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Soluble factors derived from neuroblastoma cell lines suppress dendritic cell differentiation and activation

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Dendritic cells (DC) play a key role in the initiation of both antitumor immunity and immunological tolerance. It has been demonstrated that exposure to soluble factors produced by tumor cells modulates DC functions and induces tolerogenic DC differentiation. In this study, we investigated the effects of neuroblastoma cell line-derived soluble factors on DC differentiation. Monocytes isolated from healthy volunteers were incubated with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor in the presence of culture supernatants from neuroblastoma cell lines. The culture supernatants from neuroblastoma cell lines, such as NLF and GOTO, partially blocked both downregulation of CD14 and upregulation of CD1a, and dramatically decreased IL-12 and tumor necrosis factor (TNF)-α production from mature DC, while no effect of SH-SY5Y cell supernatant was noted. In addition, IL-6 and IL-10 production from monocytes was increased by the supernatants of NLF and GOTO cells at 24 hours after incubation. Furthermore, we evaluated DC functions through stimulation of invariant natural killer T (iNKT) cells. α-Galactosylceramide-pulsed DC co-cultured with supernatants of NLF cells were unable to sufficiently stimulate iNKT cells. The decreased ability of iNKT cells to produce interferon (IFN)-γ after stimulation with neuroblastoma cell line supernatant-cultured DC was reversed by addition of IL-12. CD40 expression and IL-12 production in NLF-sup-treated DC were increased by addition of exogenous IFN-γ. These results indicate that tolero-

Abbreviations: Ab, antibody; APC, antigen-presenting cell; BxCM, BxPC-3-conditioned medium; CBA, cytometric bead array; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; IFN, interferon; Ig, immunoglobulin; IL, interleukin; iNKT cell, invariant natural killer T cell; MACS, magnetic-activated cell sorting; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; moDC, monocyte-derived dendritic cell; NB, neuroblastoma; PB, Pacific blue; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TGF, transforming growth factor; Th, T-helper; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; αGalCer, α-galactosylceramide.

genic DC are induced in the neuroblastoma tumor microenvironment and attenuate the antitumor effects of iNKT cells. Interactions between iNKT cells and αGalCer-pulsed DC have the potential to restore the immunosuppression of tolerogenic DC through IFN-γ production.

KEYWORDS

dendritic cells, immunosuppression, invariant natural killer T cell, neuroblastoma

1 | INTRODUCTION

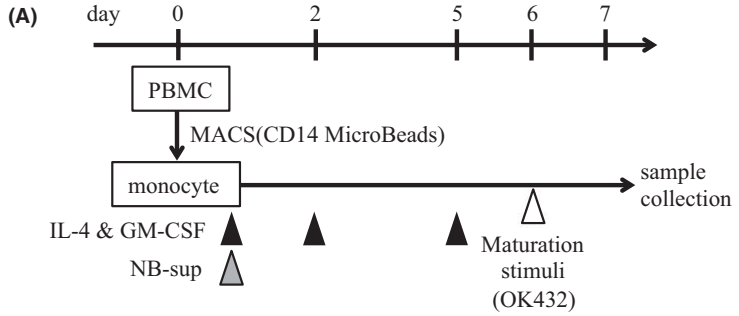
Neuroblastoma (NB), a tumor of the sympathetic nervous system, is the most common extracranial solid tumor in children. The majority of patients are assigned to the high-risk group based on their age at diagnosis, stage, histology, MYCN status and DNA ploidy. NB prognosis remains poor with a 5-year event-free survival rate of approximately 40% despite intensive myeloablative chemotherapy and bone marrow transplantation.[1] Current therapeutic regimens frequently induce a minimal residual disease condition, and relapsed tumors are often refractory towards salvage chemotherapy because of multidrug resistance.[2,3] In these patients, immunotherapy may provide an additional therapeutic strategy.[4,5]

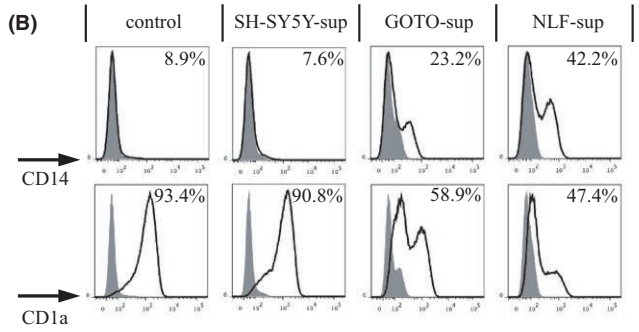
Many mechanisms underlying how tumors escape tumor immunosurveillance have been proposed. DC play a crucial role in the initiation of both antitumor immunity and immunological tolerance.[6,7] The immunogenic and tolerogenic functions of DC originate from distinct stages of differentiation.[8] Tolerogenic DC are characterized by low expression of costimulatory molecules, low production of IL-12 and resistance to maturation in response to danger signals such as Toll-like receptor ligands.[9] In humans, DC represent less than 1% of circulating cells in peripheral blood and can be obtained in vitro from monocytes through a combination of factors and cytokines.[10] It has been shown that tumor cells produce various cytokines and small molecules as well as suppressing human DC differentiation and functions. For example, renal cell carcinoma secretes IL-6 and macrophage colony-stimulating factor, and induces macrophages, thereby inhibiting DC differentiation.[11] Leukemic cell products induce secretion of IL-1β by monocytes and interfere with differentiation of human DC.[12,13] IL-10, TGF-β1 and VEGF are also reported to modulate DC functions.[14-17] In addition to cytokines, gangliosides from neuroblastoma and melanoma impair DC differentiation from monocytes.[18,19] These findings suggest that DC may be polarized to a tolerogenic phenotype through tumor cell-derived soluble factors in the tumor microenvironment. However, the mechanism through which tumor cell-derived soluble factors suppress antitumor immunity and the responsible molecules for such suppression remain unclear.

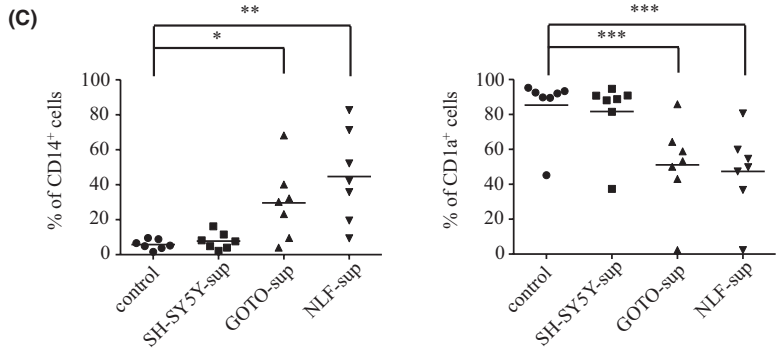
Invariant natural killer T cells play an important role in tumor immunity. They are activated by a specific glycolipid antigen, αGalCer, presented by the HLA class I-like molecule CD1d on APC. Activated iNKT cells rapidly produce high levels of cytokines, such as IFN-γ, and enhance both innate and adaptive immunities through activation of other effector cells including DC, natural killer (NK) cells and cytotoxic T cells.[20] iNKT cells reportedly exert a strong antitumor effect against various types of malignant tumors.[21,22] In murine liver and lung metastasis models, i.v. administration of αGalCer-pulsed DC activates iNKT cells and eradicates the established metastatic tumor foci.[23] However, the abovementioned functions of DC are suppressed in the tumor microenvironment, and whether tolerogenic DC can stimulate iNKT cells remains unclear.

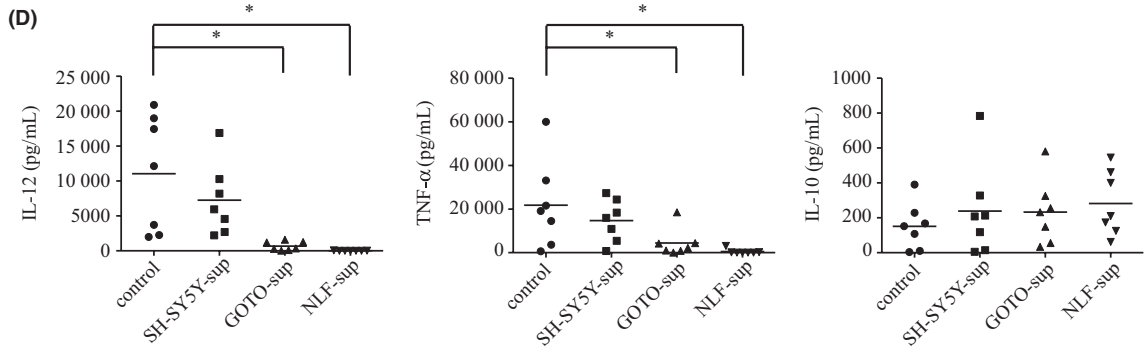
In this study, we investigated the effects of NB cell line-derived soluble factors on DC differentiation. Our results indicated that culture supernatants from neuroblastoma cell lines, such as NLF and GOTO, inhibited the differentiation of monocytes into DC and dramatically decreased IL-12 and TNF-α production in mature DC. Furthermore, αGalCer-pulsed DC that were co-cultured with the supernatant of NLF cells were unable to sufficiently stimulate iNKT cells. However, the decreased ability of iNKT cells to produce IFN-γ was reversed by addition of IL-12, and NLF-sup-treated DC

