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The Hedgehog inhibitor suppresses the function of monocyte-derived dendritic cells from patients with advanced cancer under hypoxia

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ABSTRACT

Immunotherapy using monocyte derived dendritic cells (Mo-DCs) from cancer patients has been developed; however, the Mo-DCs regularly studied have been derived from non-cancer bearing donors or mice, and evaluated in normoxic conditions. In the present study, we investigated the effects of Hedgehog (Hh) inhibitors which are being developed as molecular target drugs for cancer on the functions of Mo-DCs derived from patients with advanced cancer when cultured in a tumor-like hypoxic environment. Mo-DC induction, migration, chemotaxis, phagocytosis, maturation, IL-12 p40 or p70 secretion and the allogeneic lymphocyte stimulation activity of Mo-DCs from patients with advanced cancer were all significantly inhibited by the Hh inhibitor, cyclopamine under hypoxic conditions. Our results suggest that Hh signaling plays an important role in the maintenance and function of Mo-DCs derived from patients with advanced cancer when cultured under hypoxic conditions.

1. Introduction

Hedgehog (Hh) signaling is morphogenically important for embryonic patterning, controlling growth, and cell fate during neonatal development [1–3]. Recently, many studies have revealed that Hh signaling is activated in various types of cancer and that it contributes to the proliferation, invasion and progression of cancer [4–9]. Therefore, the Hh pathway is thought to be a potential therapeutic target in some diseases. Of the Hh inhibitors, Smoothened (Smo) inhibitors are well researched and some are under clinical trial [10,11]. For example, in January 2012, the US Food and Drug Administration approved Smo inhibitor, vismodegib for the therapy of metastatic or unresectable basal cell carcinomas of the skin [12]. Of late, research into combination therapy with chemodrugs and Hh inhibitors has also been reported [13,14]. Combination therapy with Hh inhibitors and immunotherapy using monocyte derived dendritic cells (Mo-DCs) will likely begin in the near future despite the fact that there are few reports investigating the interaction between Hh signaling and Mo-DCs in immunotherapy.

DCs are specialized antigen-presenting cells that initiate the primary T-cell immune response [15]. Re-vaccination with self-derived DCs pulsed with tumor associated antigens (TAAs), tumor specific peptide vaccination, and activated TNK lymphocytes including cytotoxic T lymphocytes are the three standard immunotherapies used today. In immunotherapy using Mo-DCs, donor-derived immature Mo-DCs (imMo-DCs) are injected locally into the tumor and mature Mo-DCs (mMo-DCs) pulsed with TAAs are administrated superficially into the lymph nodes. It has been reported that the oxygen levels are near 5.3% in mixed venous blood, 3.3–7.9% in well-vascularized organs, 1.3% in tumor tissues, and 0.5% in lymphoid organs [16,17]. Thus, DCs function in 0.5–8% O_2 conditions. Therefore, the Mo-DCs used in immunotherapy are harvested and induced under normoxic conditions *ex vivo*, are directly injected into the tumor or lymphoid tissue, and are expected to function well in these hypoxic conditions without an adjustment period [18]. In addition, some authors have shown that Hh signaling plays a pivotal role in DC function [19,20]. However, these results were ascertained using DCs from mice or non-cancerous volunteers and tested under normoxic culture conditions. Importantly, we have shown that the functions of Mo-DCs from patients with advanced cancer were impaired compared with those of Mo-DCs from healthy volunteers [21]. Thus, a better understanding of the functions of Mo-DCs derived from patients with advanced cancer and cultured under hypoxia is essential to improve the effect of immunotherapy. In the present study, we asked if Hh inhibition using Cyclopamine affected the function of Mo-DCs derived from patients with advanced cancer and cultured under hypoxic conditions.

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2. Patients and methods

2.1. Patients

Twenty-seven patients enrolled in this study underwent cancer immunotherapy at the Fukuoka General Cancer Clinic (Fukuoka, Japan). The patient characteristics are shown in Supplementary Table 1. Written informed consent was obtained from all individuals.

