

Establishment of Synergistic Chemoimmunotherapy for Head and Neck Cancer Using Peritumoral Immature Dendritic Cell Injections and Low-Dose Chemotherapies



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Abstract

The lack of available tumor antigens with strong immunogenicity, human leukocyte antigen restriction, and immunosuppression via regulatory T-cells (Tregs) and myeloid-derived suppressor cells are limitations for dendritic cell (DC)-based immunotherapy in patients with advanced head and neck cancer (HNC). We sought to overcome these limitations and induce effective antitumor immunity in the host. The effect of low-dose docetaxel (DTX) treatment on DC maturation was examined in an *ex vivo* study, and a phase I clinical trial of combination therapy with direct peritumoral immature DC (iDC) injection with OK-432 and low-dose cyclophosphamide (CTX) plus DTX was designed. Low-dose DTX did not negatively affect iDC viability and instead promoted maturation and IL-12 production. Five patients with metastatic or recurrent HNC were enrolled for the trial. All patients experienced grade 1 to 3 fevers. Intriguingly, elevated CD8⁺ effector T-cells and reduced Tregs were observed in four patients who completed two treatment cycles. All patients were judged to have progressive disease, but tumor regressions were observed in a subset of targeted metastatic lesions in two of five patients. Our results show that the combination of direct peritumoral iDC injection with OK-432 and low-dose CTX plus DTX is well tolerated and should give rise to changing the immune profile of T-cell subsets and improvement of immunosuppression in advanced HNC patients. Additionally, our *ex vivo* data on the effect of low-dose DTX treatment on DC maturation may contribute to developing new combination therapies with low-dose chemotherapy and immunotherapy.

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Introduction

Dendritic cell (DC)-based immunotherapy is anticipated to be an effective therapeutic strategy for advanced head and neck cancer (HNC). DCs play an important role in the initiation of antitumor immunity by increasing tumor antigen-specific cytotoxic T lymphocytes [1]. In general, *ex vivo* DC generation requires immunogenic epitopes of tumor antigens to elicit antitumor immunity. However, there are currently few well-defined tumor antigens for DC vaccines for HNC [2]. Direct injection of immature DCs (iDCs) into tumor tissue along with standard therapies, including chemotherapy and radiotherapy, has been used to treat advanced malignancies [3,4]. This strategy requires *in vivo* DC pulsing but has several advantages relative to *ex vivo* DC generation: 1) the expression of a specific tumor

antigen is not required, and 2) there is no human leukocyte antigen (HLA) restriction. However, no studies to date have reported therapy using direct peritumoral iDC injection in HNC.

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HNC progression is associated with immune suppression in the host [5]. In advanced-stage HNC, tumor cells acquire immune resistance, escaping from antitumor immune responses by decreasing their immunogenicity and HLA class I expression [1]. Moreover, immunosuppressive components, including regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs), support immune escape by several mechanisms: 1) inhibition of activated T cells by cell-cell contact and production of the immunosuppressive cytokines IL-10 and TGF β and 2) functional suppression of activated DCs [6,7]. Therefore, immune resistance remains a serious problem that must be resolved to achieve clinical benefit from DC-based immunotherapy.

Increasing studies are focusing on the synergistic benefits of combining cancer immunotherapy with chemotherapy [8]. OK-432, an immunotherapeutic agent derived from penicillin-killed *Streptococcus pyogenes*, received clinical consensus for HNC treatment [9]. OK-432 enhances DC secretion of proinflammatory cytokines to stimulate CD8⁺ T cells, leading to enhanced antitumor immunity in the host [10]. Moreover, low-dose chemotherapy is favored in combination with DC-based immunotherapy because high-dose exposure to chemotherapeutic agents inevitably decreases the number of neutrophils and lymphocytes, leading to a deleterious effect on the host immunity [11]. Cyclophosphamide (CTX) and docetaxel (DTX) exhibit immunomodulatory effects to upregulate antitumor immune responses in a dose-dependent manner [11]. Both drugs induce immunogenic cell death (ICD) in tumor cells, thereby initiating and stimulating the adaptive and innate immune responses [12,13]. Moreover, low-dose CTX depletes Tregs [14]. Unlike CTX, DTX can inhibit MDSCs, but not Tregs, in tumor-bearing hosts [15]. These observations indicate that direct iDC injection with OK-432 and low-dose chemotherapy might provide antitumor effects by attenuating immunosuppression. However, whether this strategy is a feasible treatment for advanced HNC has not been investigated.