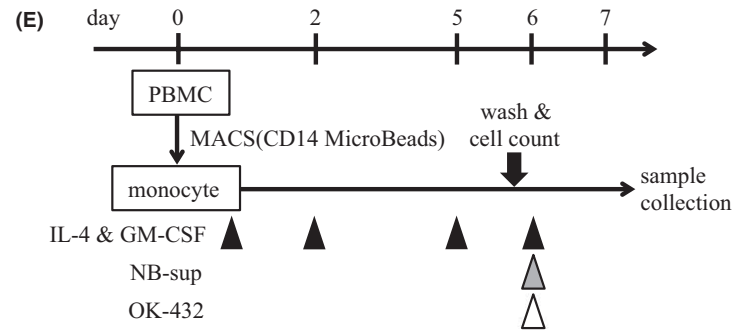
FIGURE 1 Cytokine production and surface expression of mature DC in the presence of culture supernatants from neuroblastoma cell lines (NB-sup). A, $CD14^+$ monocytes were incubated with IL-4 and GM-CSF in the presence or absence of 25% (v/v) NB-sup (NLF, GOTO or SH-SY5Y cells). After 6 days of culture, immature DC were cultured for an additional day with OK-432. On day 7, the cells and culture supernatants were harvested. B, CD14 and CD1a expression of mature DC was analyzed by flow cytometry. Representative histograms from seven experiments are shown. The gray-shaded histogram represents the isotype control; the unshaded histogram represents CD14 or CD1a. The percentages of positive cells are indicated in the upper right quadrant. C, Percentage of cells expressing CD14 (left panel) and CD1a (right panel). Horizontal lines represent the mean of seven healthy donors. D, Amounts of cytokines (IL-12, TNF-α and IL-10) secreted from mature DC were measured by ELISA and the CBA. E, $CD14^+$ monocytes were incubated with IL-4 and GM-CSF. After 6 days of culture, the cells were resuspended at 5×10^5 cells/mL in complete RPMI-1640 medium with IL-4 and GM-CSF. Then, immature DC were cultured for an additional day with OK-432 in the presence or absence of 25% (v/v) NB-sup (NLF or GOTO cells). On day 7, the culture supernatants were harvested. F, Amounts of IL-12 secreted from mature DC were measured by ELISA. *P*-values were calculated by the paired *t*-test (C, D and F). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. CBA, cytometric bead array; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor

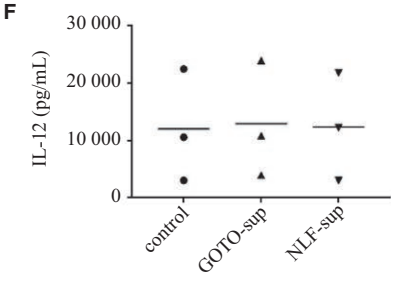

(A)
day
0
2
5
6
7
PBMC
MACS(CD14 MicroBeads)
monocyte
sample collection
IL-4 & GM-CSF
NB-sup
Maturation stimuli (OK432)


(B)
control
SH-SY5Y-sup
GOTO-sup
NLF-sup
8.9%
7.6%
23.2%
42.2%
CD14
93.4%
90.8%
58.9%
47.4%
CD1a


(C)
% of CD14+ cells
% of CD1a+ cells
control
SH-SY5Y-sup
GOTO-sup
NLF-sup


(D)
IL-12 (pg/mL)
TNF-α (pg/mL)
IL-10 (pg/mL)
control
SH-SY5Y-sup
GOTO-sup
NLF-sup


(E)
day
0
2
5
6
7
PBMC
MACS(CD14 MicroBeads)
wash & cell count
monocyte
sample collection
IL-4 & GM-CSF
NB-sup
OK-432


F
IL-12 (pg/mL)
control
GOTO-sup
NLF-sup

were able to elevate CD40 expression and increase IL-12 production by addition of IFN-γ. Based on these results, we concluded that although tolerogenic DC are induced in the NB tumor microenvironment and attenuate the antitumor effects of iNKT cells, interactions between iNKT cells and αGalCer-pulsed DC have the potential to restore the immunosuppression of tolerogenic DC through IFN-γ production.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Recombinant human IL-2 (Imunace; Shionogi, Osaka, Japan), IL-4 (PeproTech, Rocky Hill, NJ, USA), IL-6 (PeproTech), IL-12p70 (PeproTech), IFN-γ (PeproTech), GM-CSF (Gene Tech, Beijing, China), αGalCer (KRN7000; REGiMUUNE, Tokyo, Japan) and OK-432 (Chugai Pharmaceuticals, Tokyo, Japan) were used for cell culture and stimulation. An anti-human IL-6 Ab, anti-human IL-12p70 Ab, anti-mouse IgG1, and anti-mouse IgG2B were purchased from R&D Systems (Minneapolis, MN, USA). For cell purification, anti-FITC MicroBeads, CD3 MicroBeads and CD14 MicroBeads were purchased from Miltenyi Biotec (Bergisch Gladbach, German).

Fluorescein isothiocyanate-conjugated anti-TCR Vα24 (clone C15) and APC-conjugated anti-TCR Vβ11 (clone C21) Abs were purchased from Beckman Coulter (Brea, CA, USA). FITC-conjugated anti-CD14 (clone M5E2), CD83 (clone HB15e), PE-conjugated anti-CD14 (clone M5E2), CD80 (clone L307.4), PD-L1 (clone MIH1), APC-conjugated anti-CD40 (clone 5C3), CD86 (clone 2331 [FUN-1]), PB-conjugated anti-CD3 (clone UCHT1) and matching isotype controls were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). An APC-conjugated anti-CD1d (clone 51.1) Ab was purchased from eBioscience (Santa Clara, CA, USA). PB-conjugated anti-CD1a (clone HI149), CD16 (clone 3G8) and HLA-DR (clone L243) Ab were purchased from BioLegend (San Diego, CA, USA).

The surface phenotypes of PBMC and cultured cells were determined by a FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the FlowJo software program (Flowjo LLC). Live cells were analyzed by gating out propidium iodide-stained cells.

2.2 | Preparation of immature DC

Peripheral blood was collected from healthy volunteers after obtaining their informed consent. All experiments were performed in accordance with the Declaration of Helsinki and approved by the institutional review board. PBMC were separated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Fairfield, CT, USA). To generate moDC, the monocytes were purified using CD14 MicroBeads and cultured for 6 days in 12-well plates (1×10^6 cells/mL) using complete RPMI-1640 medium (containing L-glutamine, penicillin G, streptomycin, 2-mercaptoethanol, HEPES buffer and 10% heat-inactivated FCS) supplemented with 50 ng/mL recombinant human IL-4 and 800 U/mL GM-CSF.

2.3 | Tumor cell supernatants

The human NB cell line SH-SY5Y was kindly provided by Dr A. Nakagawara (Chiba, Japan).[24] GOTO and NLF cells were obtained from the Japanese Cancer Research Resources Bank.[25,26] All cell lines were cultured at 1×10^6 cells/mL in complete RPMI-1640 medium. After 24 hours of culture, supernatants were collected by centrifugation and stored at −80°C. The supernatants were used for monocyte culture. The volume of supernatants added to monocyte cultures was 1%, 5% or 25% of the final volume.

2.4 | Maturation of DC

After 6 days of culture, immature DC induced by the abovementioned method were cultured for an additional day with 0.1 KE/mL OK-432. On day 7, the cells and culture supernatants were harvested.

TABLE 1 Cell surface expression profiles of mature DC in the presence of 25% (v/v) NB-sup

	Control	SH-SY5Y-sup (25% [v/v])	GOTO-sup (25% [v/v])	NLF-sup (25% [v/v])
CD1a	1127 ± 499	1065 ± 500	425 ± 381**	337 ± 248**
CD1d	23.5 ± 8.7	19.6 ± 5.8	36.9 ± 19.4	38.6 ± 17.6
CD14	7.4 ± 3.4	12.9 ± 7.5	135 ± 115*	253 ± 188*
CD16	13.6 ± 9.1	33.0 ± 31.0	106 ± 76*	254 ± 153**
CD40	497 ± 343	500 ± 322	281 ± 166*	208 ± 63*
CD80	1338 ± 494	1400 ± 900	1087 ± 230	1090 ± 186
CD83	66.6 ± 8.4	69.5 ± 9.3	35.4 ± 19.2*	16.5 ± 10.8***
CD86	4053 ± 1040	4161 ± 903	3152 ± 1110	2041 ± 1087*
PD-L1	286 ± 74	342 ± 138	880 ± 526*	742 ± 396*
HLA-DR	884 ± 277	974 ± 327	1348 ± 449**	1508 ± 505**

Data represent the Δmean fluorescence intensity, MFI (n = 7), mean ± SD. ΔMFI = MFI (sample) − MFI (isotype).
Paired *t*-test, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.
DC, dendritic cells; PD-L1, programmed death-ligand 1; SD, standard deviation.