2.2. Cells and culture conditions

Human peripheral blood mononuclear cells (PBMCs) were collected by apheresis (Haemonetics Co, Stoughton, MA, USA) prior to immunotherapy and stored at −80 °C until used. Thawed PBMCs were cultured in RPMI-1640 (Nipro, Osaka, Japan) supplemented with 0.5% human serum, 100 μg/ml penicillin (Meijiseika, Tokyo, Japan) and 100 μg/ml streptomycin (Meijiseika) (hereafter referred to as RPMI medium). After overnight culture, the non-attached cells were removed and used as lymphocytes. Adherent cells were cultured in RPMI medium supplemented with GM-CSF (100 ng/ml, North China Pharmaceutical Group Corporation-Gene Tech, China) and IL-4 (50 ng/ml, Osteogenetics, Wuerzburg, Germany) for 5–7 days to induce imMo-DC. To induce maturation, 1 μg/ml of LPS (Sigma, St Louis. MO, USA) was added for an additional 2 days. Smo inhibitor, cyclopamin (Certificate of Analysis, North York, Canada), was diluted in 99% ethanol. As hypoxic condition, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigus incubator (Sanyo, Tokyo, Japan).

2.3. Reverse transcription (RT)-PCR

Total RNA was extracted by using High Pure RNA Isolation kit (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's instructions. The sequences of the primers used were: Gli1 forward, 5′-TACATCAACTCCGGCCAATAGG-3′, reverse, 5′-CGGCGGCTGACAGTATAGGCA-3′, Smo forward, 5′-CTGCACACACTCACCTCTAA-3′, reverse, 5′-AAGCTTTCTTGCCTGGCTGA-3′, Shh forward, 5′-ACCATTCTCATCAACCGGGT-3′, reverse, 5′-ATTTGGTAGAGCAGCTGCGA-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-CCACCCATGGCAAATTCCATGGCA-3′, reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. Amplification conditions comprised an initial denaturation step for 2 min at 95 °C followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. RT-PCR products were separated on 2% agarose gels and visualized using ethidium bromide on a Molecular Imager FX (Bio-Rad, Hercules, CA).

2.4. Random migration and chemotaxis assay

The migration of Mo-DCs was assessed by migration through transwell inserts (8.0-μm pore size; BD Biosciences, Heidelberg, Germany). Then 5×10^4 cells were added to the upper chamber and incubated for 16 h under normoxia and hypoxia. After incubation, all cells that had migrated to the lower side were counted under a light microscope (BX50; Olympus, Tokyo, Japan). For chemotaxis assays, 100 ng/ml of recombinant human RANTES (CCL5, Peprotech, Rocky Hill, NJ, USA) or 6Ckine (CCL21, Peprotech) were added to the lower chamber and a filter (8.0-μm pore size) was placed in the well. Then 5×10^4 cells were added to the upper chamber and incubated for 5 h under normoxia and hypoxia. After incubation, all cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus).

2.5. Fluorescence activated cell sorting (FACS) analysis

Mo-DCs were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-CD83 and HLA-ABC mAbs or Phycoerythrin (PE)-conjugated anti- CD86 and HLA-DR mAbs (BD Pharmingen, San Diego, CA, USA). Mouse IgG was used as an isotype control (BD Pharmingen). The fluorescence intensity of the gated Mo-DC population was measured using a FACSCalibur flow cytometer (BD Pharmingen) and analyzed with CellQuest software (BD Pharmingen).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Mo-DCs were cultured with 0.05 KE/ml of streptococcal preparation, OK-432 (Chugai Pharma Co., Tokyo, Japan) for 16 h under normoxia and hypoxia. Then the concentrations of IL-12 p40 and IL-12 p70 in the supernatant were evaluated by ELISA according to the manufacturer's instructions (Biosource, Carlsbad, CA, USA). The detection limit of the assay was 0.5 pg/ml.

2.7. Evaluation of phagocytosis

Fluorescent polystyrene latex microspheres (1.00 μm, Cosmo Bio Co., Ltd., Tokyo, Japan), first coated in serum (opsonization), were added to the RPMI medium for 3 h at 37 °C or 4 °C under normoxia and hypoxia. The Mo-DCs were washed, and the fluorescence intensity in the gated Mo-DC population was measured by FACS analysis.

2.8. Allogeneic T-cell proliferation assay

Lymphocytes were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, Netherlands) in PBS at 37 °C for 10 min. Then lymphocytes were co-cultured with allogeneic Mo-DCs at a 1:7.5, 1:15, or 1:30 Mo-DC/lymphocytes ratio for 5 days under normoxic and hypoxic conditions. Lymphocyte proliferation was assessed by the CFSE dilution method.

2.9. Statistical analysis

An unpaired two-tailed Student's *t*-test was used for statistical analysis. A *p*-value of <0.05 was considered significant.