To overcome the limitations of the performance of DC-based immunotherapy in HNC, we initiated combination therapy with direct peritumoral iDC injections with OK-432 and low-dose CTX plus DTX as a clinical trial. This combination was shown to be acceptably safe and should alter the immune profile of T-cell subsets with improvement of immunosuppression in patients with advanced HNC. In addition, our *ex vivo* data on the effect of low-dose DTX treatment on DC maturation may contribute to developing new combination therapies with low-dose chemotherapy and immunotherapy.

Materials and Methods

Cell Lines, Cell Culture, and Cell Viability Assay

The HSC4 and Ca9-22 human oral squamous cell carcinoma cell lines, which expressed HLA-A*2402 by HLA typing, were cultured as previously described [16,17]. Cells were treated with DMSO or DTX (3.125 μ M or 12.5 μ M) for 48 hours. The culture supernatant (CS) was collected for *ex vivo* co-culture assays. Cell viability was determined by scoring Trypan blue uptake.

Preparation of iDCs, DC Maturation, and DC Activation

Peripheral blood mononuclear cells (PBMCs) from HLA-typed healthy donors were separated by centrifugation over Ficoll-Paque Plus (GE Healthcare), and monocytes were enriched by adherence to a plastic culture flask for 60 minutes at 37°C. For induction of iDCs,

adherent cells were cultured for 5 days with 1000 U/ml GM-CSF (CellGenix) and IL-4 (COSMO BIO) in AIM-V (Thermo Fisher Scientific). Thereafter, iDCs were incubated in the CS of low-dose DTX-treated tumor cells for 24 hours to promote maturation. DC activation was induced by culture in AIM-V with 0.1 KE/ml OK-432 (Chugai Pharmaceutical).

For generation of iDCs under GMP conditions, blood samples were collected by leukapheresis (Fresenius Kabi) 1 month after the termination of conventional therapy. All subsequent operations were performed under GMP conditions in the Cell Processing Center. Mononuclear cells from 50 ml of the leukapheresis products were separated by Ficoll-Paque Premium gradient centrifugation (GE Health Care), washed in PBS (Thermo Fisher Scientific), and plated in flasks. After 1 hour, nonadherent cells were removed by PBS washing. Adherent monocytes were cultured for 5 days in AIM-V medium with 5 ng/ml of GM-CSF and 5 ng/ml of IL-4 at 37°C in a humidified incubator containing 5% CO₂. The iDCs were harvested, washed in PBS, and resuspended in AIM-V medium. Inspection of endotoxin contamination was also performed in each collected sample.

Flow Cytometry

Cells were analyzed for the expression of cell-surface markers on a BD Accuri C6 flow cytometer or Attune Acoustic Focusing Cytometer. After Fc blocking, PBMCs were labeled with specific antihuman monoclonal antibodies against the following molecules: 1) CD11c, CD80, CD86, CD83, and HLA-DR or 2) CD3, CD4, CD8, CD11b, CD14, CD45RO, CD25, CD62L, and CD127. All antibodies were directly conjugated to FITC, PE, PE-Cy7, or APC. We used 7-amino-actinomycin D (7-AAD) for exclusion of nonviable cells. Appropriate isotype control antibodies, such as IgG1 κ , IgG2a, and IgG2b, were included in all assays. Data were analyzed using the FlowJo software V9.