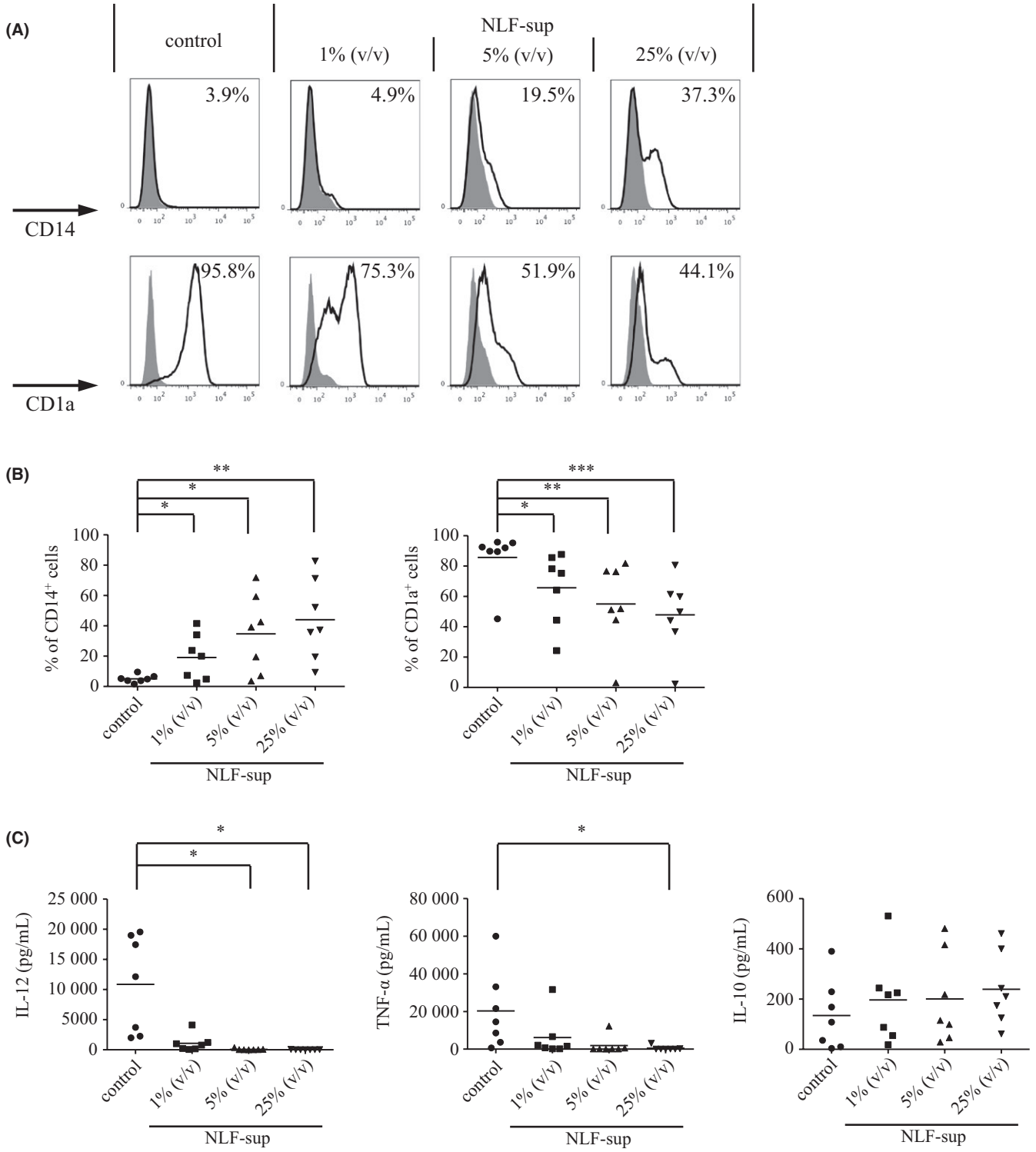


FIGURE 2 Cytokine production and cell surface molecule expression of mature DC in the presence of 1% (v/v), 5% (v/v) or 25% (v/v) NLF-sup. A, moDC were cultured under the same conditions described in Figure 1A. CD14 and CD1a expression of mature DC in the presence of 1% (v/v), 5% (v/v) or 25% (v/v) NLF-sup was analyzed by flow cytometry. Representative histograms from seven experiments are shown. The gray-shaded histogram represents the isotype control; the unshaded histogram represents CD14 or CD1a. Percentages of positive cells are indicated in the upper right quadrant. B, Percentages of cells expressing CD14 (left panel) and CD1a (right panel). Horizontal lines represent the mean of seven healthy donors. C, Amounts of cytokines (IL-12, TNF-α and IL-10) secreted from mature DC were measured by ELISA and the CBA. *P*-values were calculated by the paired *t*-test (B and C). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. CBA, cytometric bead array; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF, tumor necrosis factor

TABLE 2 Cell surface expression profiles of mature DC in the presence of NLF-sup

	Control	NLF-sup (1% [v/v])	NLF-sup (5% [v/v])	NLF-sup (25% [v/v])
CD1a	1167 ± 515	645 ± 404**	483 ± 359**	337 ± 248**
CD1d	22.7 ± 8.6	23.5 ± 7.6	34.5 ± 16.7	38.5 ± 17.6
CD14	7.4 ± 3.4	59.8 ± 45.3*	183 ± 176*	246 ± 191*
CD16	13.0 ± 9.2	101 ± 91*	167 ± 128*	259 ± 155**
CD40	506 ± 340	404 ± 189	298 ± 137	214 ± 62*
CD80	1361 ± 514	1487 ± 304	1241 ± 288	1116 ± 183
CD83	66.0 ± 8.7	44.1 ± 15.0*	24.3 ± 16.6**	15.8 ± 10.6***
CD86	4059 ± 1048	3402 ± 946	2414 ± 1221*	2222 ± 1183*
PD-L1	282 ± 72	558 ± 247*	730 ± 376*	749 ± 397*
HLA-DR	888 ± 280	1449 ± 583**	1546 ± 571**	1505 ± 506**

Data represent the ΔMFI (n = 7), mean ± SD. ΔMFI = MFI (sample) − MFI (isotype).
Paired *t*-test, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.
DC, dendritic cells; PD-L1, programmed death-ligand 1.

2.5 | Mixed leukocyte reaction assay

Functional activity of DC was determined by an allogeneic MLR assay using human T lymphocytes as responder cells. Allogeneic T cells were purified using CD3 MicroBeads. The MLR assays were carried out on a 96-well plate. A total of 1×10^5 T cells were stimulated by 2×10^4 immature DC. The culture supernatants were collected after 48 hours.

2.6 | Activation of iNKT cells

Peripheral blood mononuclear cells were cultured in complete RPMI-1640 medium in the presence of 100 U/mL recombinant IL-2 and 200 ng/mL αGalCer for 7 days. iNKT cells were purified using an FITC-labeled anti-Vα24 Ab and anti-FITC MicroBeads by MACS sorting. The purified iNKT cells were cultured in complete RPMI-1640 medium in the same concentration of recombinant IL-2 for 7 days after separation.

On day 14, iNKT cells were collected and cultured in a 96-well plate. For iNKT cell activation, 200 ng/mL αGalCer was added to the culture medium of immature DC, and 1×10^5 iNKT cells were stimulated with 2×10^4 αGalCer-pulsed DC. The supernatants were collected after 24 hours.

2.7 | Cytokine measurement

Culture supernatants were collected and stored at −80°C until measurements of cytokine levels. The levels of IFN-γ, IL-12p70 and TGF-β1 were determined using ELISA kits (BD Biosciences). IL-1β, IL-4, IL-6, IL-10, TNF-α and VEGF levels were determined using a BD Cytometric Bead Array System (BD Biosciences).

2.8 | Statistical analyses

Statistical analysis was performed using GraphPad Prism software, version 5.04. Error bars represent the standard derivation in all graphs. All *P*-values were two-sided, and *P*-values of less than 0.05 were considered to be statistically significant.

3 | RESULTS

3.1 | Effects of culture supernatant from NB cell lines (NB-sup) on DC differentiation

Monocytes isolated from healthy volunteers were incubated with IL-4 (50 ng/mL) and GM-CSF (800 U/mL) in the presence or absence of 25% (v/v) NB-sup (NLF, GOTO or SH-SY5Y cells). After 6 days of culture, immature DC were cultured for an additional day with 0.1 KE/mL OK-432. On day 7, the cells and culture supernatants were harvested (Figure 1A).