3. Results

3.1. The Hh inhibitor, cyclopamine, decreased proliferation and migration of Mo-DCs

First, the expressions of Hh signaling related molecules in Mo-DCs were evaluated by RT-PCR. *Gli1* and *Smo* mRNA were detected but *Shh* mRNA was not detected (Fig. 1A). Morphologically, cyclopamine did not affect imMo-DC morphology. The Mo-DCs showed a spindle-like morphology during the LPS treatment; however, cyclopamine-treated Mo-DCs remained round even during LPS treatment (Fig. 1B). Next we investigated whether cyclopamine affected the number of induced Mo-DCs. Cyclopamine significantly affected the number of generated Mo-DCs both under normoxic and hypoxic conditions (Fig. 1C).The ability to migrate is also an important function of Mo-DCs. As such, we evaluated nondirectional migration and chemotaxis in hypoxic conditions. Cyclopamine significantly decreased the capacity of random migration of imMo-DCs (Fig. 1D) and mMo-DCs (Fig. 1E) both under normoxic and hypoxic conditions. Then we used CCL5 and CCL21 as chemotaxic factors for the imMo-DCs and mMo-DCs, respectively.

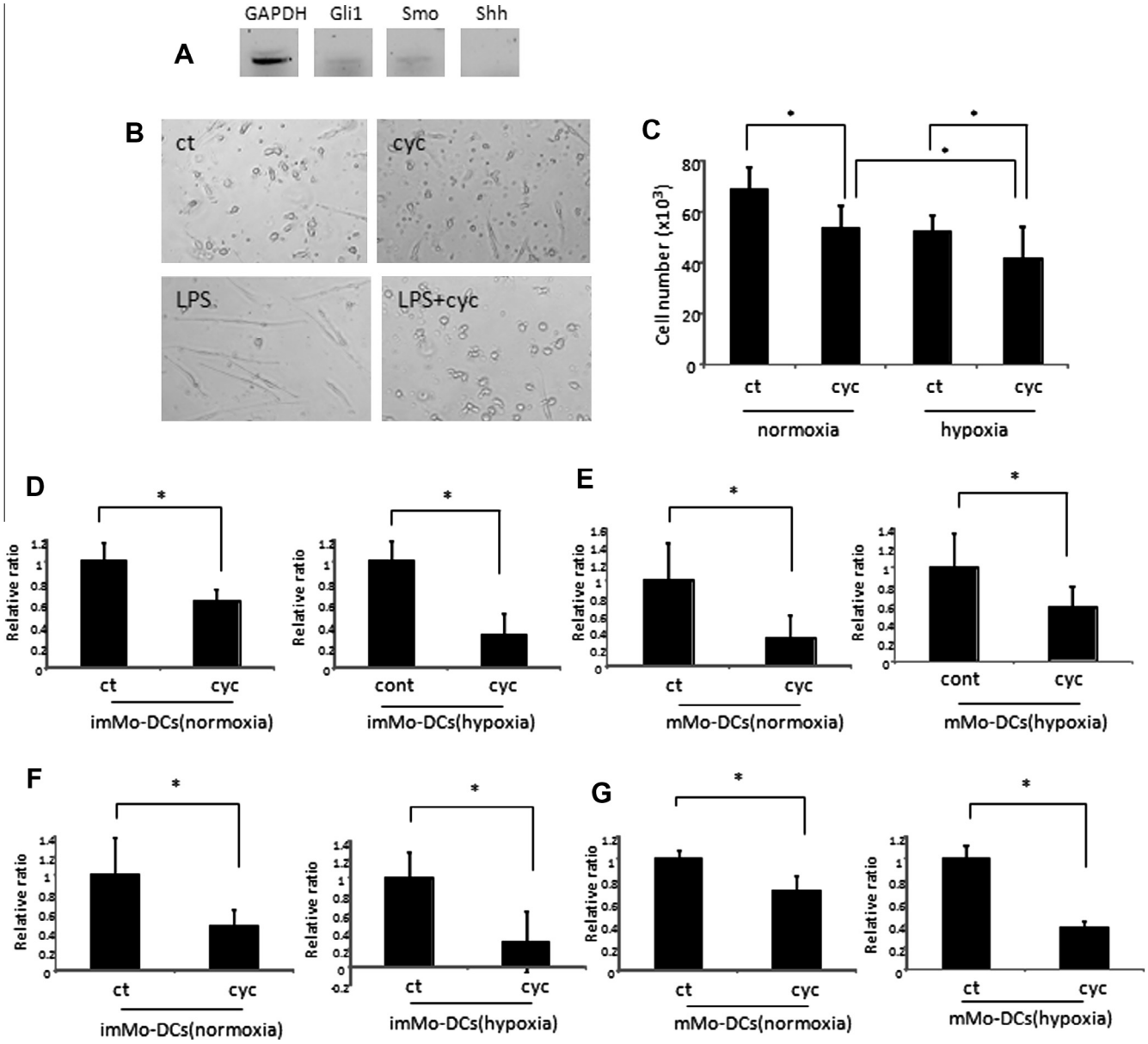

Fig. 1. Proliferation and migration were impaired in cyclopamine-treated Mo-DCs in hypoxia. (A) Representative results of Hh signaling molecule expression (Gli1, Smo, and Shh) by RT-PCR. (B) Representative images of cyclopamine-treated imMo-DCs and mMo-DCs. After the induction of Mo-DCs for 5 days, 10 μM of cyclopamine and/or 1 μg/ml of LPS were added to the culture for an additional two days under hypoxia. Pictures were obtained by phase contrast microscopy. Original magnification is ×200. (C) Induced imMo-DCs. After monocytes were co-cultured with 100 ng/ml of GM-CSF and 50 ng/ml of IL-4 for 5 days, 10 μM of cyclopamine or 99% ethanol were added to the culture. After an additional 3 days of culture under normoxia and hypoxia, cell number was counted by light microscopy. (D and E) Random migration ability of Mo-DCs. Fifty thousand imMo-DCs (D) or mMo-DCs (E) were applied to the upper chamber and were cultured for 16 h under normoxia and hypoxia. Cells that had migrated from the upper to the lower side of the filter were counted using a light microscope. (F and G) Chemotaxis in Mo-DCs. Recombinant human RANTES (100 ng/ml, CCL5) and 6Ckine (100 ng/ml, CCL21) were added to the lower chamber. Then 50,000 imMo-DCs (F) or mMo-DCs (G) were applied to the upper chamber and were cultured for 5 h under normoxia and hypoxia. Cells that had migrated from the upper to the lower side of the filter were counted using a light microscope. The graph shows mean ± SD. $^{*}p < 0.05$.

In the chemotaxis assay, the number of cells that migrated was significantly reduced by cyclopamine treatment in both imMo-DCs (Fig. 1F) and mMo-DCs (Fig. 1G) both under normoxic and hypoxic conditions.

3.2. Cyclopamine reduced antigen presentation-related molecules of Mo-DCs

Antigen presentation-related molecules such as HLA-ABC, HLA-DR, and CD86 are important for Mo-DC antigen presentation to T cells, and CD83 is used as an index of maturation. Thus, HLA-ABC, HLA-DR, CD83, and CD86 expressions in imMo-DCs and mMo-DCs were analyzed by FACS. First, Mo-DC cell size and complexity were evaluated by forward scatter (FSC) and side scatter (SSC), respectively. Cyclopamine significantly decreased cell size as determined by FSC; however, it did not affect the complexity of the cells as determined by SSC (Fig. 2A). LPS treatment successfully increased CD86, CD83, and HLA-ABC expression under hypoxia. Cyclopamine treatment decreased CD86 expression on imMo-DCs and both CD86 and HLA-DR expression on mMo-DCs under hypoxia (Fig. 2B). These results were almost the same as those under normoxia (Supplementary fig. 1). Interestingly, LPS treatment decreased HLA-DR expression on cyclopamine-treated Mo-DCs and cyclopamine treatment increased CD83 expression on mMo-DCs under hypoxia, but not under normoxia (Fig. 2B and Supplementary fig. 1).