Real-Time RT-PCR

Quantitative PCR analysis was performed as previously described [17]. The primers used were as follows: human *GAPDH* (sense, 5'-GCACCGTCAAGGCTGAGAAC-3'; antisense, 5'-ATGGTGGTGAAGACGCCAGT-3'), human *IL12A* (sense, 5'-GCACAGTGGAGGCCTGTTTA-3'; antisense, 5'-GCCAGGCAACTCCCATTAGT-3'), and human *IL12B* (sense, 5'-AGA ACTTGCAGCTGAAGCCA-3'; antisense, 5'-CCTGGACCTGAACGCAGAAT-3'). mRNA levels were normalized to GAPDH mRNA levels in the same sample. The relative expression levels of target genes were determined by the 2^{- $\Delta\Delta$ CT} method.

Enzyme-Linked Immunosorbent Assay (ELISA)

CS was collected from iDCs cultured in CS of DMSO or low-dose DTX-treated head and neck squamous cell carcinoma (HNSCC) cells for 24 hours and then used for ELISA. IL-12 was measured using the Human IL-12 p70 Quantikine ELISA kit (R&D System, Minneapolis, MN). All ELISA samples were read on the Multi-Microplated Reader (CORONA, Ibaragi, Japan).

Study Design of the Phase I Trial

A phase I trial study was performed at the University of Yamanashi Hospital, Japan. The primary endpoint was adverse events graded according to the NCI-Common Toxicity Criteria version 3.0. The secondary endpoint was immune induction after chemoimmunotherapy.

The clinical study was approved by the ethical committee of University of Yamanashi (Institutional Review Board number: 631), and written informed consent was obtained from all subjects. The trial was carried out in accordance with the Declaration of Helsinki on experimentation on human subjects and registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN000003725) and ClinicalTrials.gov (NCT01149902).

Patients

For the clinical trial, inclusion criteria for patients were as follows: a) relapsed and refractory HNC; b) ECOG performance status of 0 or 1; c) no therapy 4 weeks before the initiation of the trial; d) age \geq 20 years; e) tumor lesions accessible to intratumoral injection of DCs; f) adequate oral intake; and g) normal organ and marrow functions. Exclusion criteria were a) age $<$ 20; b) presence of pulmonary, cardiac, or other systemic diseases limiting patient survival; c) history of systemic steroid treatment; d) positive status for hepatitis B or C viruses or HIV; e) clinically active infection; or f) psychiatric illness.

Therapeutic Regimen for Clinical Trial

Low-dose CTX (50 mg/day), used to achieve immune suppression in the host, was orally administered from day 1 to day 7. Low-dose DTX (30 mg/m²) was administered by intravenous drip infusion for 1 h on day 5. Patients received iDC injections on days 6 and 13. The first injection was performed 1 day after low-dose DTX administration. To promote DC activation, OK-432 (5 KE/body) was injected into the same sites where the iDCs were injected. One week elapsed between the starting points of each cycle. Enrolled patients received up to two cycles of treatment.

Clinical Assessment

To establish the baseline, all patients underwent a complete history examination, physical examination, computed tomography (CT), magnetic resonance imaging (MRI), and laboratory blood tests before treatment was initiated. Pathological parameters were expressed according to the TNM classification of the International Union Against Cancer. Radiological imaging was repeated after the second cycle of chemioimmunotherapy. Clinical response in each patient was determined by otolaryngological examination, CT, and/or MRI assessments.

Delayed-Type Hypersensitivity Test

For immunological monitoring of cell-mediated immunity, the skin reaction of delayed-type hypersensitivity (DTH) was assessed using tuberculin-purified protein derivative (PPD). The diameters of induration were measured 48 hours after intradermal injection of tuberculin PPD at baseline and 1 week after each cycle. Positive skin reaction was defined as \geq 5 mm of induration.

Collection of PBMCs

Blood samples of patients (50 ml) were collected at baseline, after the first cycle, and after the second cycle. Blood was drawn into heparinized tubes and centrifuged on Ficoll-Paque gradients. PBMCs were used for analyzing different subsets of T cells.

