ketamine for pediatric procedural sedation in the emergency department. Pediatr Emerg Care. 2008;24:529–533.
40 Dallimore D, Anderson BJ, Short TG, Herd DW. Ketamine anesthesia in children-exploring infusion regimens. Paediatr Anaesth. 2008;18:708–714.
41 Atallah MM, el-Mohayman HA, el-Metwally RE. Ketamine-midazolam total intravenous anaesthesia for prolonged abdominal surgery. Eur J Anaesthesiol. 2001;18:29–35.
42 Grant IS, Nimmo WS, McNicol LR, Clements JA. Ketamine disposition in children and adults. Br J Anaesth. 1983;55: 1107–1111.
43 Kadowaki N. The divergence and interplay between pDC and mDC in humans. Front Biosci. 2009;14:808–817.
44 Naik SH, Corcoran LM, Wu L. Development of murine plasmacytoid dendritic cell subsets. Immunol Cell Biol. 2005;83:563–570.
45 Honda K, Taniguchi T. Toll-like receptor signaling and IRF transcription factors. IUBMB Life. 2006;58:290–295.
46 Paun A, Pitha PM. The IRF family, revisited. Biochimie. 2007; 89:744–753.
47 Tamura T, Tailor P, Yamaoka K, Kong HJ, Tsujimura H, O'Shea JJ, et al. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. J Immunol. 2005;174:2573–2581.
48 Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature. 2005;434:772–777.
49 Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004;16:3–9.