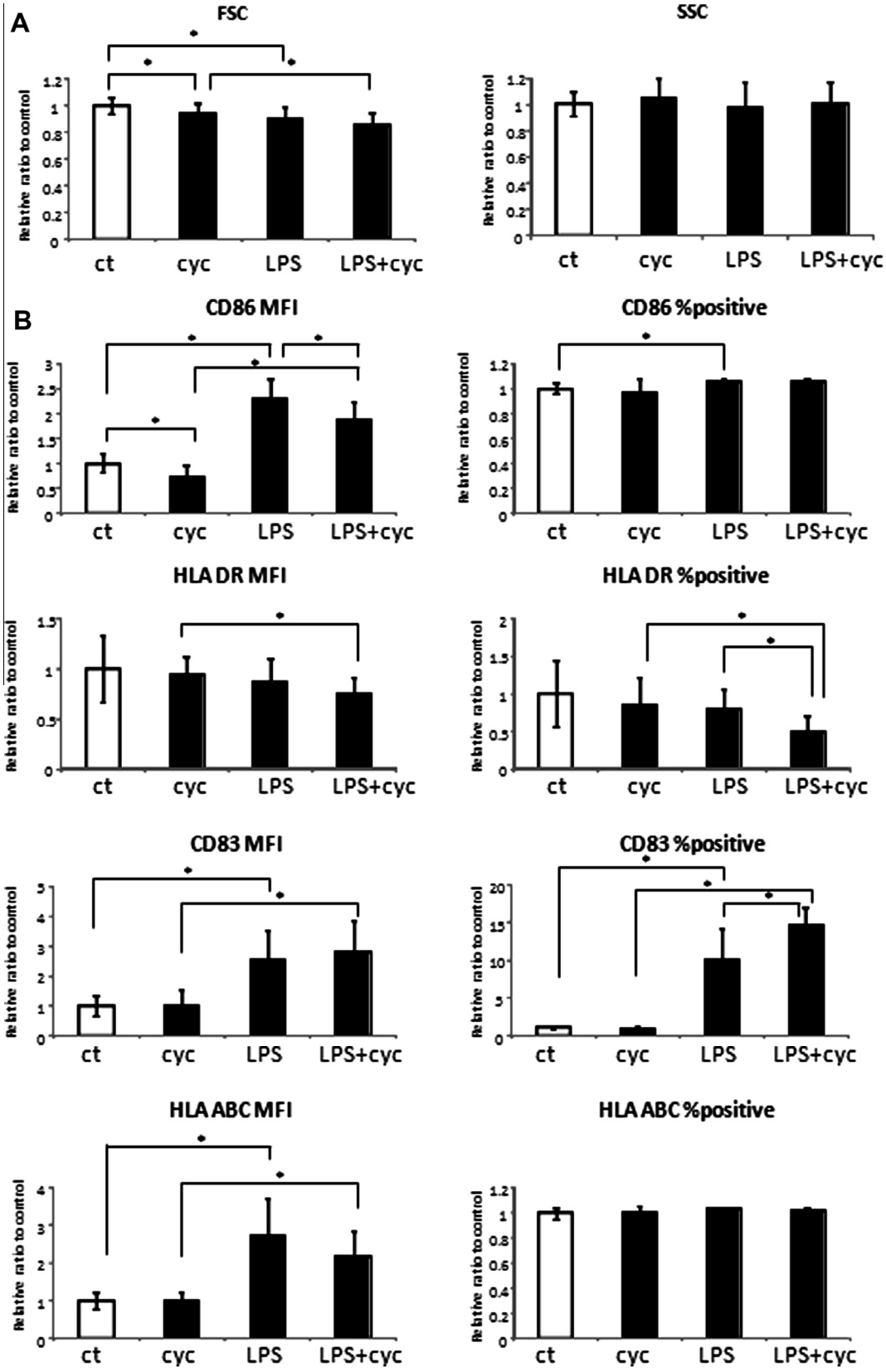

Fig. 2. Expression of antigen presentation-related molecules was impaired in cyclopamine-treated Mo-DCs under hypoxia. (A and B) Size and complexity of Mo-DCs. After the induction of Mo-DCs for 5 days, 10 μM of cyclopamine and/or 1 μg/ml of LPS were added to the cultures for an additional two days under hypoxia. Then FSC (A) and SSC (A), and expressions of CD86 (B), HLA-DR (B), CD83 (B), and HLA-ABC (B) were evaluated by FACS. The graph shows mean ± SD. $^{*}p < 0.05$.