Statistical Analysis

All averages were calculated as mean. Paired samples were evaluated by Wilcoxon paired signed-rank test, and correlations were calculated by the Spearman test in GraphPad Prism 7.

Results

Effect of Low-Dose DTX on iDCs

To supply tumor antigens to iDCs *in vivo*, we selected low-dose DTX treatment to induce tumor apoptosis because low-dose CTX does not show cytotoxicity against HNC [18–20]. To determine the optimal dose in *ex vivo* experiments, we first investigated the cytotoxicity of low-dose DTX on iDCs. Two different human HNC cell lines (HSC4 and Ca9-22) that show HLA-A24 expression were exposed to various DTX concentrations for 24 hours. Following treatment with 3.125 μ M DTX, the viabilities of HSC4 and Ca9-22 cells were 79.3% and 92.5%. However, most tumor cells were killed upon treatment with 12.5 μ M DTX (Supplementary Figure 1A). We thus defined low-dose and high-dose concentrations as 3.125 μ M and 12.5 μ M, respectively.

To clarify the cytotoxicity of DTX on iDCs, we collected iDCs from healthy donors with HLA-A24 and cultured them with CS from HSC4 cells treated with DMSO or various concentrations of DTX for 24 hours (Figure 1A). Compared with CS from control DMSO-treated HSC4 cells, CS from low-dose DTX-treated HSC4 cells significantly increased the proportion of iDCs. Interestingly, CS from high-dose DTX-treated HSC4 cells reduced the proportion of iDCs compared with CS from low-dose DTX-treated HSC4 cells (Figure 1B). Similarly, CS from low-dose DTX-treated Ca9-22 cells increased the iDC proportion compared with control CS, whereas CS from high-dose DTX-treated Ca9-22 reduced the iDC proportion (Supplementary Figure 1B). Next, we examined the viability of iDCs by 7-AAD staining. Intriguingly, iDCs exhibited higher viability in CS from low-dose DTX-treated HSC4 cells than in control CS (Figure 1C). The iDCs cultured in CS from low-dose DTX-treated HSC4 cells also maintained the membranous projections that are characteristic of iDCs (Figure 1D). These data suggested that low-dose DTX did not compromise iDC viability and instead prolonged the survival of these cells.

The human iDC population can be identified by CD11c, CD80, CD86, HLA-DR, and CD83 expression [21]. We next examined cell surface marker expressions on iDCs by flow cytometry. CD80 and CD86 levels were significantly elevated in iDCs cultured in CS from low-dose DTX-treated HSC4 cells compared with iDCs cultured in control CS, whereas CS from high-dose DTX did not change the expression levels of either marker (Figure 1E). In addition, the CS from low-dose DTX-treated Ca9-22 cells significantly upregulated only CD80 but not CD86 (Supplementary Figure 1C). These results suggest that CS from low-dose DTX-treated HNC cells could support and enhance DC maturation.

Next, we examined IL12 production, which is related to activation of the adaptive immune system in the host. *IL12A* and *IL12B* mRNA levels were significantly increased in iDCs cultured in CS from low-dose DTX-treated HSC4 cells or Ca9-22 cells compared with control CS (Figure 1F, Supplementary Figure 1D). Additionally, IL-12 p70 production was higher in iDCs cultured in CS from low-dose DTX-treated HSC4 or Ca9-22 cells than that in iDCs cultured in control CS (Figure 1G, Supplementary Figure 1E). Together, these indicate that the CS from low-dose DTX-treated HNSCC cells positively affects a functional phenotype of iDCs for activating the adaptive immune system in the host.

OK-432 Promotes Maturation of DCs with Low-Dose DTX Treatment

The combination of chemotherapy and OK-432 exerts a beneficial effect on patient survival [22]. OK-432-treated DCs secrete higher quantities of proinflammatory cytokines, such as IL-12 and IL-15,

and strongly stimulate both CD4+ and CD8+ T cells, leading to enhanced antitumor immunity in the host [10].