In the control culture, mature DC lost their CD14 expression (MFI, 7.4 ± 3.4) and showed upregulated CD1a expression (MFI, 1127 ± 499). However, the presence of GOTO cell supernatant (GOTO-sup) and NLF cell supernatant (NLF-sup) partially blocked both the downregulation of CD14 (135 ± 115 in GOTO-sup and 253 ± 188 in NLF-sup) and upregulation of CD1a (425 ± 381 in GOTO-sup and 337 ± 248 in NLF-sup), whereas the SH-SY5Y cell supernatant (SH-SY5Y-sup) had no effect (Figure 1B,C, and Table 1). Furthermore, GOTO-sup and NLF-sup downregulated CD40, CD83 and CD86 expression and upregulated CD16, PD-L1 and HLA-DR (Table 1, Figures S1,S2). We next measured the cytokine secretion by DC following stimulation with OK-432. Exposures to NLF-sup and GOTO-sup dramatically decreased IL-12 and TNF-α production, whereas no significant changes were found in the levels of IL-10 (Figure 1D).

To demonstrate the direct effect of the culture supernatant from tumor cells on DC, monocytes were incubated with IL-4 and GM-CSF without NB-sup. After 6 days of cultivation, the cells were resuspended in complete RPMI-1640 medium with IL-4 and GM-CSF. Then, immature DC were cultured for an additional day with OK-432 in the presence or absence of 25% (v/v) NB-sup (NLF or GOTO cells), and IL-12 secretion from

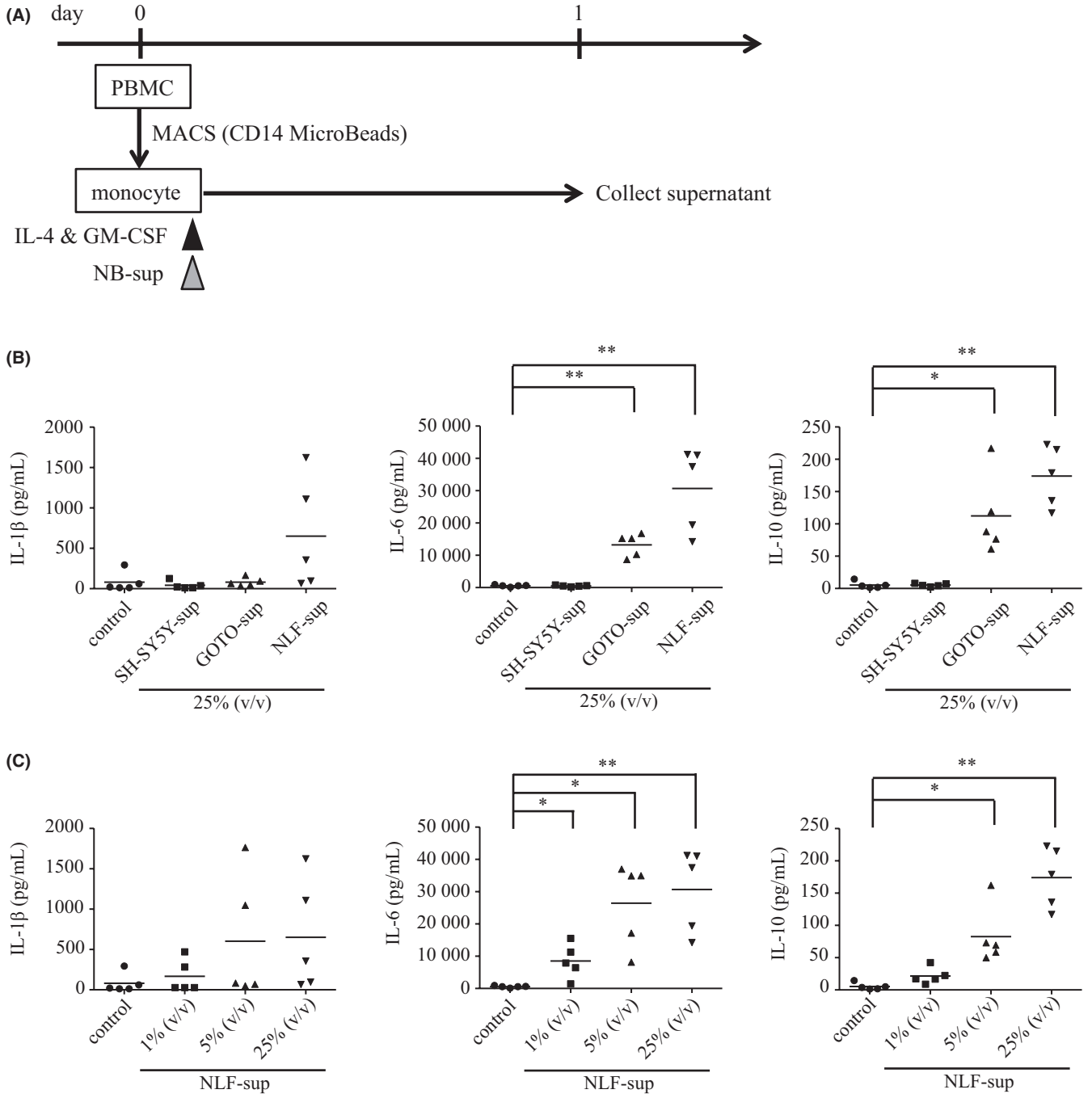


FIGURE 3 Cytokine production by monocytes in the presence of NB-sup. A, Monocytes isolated from healthy volunteers were incubated with IL-4 and GM-CSF in the presence of NB-sup for 24 h. The supernatants of 25% (v/v) NB-sup (NLF, GOTO or SH-SY5Y cells-treated monocytes (B) and 1% (v/v), 5% (v/v) or 25% (v/v) NLF-sup-treated-monocytes (C) were harvested, and the amounts of cytokines (IL-1β, IL-6 and IL-10) were measured by the CBA. *P*-values were calculated by the paired *t*-test (B and C). $^{*}P < 0.05$ and $^{**}P < 0.01$. CBA, cytometric bead array; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MACS, magnetic-activated cell sorting; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor

OK-432-stimulated DC was measured. Despite the exposure to NLF-sup and GOTO-sup, IL-12 production was not decreased (Figure 1E,F).

Next, we investigated the influence of the NLF-sup concentration on monocyte cultures. The loss of CD14 expression and upregulation of CD1a were abrogated by addition of 1% (v/v) NLF-sup (Figure 2A,B). In addition, IL-12 production after stimulation with OK-432 was clearly decreased (Figure 2C). TNF-α production tended to decrease in 1% (v/v) and 5% (v/v) NLF-sup groups, but not significantly. The other cell surface profiles of mature DC are shown in Table 2 and Figures S3 and S4. Based on these results, we concluded that NLF-sup and GOTO-sup inhibited the differentiation of monocytes to DC and decreased IL-12 and

TNF-α production. Furthermore, the inhibitory effect of NLF-sup was confirmed to be dose-dependent.

3.2 | Analyses of soluble factors in NB-sup

Some tumor cells produce pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which are associated with tumor development and progression.[27] IL-10, TGF-β1 and VEGF have been reported to hamper the differentiation of DC.[14-17] We measured the concentrations of these cytokines in NB-sup by the CBA. IL-1β, IL-6, TNF-α, IL-10 and TGF-β1 were not detected in NLF-sup, GOTO-sup or SH-SY5Y-sup. VEGF was detected in the tumor cell supernatants, but its concentration was almost the same in all three cell line supernatants (NLF-sup, 403.7 pg/mL; GOTO-sup, 66.2 pg/mL; SH-SY5Y-sup, 169.8 pg/mL).