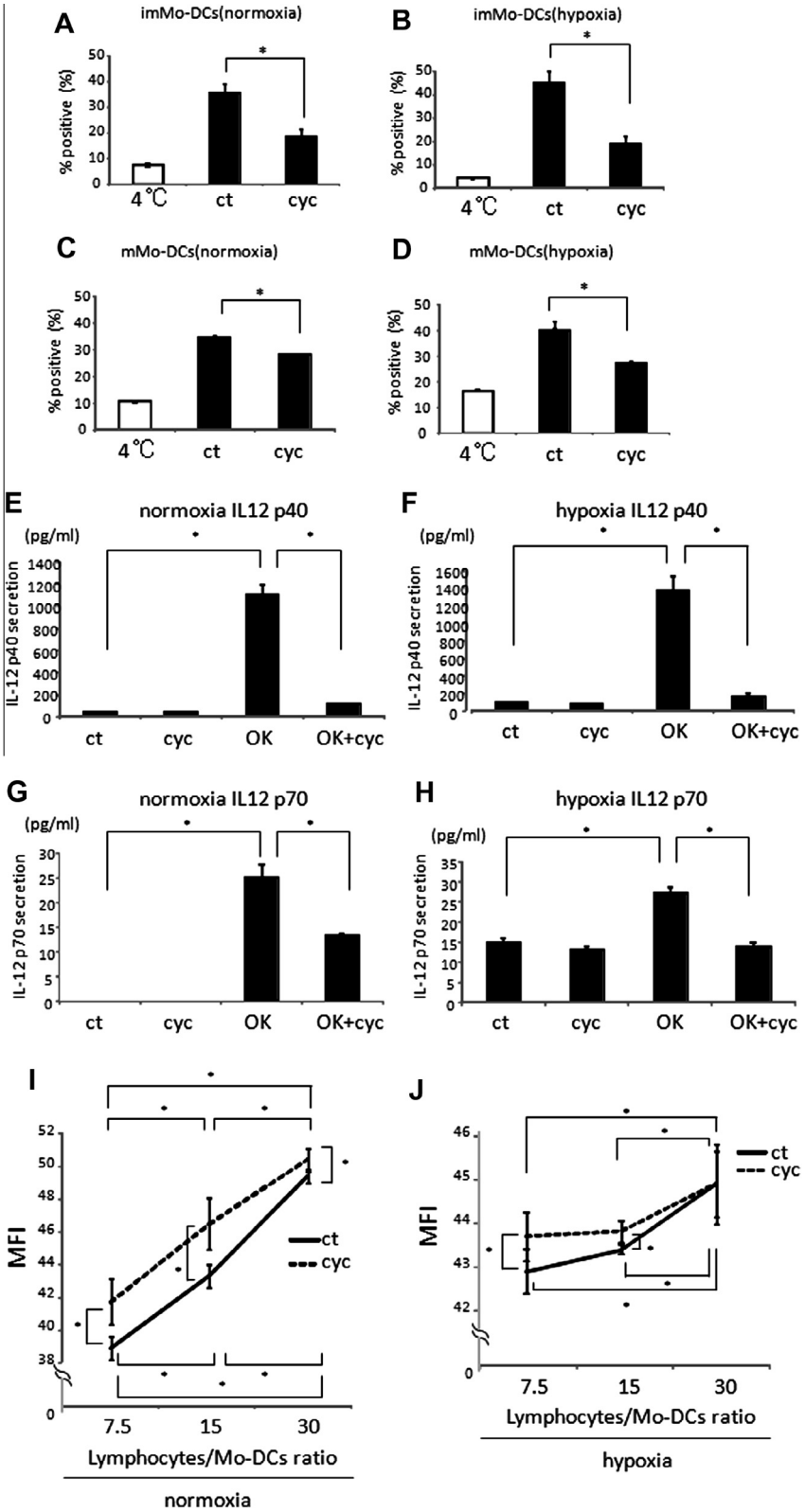

Fig. 3. Phagocytic ability, IL-12 secretion and proliferative capacity of allogeneic lymphocytes were impaired in cyclopamine-treated Mo-DCs. (A-D) Phagocytic ability of Mo-DCs. After the induction of Mo-DCs for 5 days, 10 μM of cyclopamine and/or 1 μg/ml of LPS were added to the culture for an additional 2 days under normoxia and hypoxia. Immature Mo-DCs and mMo-DCs were cultured with opsonized FITC beads for 5 h at 37 °C or 4 °C under normoxia (A and C) and hypoxia (B and D). Then the amount of FITC positive Mo-DCs was expressed as percent-positive Mo-DCs by FACS. The results from the 4 °C culture were used as control. A graph of percent-positive cells from the imMo-DCs (A and B) and mMo-DCs (C and D), respectively, are shown. (E-H) IL-12 secretion by cyclopamine-treated Mo-DCS. After imMo-DCs were co-cultured with 10 μM of cyclopamine and/or 0.05 KE/ml of OK-432 for 16 h under normoxia (E and G) and hypoxia (F and H), IL-12 p40 (E and F) and IL-12 p70 (G and H) concentrations in the supernatant were evaluated by ELISA. (I and J) Mature Mo-DCs were co-cultured with CFSE labeled allogeneic lymphocytes at a ratio of 1:7.5, 1:15, or 1:30 (Mo-DCs:lymphocytes) for 5 days under normoxia (I) and hypoxia (J). The mean value of proliferated allogeneic lymphocytes (CFSE-reducing lymphocytes) was measured by FACS. The graph shows mean ± SD. $^{*}p < 0.05$.