To determine whether OK-432 could activate iDCs cultured in CS from low-dose DTX-treated HNC cells, we stimulated iDCs with

OK-432 for 24 hours and examined viability and the expression of CD83, a marker of DC activation. OK-432 had no impact on the proportion of iDCs cultured in CS from low-dose DTX-treated HNC cells (Figure 2A) or cell viability (Figure 2B). However,

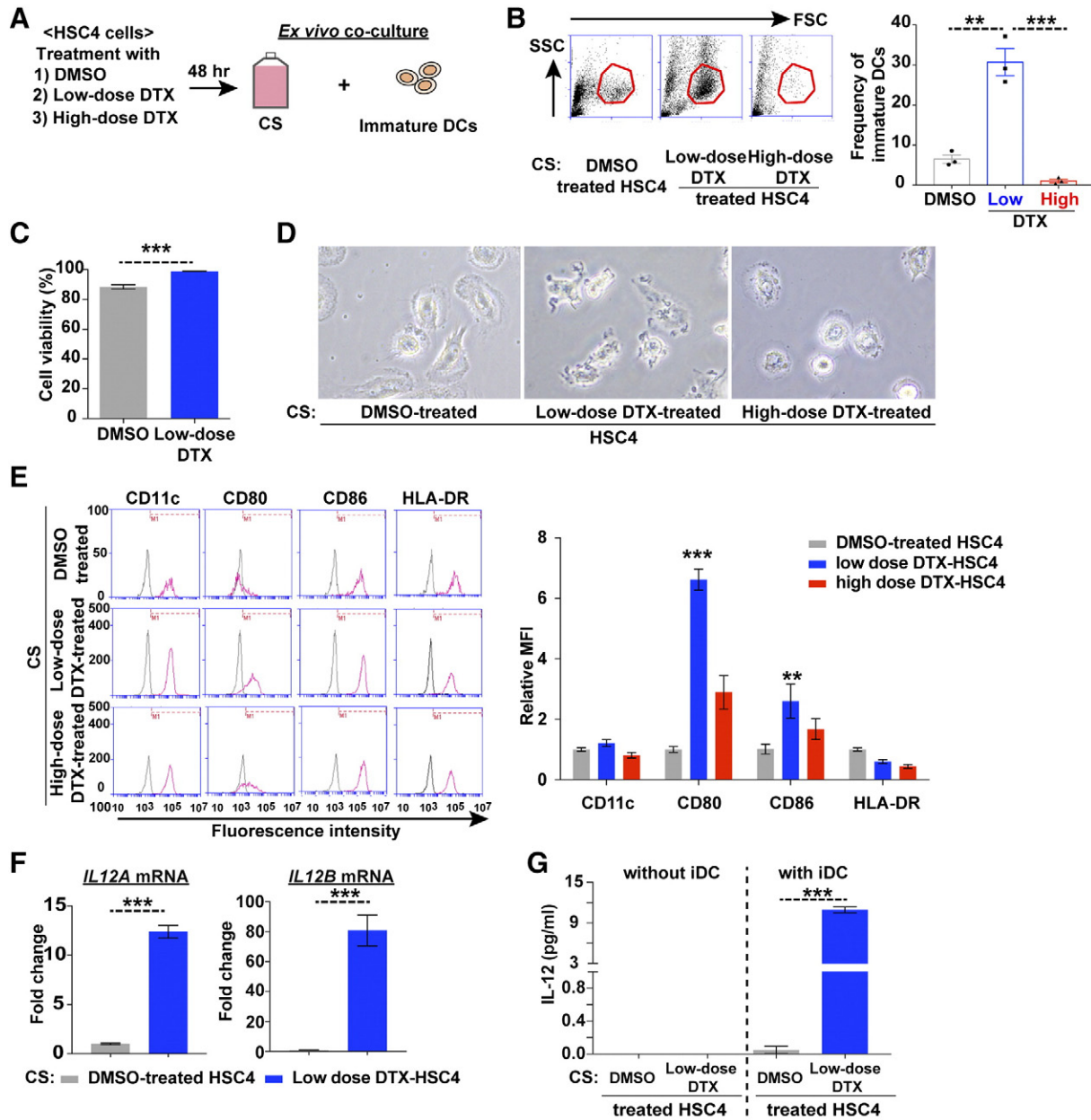


Figure 1. Culture supernatant from low-dose DTX-treated HSC4 cells supports and enhances immature DC differentiation without a cytotoxic influence on viability. (A) Schematic of the experimental design. CS was collected from HSC4 cells treated with DMSO, low-dose DTX (3.125 μ M), or high-dose DTX (12.5 μ M). iDCs were induced from PBMCs prepared from three healthy donors as described in the “Materials and Methods.” (B) Representative FACS plots of iDC populations after co-culture with CS from HSC4 cells treated with DMSO, low-dose DTX, or high-dose DTX. Left panels show representative images of treated PBMCs from one healthy donor. Quantitative data from three healthy donors are on the right. Values are means \pm SEM ($n = 3$). (C) Cell viability of iDCs cultured in CS from DMSO or low-dose DTX-treated HSC4 cells for 24 hours. The percentages of 7-AAD^{neg} iDCs are presented. Values are means \pm SEM ($n = 3$). (D) Phase-contrast images of morphological changes in iDCs co-cultured in CS from HSC4 cells treated with DMSO, low-dose DTX, or high-dose DTX for 24 hours. (E) CD11c, CD80, CD86, and HLA-DR expressions on iDCs from three healthy donors. Representative histograms on the left show data from one healthy donor. On the right, relative MFI levels of the indicated markers are presented relative to levels in iDCs cultured in CS from DMSO-treated HSC4 cells as control. Values are means \pm SEM ($n = 3$). (F & G) iDCs were cultured in CS from DMSO-treated or low-dose DTX-treated HSC4 cells for 24 hours. Total RNA and CS were collected from cultured iDCs for qPCR assay (F) and IL-12 p70 ELISA assay (G). Values are means \pm SEM ($n = 3$). All P values were determined by Student's t test. * $P < .05$, ** $P < .01$, *** $P < .001$.