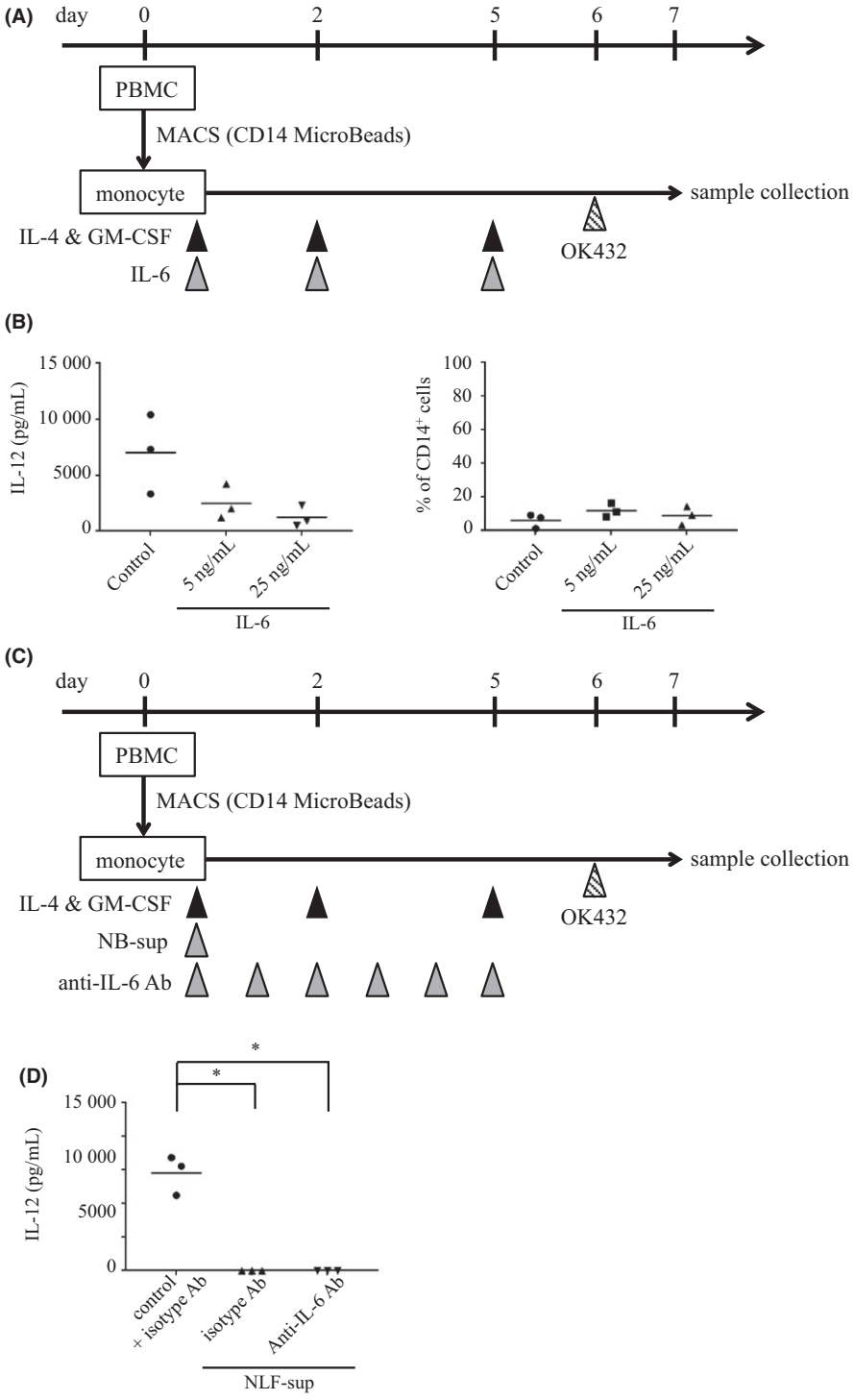


FIGURE 4 Effects of IL-6 on moDC. A, moDC were incubated in the presence or absence of recombinant human IL-6. After 6 days of culture, immature DC were cultured for an additional day with OK-432. On day 7, the cells and culture supernatants were harvested. B, Amounts of IL-12 secreted from mature DC were measured by ELISA, and CD14 expression in mature DC was analyzed by flow cytometry. C, moDC were incubated in the presence of 25% (v/v) NB-sup (NLF cells) with a neutralizing anti-IL-6 Ab (10 μg/mL) or isotype control mouse IgG2B Ab. D, Amounts of IL-12 secreted from mature DC were measured by ELISA. *P*-values were calculated by the paired *t*-test (B and D). **P* < 0.05. Ab, antibody; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ig, immunoglobulin; IL, interleukin; MACS, magnetic-activated cell sorting; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor

3.3 | Cytokine production by monocytes in the presence of NB-sup

As shown in Figure 3A, monocytes isolated from healthy volunteers were incubated with IL-4 (50 ng/mL) and GM-CSF (800 U/mL) in the presence of 1% (v/v), 5% (v/v) or 25% (v/v) NB-sup (NLF, GOTO or SH-SY5Y cells). After 24 hours of incubation, the supernatants were harvested, and the levels of cytokines (IL-1β, IL-6 and IL-10) secreted from monocytes were measured by the CBA. We found that monocytes produced increased levels of IL-6 and IL-10 after stimulation with NLF-sup and GOTO-sup, whereas SH-SY5Y-sup had no effect (Figure 3B). Furthermore, IL-6 and IL-10 secretion from monocytes in response to NLF-sup was dose-dependent (Figure 3C). In contrast, IL-1β production was more than 1000 pg/mL in two of five healthy donors when cultured in the presence of 5% or 25% (v/v) NLF-sup, but the remaining three donors showed markedly less IL-1β production (<500 pg/mL).

3.4 | Effects of IL-6 on moDC

Next, we investigated the effects of IL-6 on moDC. As shown in Figure 4A, moDC were incubated in the presence or absence of recombinant human IL-6. IL-6 addition to monocyte cultures tended to decrease IL-12 production after stimulation with OK-432, but CD14 expression was comparable with that in control DC (Figure 4B).

We also evaluated the effects of IL-6 neutralization in the moDC culture. As shown in Figure 4C, moDC were incubated in the presence of 25% (v/v) NLF-sup with a neutralizing anti-IL-6 Ab (10 μg/mL) or matching isotype control. IL-6 blockade in monocyte cultures with NLF-sup did not recover IL-12 production after stimulation with OK-432 (Figure 4D). Therefore, our data suggested that IL-6 was not the only factor that modulated DC functions.

3.5 | Cytokine production by T cells in the MLR assay with NB-sup-treated DC

Previous studies have shown that tolerogenic DC have the capacity to induce anergy in tetanus toxoid-specific memory $CD4^+$ T cells.[14] We examined the functional activity of NB-sup-treated DC by an allogeneic MLR assay using human T cells as responders. moDC were cultured under the same conditions described in Figure 1A. On day 7, T cells were purified from PBMC using CD3 MicroBeads. Allogeneic T cells and moDC were mixed at a ratio of 5:1, and the culture supernatants were collected after 48 hours (Figure 5A). The IFN-γ production of allogeneic T cells after stimulation with NLF-sup-treated DC was decreased significantly (Figure 5B).