3.3. Cyclopamine decreased phagocytosis, IL-12 secretion, and allogeneic lymphocyte proliferation ability

Phagocytosis is another important function in the presentation of antigens. The phagocytic ability of Mo-DCs was analyzed and was significantly decreased in imMo-DCs and mMo-DCs after cyclopamine treatment under normoxia and hypoxia (Fig. 3A–D). IL-12 secretion is also an important function of imMo-DCs required to induce Th1 immunity. The IL-12 p70 heterodimer, which is composed of a p35 and a p40 subunit, is the biologically active form of IL-12 [22]. When imMo-DCs were stimulated by streptococcal preparation, OK-432 under normoxia and hypoxia, IL-12 p40 and IL-12 p70 secretion significantly increased. However cyclopamine treatment significantly abrogated IL-12 p40 and IL-12 p70 secretion (Fig. 3E–H). Next, to estimate the capacity of mMo-DCs to stimulate alloreactive lymphocytes, mMo-DCs were co-cultured with CFSE-labeled allogeneic lymphocytes. The capacity of proliferating allogeneic lymphocytes (CFSE-reducing lymphocytes) in cyclopamine-treated mMo-DC co-culture was significantly lower than that in control both under normoxia (Fig. 3I) and hypoxia (Fig. 3J).

4. Discussion

In the present study, we focused on characterizing Mo-DCs isolated from patients with advanced cancer and cultured under hypoxic conditions. We used Gli1 as an index of Hh signaling because Gli1 is both a transcription factor and a target gene of the pathway. Importantly, the Hh-related molecules Gli1 and Smo were detected in induced Mo-DCs. However, Shh was not detected in the Mo-DCs despite having been reported to be expressed in human thymic DCs [19,20]. To activate the Mo-DCs that we induced and administered *in vivo* as immunotherapy, Shh stimulation by other cell types such as tumor infiltrating macrophage or the cancer cell itself may be occurring [5,23].

The expressions of antigen presentation-related molecules such as CD83, CD86, and HLA-ABC were significantly increased by LPS treatment, suggesting that Mo-DC maturation was successfully induced. Interestingly, LPS decreased HLA-DR expression on cyclopamine-treated Mo-DCs. In addition, in contrast to the findings of Ogino et al. [18], HLA-DR expression did not significantly increase from LPS treatment in our study. Our results regarding HLA-DR expression are more consistent with Scott-Taylor's results, which demonstrated that Mo-DCs retained their MHC class II in the cytoplasm during the maturation process [24]. Therefore, one reason our results regarding HLA-DR expression may differ is that the HLA-DR molecule is internalized by LPS treatment. Another reason could be that our Mo-DCs were derived from patients with advanced cancer and were frozen as PBMCs before use. Interestingly, our finding that cyclopamine increased CD83 expression in mMo-DCs was novel. The function of CD83 is still controversial; however, it was reported that the loss of CD83 resulted in the impairment of T-cell activation by DCs [25,26]. We have shown that silencing of HIF-1α in hypoxia-induced mMo-DCs reduced CD83 expression [18]. The reason why CD83 is induced by cyclopamine in mMo-DCs is still unclear.

In our phagocytosis assays, the mMo-DCs showed increased nonspecific bead binding which is shown at 4 °C results, compared with imMo-DCs, suggesting that more co-stimulatory molecules were expressed in mMo-DCs and that the beads bound nonspecifically. When we induce Mo-DCs pulsed with TAAs, we first co-culture imMo-DCs with the TAAs to allow phagocytosis, and then we induce maturation and administer the cells *in vivo*. In some institutions, mMo-DCs only pulsed with TAAs are administered *in vivo*. Because imMo-DCs show high levels of phagocytosis and mMo-DCs revealed robust expressions of co-stimulatory molecules that bind TAAs, it is likely that both methods are useful for the induction of Mo-DCs *in vivo*.