OK-432 stimulation of iDCs cultured in CS from low-dose DTX-treated HSC4 cells significantly increased CD83 expression compared with iDCs without OK-432 treatment (Figure 2C). These results indicate that OK-432 can activate iDCs cultured in CS from low-dose DTX-treated HNC cells without affecting cell viability.

Combination of Direct Peritumoral iDC Injection with OK-432 and Low-Dose CTX Plus DTX

We next initiated a phase I trial of combination therapy with direct peritumoral iDC injections with OK-432 and low-dose CTX plus DTX in HNC patients.

Patient Characteristics

Five patients, who received standard treatments for the purpose of complete cure, were consecutively enrolled in this trial. Patient characteristics are shown in Table 1. Eligible patients had histologically confirmed progressive or recurrent squamous cell carcinoma (four cases) or adenoid cystic carcinoma (one case). All patients had injectable tumor lesions: locally recurrent lesion (patient no. 1), skin metastases (nos. 2 and 3), or metastatic cervical lymph nodes (nos. 4 and 5). Performance status in each eligible patient was evaluated as score 0 or 1 (Table 1).

Treatment and Adverse Events. A schematic for the combination therapy regimen is shown in Figure 3A. Patients received two peritumoral DC injections on day 6 and day 13. The average number of injected iDCs was 23.0×10^6 (range, 12.9 – 40.3×10^6) (Table 2). To test whether cell-mediated immunity was activated, the DTH skin reaction was evaluated after each treatment cycle. Among the five cases, which were negative before treatment, two cases were positive, one was weakly positive, and two were negative after the treatment cycles (Table 2). Thus, this combination therapy evoked cell-mediated immunity in 60% patients.