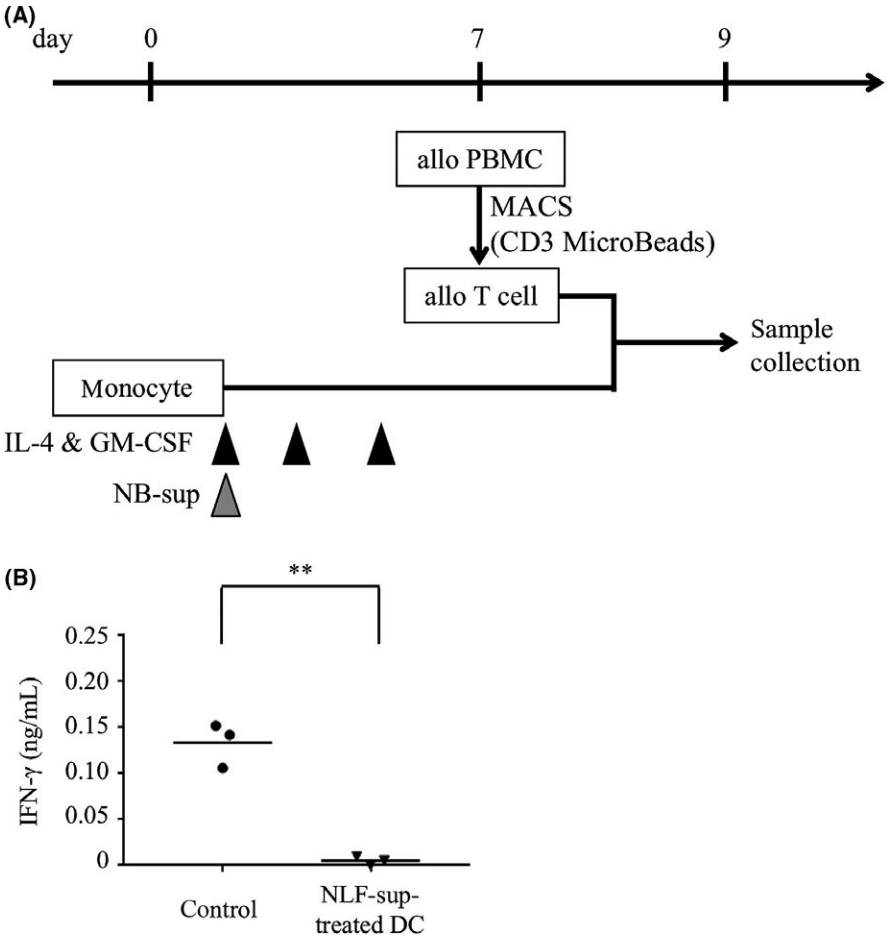
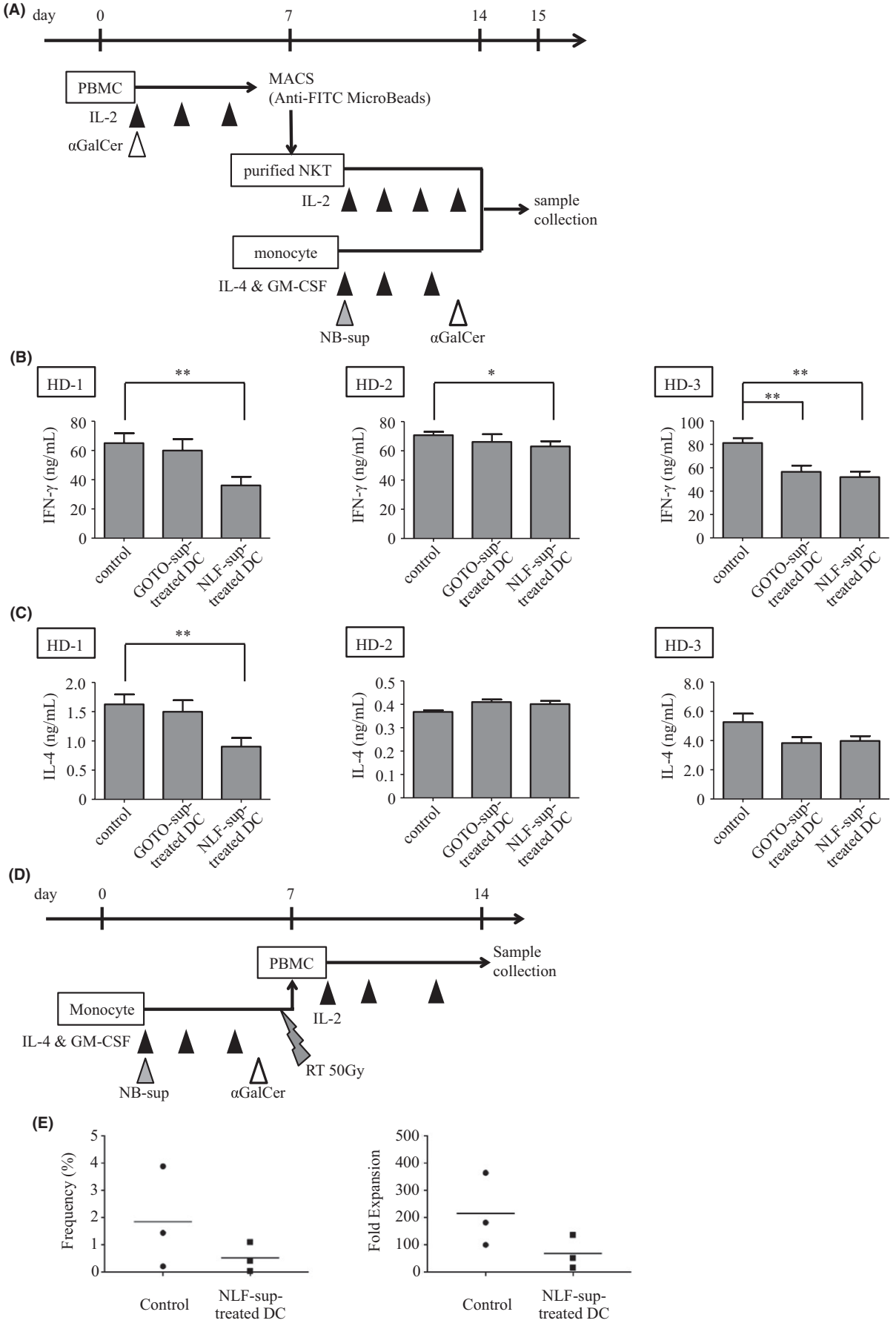


FIGURE 5 Cytokine production by T cells in the MLR assay with NB-sup-treated DC. A, moDC were cultured under the same conditions described in Figure 1A. Allogeneic T cells and moDC were mixed at a ratio of 5:1, and the culture supernatants were collected after 48 h. B, IFN-γ concentrations in culture supernatants were measured by ELISA. *P*-values were calculated by the paired *t*-test. $^*P < 0.05$ and $^{**}P < 0.01$. DC, dendritic cells; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; MLR, mixed lymphocyte reaction


(A)
day
0
7
14
15
PBMC
IL-2
αGalCer
MACS
(Anti-FITC MicroBeads)
purified NKT
IL-2
sample
collection
monocyte
IL-4 & GM-CSF
NB-sup
αGalCer
(B)
HD-1
HD-2
HD-3
IFN-γ (ng/mL)
control
GOTO-sup-treated DC
NLF-sup-treated DC
(C)
IL-4 (ng/mL)
(D)
PBMC
Sample collection
Monocyte
IL-2
IL-4 & GM-CSF
RT 50Gy
NB-sup
αGalCer
(E)
Frequency (%)
Fold Expansion
Control
NLF-sup-treated DC

3.6 | iNKT cell activation by NB-sup-treated, αGalCer-pulsed DC

We further evaluated whether NB-sup-treated DC could stimulate iNKT cells. As shown in Figure 6A, PBMC obtained from healthy donors were cultured with 100 U/mL IL-2 and 200 ng/mL αGalCer for 7 days. Representative profiles of iNKT cells are shown in Figure S5. After incubation with IL-2 and αGalCer, the population of $V\alpha24^+V\beta11^+$ cells was expanded efficiently, and $V\alpha24^+$ cells were purified by MACS sorting. Purified iNKT cells were cultured for a further 7 days and then stimulated with NB-sup-treated, αGalCer-pulsed DC in a 96-well plate. After 24 hours, the culture supernatant was collected, and IFN-γ concentrations were measured by an ELISA.

The IFN-γ production of iNKT cells after stimulation with NLF-sup-treated DC was decreased in all three donors. In contrast, IFN-γ production induced by GOTO-sup-treated DC was decreased in only one of three donors (Figure 6B). Because iNKT cells secrete both Th1 and Th2 cytokines, we measured IL-4 concentrations by the CBA. In NLF-sup-treated DC, IL-4 production was decreased in only one of three donors, and there were no significant changes in GOTO-sup-treated DC (Figure 6C). These results indicated that NLF-sup-treated DC had a decreased ability to activate iNKT cells, and IFN-γ, but not the IL-4, production was reduced in iNKT cells. Although the inhibitory effects of GOTO-sup on DC were similar to those of NLF-sup (Figure 1), the ability of GOTO-sup-treated DC to activate iNKT cells was not decreased.

Next, we examined the effect of NLF-sup-treated DC on primary iNKT cells. As shown in Figure 6D, PBMC and irradiated αGalCer-pulsed DC were co-cultured at a ratio of 10:1. After 7 days of co-culture, the cells were collected and the percentages of $V\alpha24^+V\beta11^+$ iNKT cells were analyzed by flow cytometry. In all three donors, iNKT cells stimulated with NLF-sup-treated, αGalCer-pulsed DC tended to decrease. However, the decrease did not reach statistical significance (Figure 6E).

3.7 | Effect of IL-12 on IFN-γ production of iNKT cells after stimulation with αGalCer-pulsed DC

The production of IFN-γ by iNKT cells in response to αGalCer requires IL-12 produced by DC and direct contact between iNKT cells and DC through CD40/CD40L interactions.[28] Figures 1D and 2D show that IL-12 secretion by NLF-sup or GOTO-sup-treated DC was dramatically decreased compared with control DC. Figure 6B shows that the IFN-γ production of iNKT cells after stimulation with NLF-sup-treated, αGalCer-pulsed DC was reduced to 70% on average in three donors compared with control DC.

We analyzed the effect of IL-12 addition on iNKT cells stimulated with αGalCer-pulsed DC. The culture conditions of iNKT cells and moDC remained unchanged. On day 14, iNKT cells were stimulated with NLF-sup-treated, αGalCer-pulsed DC and various concentrations of IL-12 (0.1, 0.5 and 2.5 ng/mL). As shown in Figure 7A, the presence of IL-12 increased the IFN-γ production of iNKT cells in all three donors. Without IL-12, there were significant differences in IFN-γ production between control DC and NLF-sup-treated DC. By addition of IL-12, the IFN-γ secretion of iNKT cells stimulated with NLF-sup-treated DC was significantly increased to a level comparable with that under stimulation with control DC. These data indicated that IL-12 improved the ability of NLF-sup-treated DC to activate iNKT cells to the same level as control DC.

Next, we evaluated the effects of IL-12 neutralization on iNKT cell stimulation. On day 14, iNKT cells were stimulated with NLF-sup-treated, αGalCer-pulsed DC in the presence of a neutralizing anti-IL-12p70 Ab (10 μg/mL) or matching isotype control. By addition of the anti-IL-12p70 Ab, IFN-γ production of iNKT cells after stimulation with αGalCer-pulsed DC was decreased in both control and NLF-sup-treated DC (Figure 7B). Furthermore, we examined the IL-12 concentration in the culture supernatant of iNKT cells after stimulation with αGalCer-pulsed DC. IL-12 was reduced in the NLF-sup-treated DC of all three donors (Figure 7C). These results indicated that IL-12 had a pivotal role in IFN-γ production of iNKT cells.