Fig. 4 summarizes the results in this study. After antigen capture and processing, imDCs move to the lymph nodes and stimulate naive T cells [15]. Cyclopamine inhibited almost all of the steps required for antigen presentation in naive T cells under hyp-

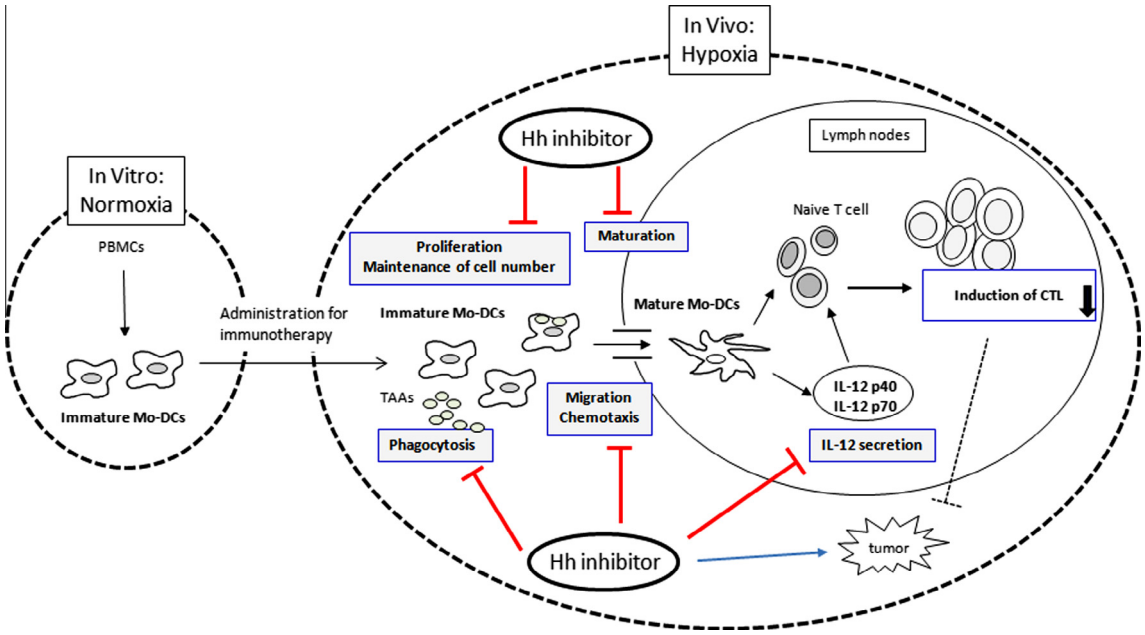

Fig. 4. Schematic representation of this study's results. The Hh inhibitor cyclopamine inhibits proliferation, phagocytosis, migration, chemotaxis, maturation, IL-12 secretion, and the allogeneic lymphocyte stimulatory effects of Mo-DCs (red lines). These decreased functions may lead to a decrease in anti-tumor effects (dotted line). On the other hand, the Hh inhibitor is reported to have its own anti-tumor effect (blue arrow); thus, the Hh inhibitor may act as an inhibitor and stimulant in some cancers. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

oxic conditions. Cyclopamine may elicit anergy and induce immunity to Th2 in cancer patients. Some authors have reported that Shh treatment upregulated Hh signaling in DCs [19,20]. Therefore, Shh administration may be an option in cyclopamine treatment. However, because several kinds of cancer also show activated Hh signaling and cyclopamine itself has anti-tumor effects [27,28]. Thus, Shh administration seems counterintuitive. Molecular target drugs may be used with chemodrugs in anticipation of a synergic effect. However, previously we showed that cyclopamine decreased the chemosensitivity of 5-FU and gemcitabine during combination use [13]. Because of this and our results, synergic effects between combination immunotherapy and cyclopamine appear unlikely. Because the Hh inhibitor negatively affects the beneficial functions of Mo-DCs, administration of Mo-DCs after therapy with the Hh inhibitor or administration of mMo-DCs may be better in immunotherapy.

In the present study, our results suggest that Hh signaling plays pivotal role in the maintenance and functions of Mo-DCs derived from patients with advanced cancer. Because cyclopamine may act as both inhibitor and stimulant of cancer cells, we should use caution when using combination therapies with Hh inhibitors, particularly during immunotherapy using Mo-DCs derived from patients with advanced cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.057.

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