3.8 | Effect of IFN-γ on moDC

It is well known that CD40 expression on DC is needed for IL-12 production by DC. Moreover, αGalCer-pulsed DC upregulate the expression of CD40L on iNKT cells, which in turn stimulates CD40 on DC to produce IL-12 that induces IFN-γ production from iNKT cells.[29] On the other hand, IFN-γ has been previously shown to be a strong CD40 inducer.[30]

Therefore, we investigated the influence of IFN-γ on moDC. As shown in Figure 8A, IFN-γ (10 ng/mL) was added to the culture

FIGURE 6 iNKT cell activation by NB-sup-treated, αGalCer-pulsed DC. A, On day 0, PBMC were cultured with IL-2 and αGalCer for 7 days. On day 7, Vα24-FITC-stained cells were purified by autoMACS Pro Separator positive selection. The purified iNKT cells were cultured for an additional 7 days. moDC were cultured under the same conditions described in Figure 1A and 200 ng/mL αGalCer was added to the culture medium of DC on day 13. iNKT cells and moDC were mixed at a ratio of 5:1, and the supernatants were collected after 24 h. Stimulations were performed in triplicate. Three independent experiments were performed. B and C, IFN-γ and IL-4 concentrations in supernatants were measured by ELISA and the CBA. Error bars represent the standard deviation. *P*-values were calculated by the unpaired *t*-test. $^*P < 0.05$ and $^{**}P < 0.01$. D, moDC were cultured under the same conditions described in Figure 1A, and 200 ng/mL αGalCer was added to the culture medium of DC on day 6. PBMC and irradiated αGalCer-pulsed DC were co-cultured at a ratio of 10:1 for 7 days. The cells were collected and counted on day 14, and the percentages of $V\alpha24^+V\beta11^+$ iNKT cells were analyzed by flow cytometry. E, Percentages and fold expansion of iNKT cells. *P*-values were calculated by the paired *t*-test. CBA, cytometric bead array; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; iNKT cells, invariant natural killer T cells; MACS, magnetic-activated cell sorting; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor

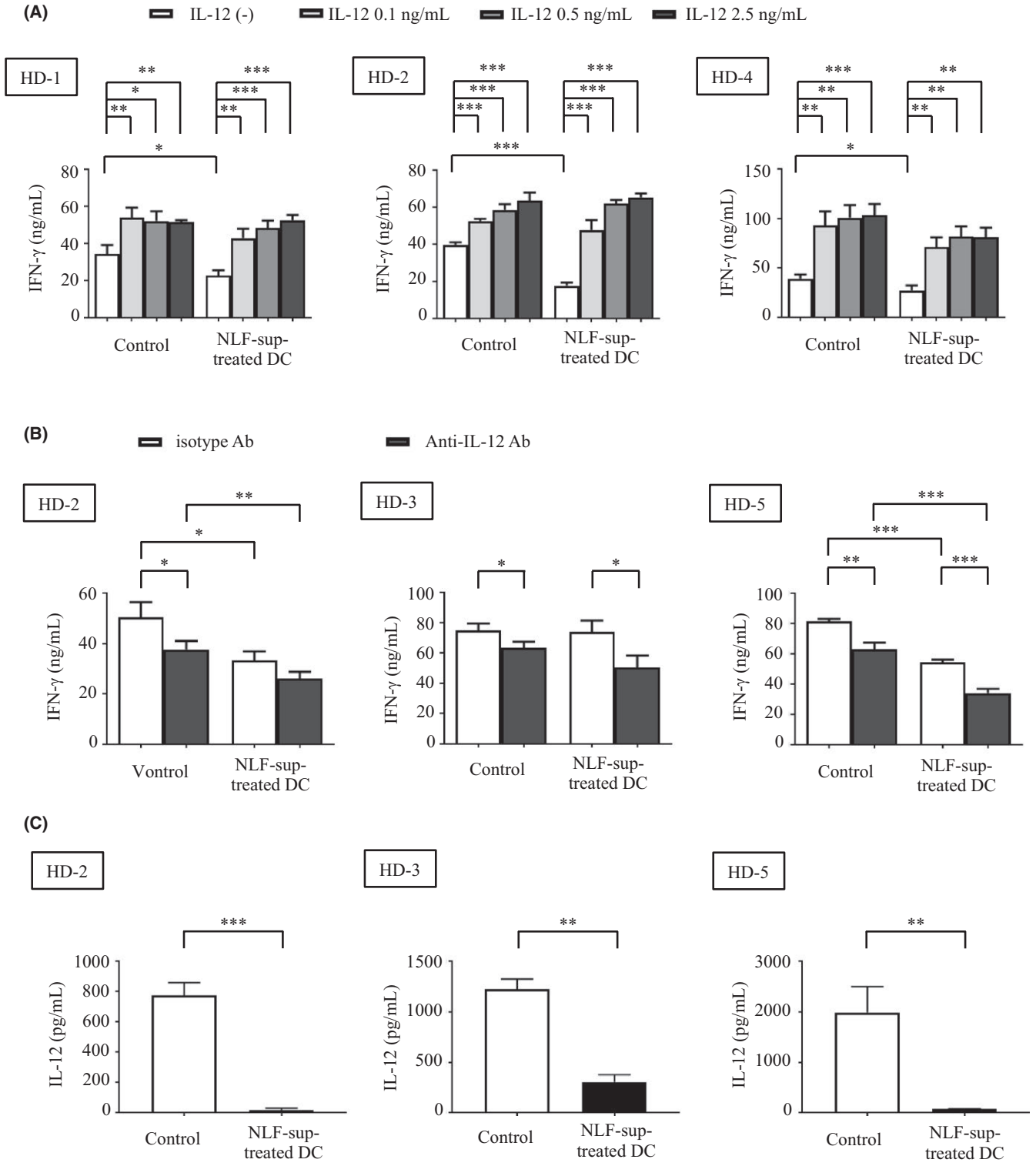


FIGURE 7 Effect of IL-12 on IFN-γ production by iNKT cells after stimulation with αGalCer-pulsed DC. iNKT cells and moDC were cultured under the same conditions described in Figure 6A. A, iNKT cells and moDC were mixed at a ratio of 5:1 with various concentrations of IL-12. The supernatants were collected after 24 hours. B, iNKT cells and moDC were mixed at a ratio of 5:1 with anti-IL-12p70 Ab (10 μg/mL) or isotype control mouse IgG1. The culture supernatants were collected after 24 hours. C, iNKT cells and moDC were mixed at a ratio of 5:1, and the culture supernatants were collected after 24 hours. A-C, Stimulations were performed in triplicate. Three independent experiments were performed. The IFN-γ concentration in culture supernatants was measured by an ELISA. Error bars represent the standard deviation. *P*-values were calculated by the unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; Ig, immunoglobulin; IL, interleukin

medium of moDC on day 5. Addition of IFN-γ to NLF-sup-treated DC increased IL-12 production (510-1356 pg/mL) after stimulation with OK-432. Furthermore, CD40 expression was significantly elevated and comparable with that in control DC (Figure 8B). These findings suggest that, in the interactions between iNKT cells and NLF-sup-treated DC, IFN-γ secreted by iNKT cells elevates CD40 expression in NLF-sup-treated DC and CD40 upregulation induces IL-12 production by NLF-sup-treated DC. Therefore, IFN-γ production from iNKT cells after stimulation with NLF-sup-treated, αGalCer-pulsed DC was reduced to only 70% on average of the level in control DC (Figure 6B), despite the fact that they were resistant to maturation stimuli.

4 | DISCUSSION

Dendritic cells play key roles in initiating and maintaining antitumor immunity, bridging innate and adaptive immune responses, and sustaining immune tolerance. As professional APC, immunogenic DC activate T cells through costimulatory molecules such as CD80 and CD86. In contrast, tolerogenic DC contribute to tolerance rather than immunity through induction of T-cell anergy and the generation of regulatory T cells.[31] It has been shown that several factors, including growth factors, cytokines, chemokines, tumor antigens, gangliosides, prostanoids, lactic acid and exposure to soluble factors produced by tumor cells modulate DC functions and induce tolerogenic DC differentiation.[11-17, 32] However, the cell surface expression profiles of tolerogenic DC have been controversial. Torres-Aguilar et al[14] reported that mature tolerogenic DC had similar expression of HLA-DR, CD1a, CD80, CD83 and CD86, lower expression of CD40, and higher expression of CD14 and CD16 than control DC. Motta et al[12,13] showed that expression of CD14, CD16 and CD68 was higher and expression of CD1a was lower in the presence of K562 cell supernatants than in their absence. Furthermore, DC activated with TNF-α had lower expression of CD80 and CD83 than control DC.[12,13] Lindenberg et al[15] reported that IL-10-conditioned mature DC expressed lower levels of CD80, CD83 and CD86, whereas expression of PD-L1 was elevated compared with control mature DC. In terms of cytokine secretion, all three authors showed that mature tolerogenic DC released lower levels of IL-12 than control mature DC.

In the current study, we found that expression of CD14, CD16, PD-L1 and HLA-DR on GOTO-sup- and NLF-sup-treated DC was higher, and CD1a, CD40, CD83 and CD86 expression was lower than on control DC, while there was no significant difference in their expression between SH-SY5Y-sup-treated and control DC (Figure 1B,C, Figures S1,S2). In addition, the exposures to NLF-sup and GOTO-sup dramatically decreased IL-12 and TNF-α production in DC (Figure 1D). Human monocytes are generally defined as $CD14^+$ cells, whereas DC are defined as $CD1a^+$ cells.[12] When monocytes were incubated with IL-4, GM-CSF and tumor supernatants, an increase in the $CD14^+$ population was observed on average. Despite the downregulation of CD14 and upregulation of CD1a even in the presence of NB-sup in some cases, IL-12 production was dramatically suppressed compared with control DC. These findings indicated that the expression of surface molecules on tolerogenic DC differed among individuals, while tolerogenic DC produced low levels of IL-12 in all subjects.

Next, we attempted to identify the inhibitory factors in NB-sup. Although the tumor microenvironment has been shown to be abundant with cytokines that modulate DC functions, IL-1β, IL-6, TNF-α, IL-10 and TGF-β1 were not detected in NLF-sup or GOTO-sup. Only VEGF was detected in NB-sup, which has no inhibitory effect on DC

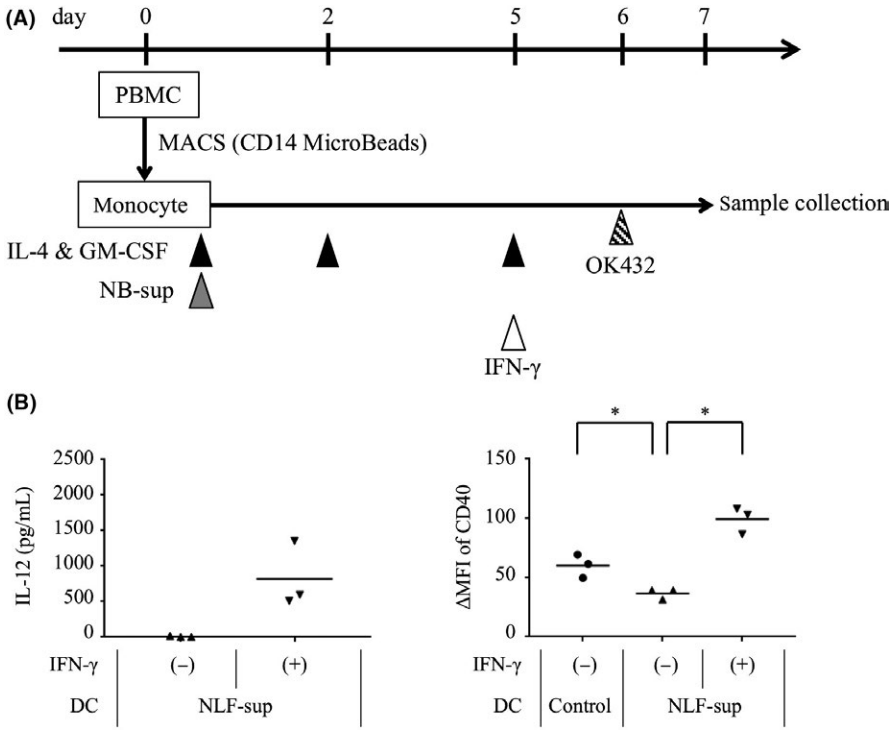


FIGURE 8 Effect of IFN-γ on moDC. A, moDC were cultured under the same conditions described in Figure 1A. On day 5, IFN-γ (10 ng/mL) was added to the culture medium of moDC and 24 hours later, cells were treated with OK-432 for an additional day. On day 7, the cells and culture supernatants were harvested. B, Amounts of IL-12 secreted from mature DC were measured by ELISA, and CD40 expression in mature DC was analyzed by flow cytometry. *P*-values were calculated by the paired *t*-test. $^*P < 0.05$. DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; MACS, magnetic-activated cell sorting; PBMC, peripheral blood mononuclear cells

differentiation. Thus, these cytokines were not the factors underlying the inhibition of DC by NB cell lines.

Motta et al[12] reported that TNF-α and IL-1β production by monocytes during differentiation increases in the presence of tumor cell supernatants. Furthermore, the presence of IL-1β during monocyte differentiation partially prevents the loss of CD14 and inhibits the appearance of CD1a. Neutralization of IL-1β in such cultures partially reverses CD14 expression but is not sufficient to modify CD1a expression. We measured the amount of cytokines secreted from monocytes cultured with NB-sup (Figure 3). When cultured with NLF-sup or GOTO-sup, the concentration of IL-6 was more than 10 ng/mL, and that of IL-10 was approximately 100 pg/mL. In contrast, IL-1β secretion varied among individuals (Figure 3B), and TNF-α secretion was comparable with that in control DC (data not shown). IL-6 has been shown to act as an immunosuppressive cytokine in DC differentiation through STAT3 activation.[33] Moreover, Bharadwaj et al[34] showed that human pancreatic cell line BxCM contains high levels of IL-6 and G-CSF. Depleting IL-6 or G-CSF from BxCM and blocking BxCM-induced STAT3 activation reverses the DC-inhibitory properties.[34] In our study, IL-6 addition to moDC cultures decreased IL-12 production after maturation stimuli, but CD14 expression was comparable with that in control DC (Figure 4B). Furthermore, IL-6 blockade in monocyte cultures with NB-sup did not recover IL-12 production after stimulation with OK-432 (Figure 4D). Therefore, our data suggest that IL-6 may be an important factor, but not the only factor, which modulates DC functions.

We also evaluated DC functions by stimulation of iNKT cells and allogeneic T cells. iNKT cells rapidly produce high amounts of both Th1 and Th2 cytokines upon stimulation, playing an important role in autoimmunity, infections and antitumor immunity.[35] αGalCer-activated iNKT cells exert a strong antitumor effect against various types of malignant tumors.[21] iNKT cell infiltration into primary tumors serves as a prognostic factor for a favorable outcome in a subset of patients with metastatic NB.[36] However, reports have shown selective decreases in the number and functions of iNKT cells in patients with advanced cancer.[37,38] IFN-γ acts on NK cells to eliminate MHC-negative tumors and on CD8 cytotoxic T cells to kill MHC-positive tumors, resulting in tumor eradication.[39] The production of IFN-γ by iNKT cells in response to αGalCer requires IL-12 production by DC and direct contact between iNKT cells and DC through CD40/CD40L interactions.[28,29] We hypothesized that NB-sup-treated DC could not stimulate iNKT cells sufficiently because only a small amount of IL-12 was secreted from NB-sup-treated DC. In contrast to our expectations, IFN-γ production in iNKT cells after stimulation with NLF-sup-treated αGalCer-pulsed DC was reduced to only 70% on average of the level in control DC. Furthermore, in GOTO-sup-treated DC, IFN-γ production was decreased in only one of three donors (Figure 6B). Tolerogenic DC expressed low levels of costimulatory molecules and high levels of co-inhibitory molecules. The differences in the expression of these molecules might have inhibited the secretion of IFN-γ by iNKT cells. By comparing the expressed molecules in NLF-sup- and GOTO-sup-treated DC, the former had lower levels of CD86 expression than the latter. Therefore, IFN-γ production in iNKT cells might have been suppressed after stimulation with NLF-sup-treated, αGalCer-pulsed DC but not after stimulation with GOTO-sup-treated αGalCer-pulsed DC.

We next analyzed the effect of IL-12 addition on iNKT cell stimulation with αGalCer-pulsed DC. The addition of exogenous IL-12 rescued IFN-γ production by iNKT cells when stimulated with NLF-sup-treated DC (Figure 7A). Moreover, inhibition of IL-12 downregulated IFN-γ production from ligand-activated iNKT cells (Figure 7B). However, data in Figure 8 suggested that IFN-γ secreted by iNKT cells elevated CD40 expression in DC and CD40 upregulation induced IL-12 production by DC, which may improve the functions of tolerogenic DC.

In summary, treatment with NLF-sup and GOTO-sup induces the secretion of IL-6 and IL-10 by monocytes and generates tolerogenic DC. Tolerogenic DC secrete little IL-12 and TNF-α after maturation stimuli and have a decreased ability to activate iNKT cells. Furthermore, NLF-sup-treated DC elevate CD40 expression and increase IL-12 production by addition of IFN-γ, and the interactions between iNKT cells and αGalCer-pulsed DC have the potential to recover the immunosuppression of tolerogenic DC.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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