A variety of adverse events were reported in all patients irrespective of causal relationship with trial therapy. Although all patients experienced grade 1 to 3 fever, none had febrile neutropenia. Only one patient experienced grade 2 hypotension with fever. Tumor hemorrhage was considered to be possibly related to trial therapy. Ulnar paresis, facial edema, aspiration pneumonia, and spontaneous pneumomediastinum were reported as unrelated events to trial

Table 1. Patient Characteristics

Patient Number	Age	Gender	Primary Site	Standard Therapy	Site of Metastasis	PS
1	76	M	Maxillary sinus (SCC)	ST, CT, RT	Primary site	0
2	34	M	Oral cavity (SCC)	ST, CT, RT, BNCT	cLNs, lung, skin	0
3	70	M	Oropharynx (SCC)	CT, RT	cLNs, lung, skin	1
4	49	M	Submandibular gland (ACC)	ST	cLNs, lung, brain, bone, adrenal gland	0
5	59	M	Supraglottis (SCC)	ST, CT, RT	cLNs, lung	0

PS, performance status; SCC, squamous cell carcinoma; ACC, adenoid cystic carcinoma; ST, surgical therapy; CT, chemotherapy; RT, radiotherapy; BNCT, boron neutron capture therapy; cLNs, cervical lymph nodes.

therapy. In one patient (no. 3), the treatment was considered inadequate and unsafe due to diagnosis of both grade 2 spontaneous pneumomediastinum and grade 3 aspiration pneumonia after one treatment cycle (Table 2).

Clinical responses were evaluated by CT after combination therapy. Unfortunately, all patients who received two treatment cycles were comprehensively judged as having progressive disease (Table 2). However, two of the patients (nos. 3 and 4) presented with tumor regressions in a subset of targeted metastatic lesions where iDCs were injected into peritumoral tissues 2 or 4 weeks after combination therapy (Figure 3B). These results suggested that our combination therapy might promote partial tumor regression.

Immunological Response. To analyze the effect of the combination therapy on the immune system, CD8⁺T-cell subsets, naïve T cells (T_{NAI}), effector T cells (T_{EFF}), central memory T cells (T_{CM}), and effector memory T cells (T_{EM}) were examined in PBMCs collected pre- and posttreatment from the four patients who completed two treatment cycles (Figure 4A). Among these subsets, only the T_{EFF} population was significantly elevated after the combination treatment, suggesting that the treatment was able to alter an immune profile of T-cell subsets (Figure 4B).

Next, we investigated whether changes in Treg and MDSC populations were elicited by the combination treatment. Treg and MDSC populations were detected as CD4⁺CD25⁺CD127⁻ and CD14⁺HLA-DR⁻ populations, respectively (Figure 4, C and E). The

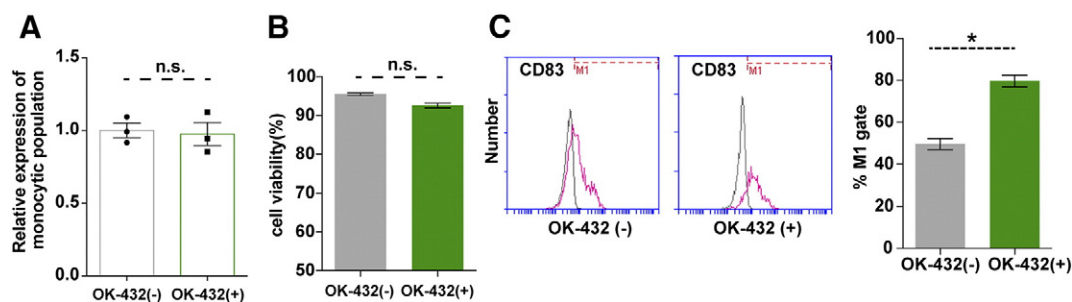


Figure 2. OK-432 can activate iDCs cultured in the CS from HSC4 cells treated with low-dose DTX without cytotoxicity. (A) Frequency of iDCs cultured in CS from HSC4 cells treated with low-dose DTX with or without OK-432 (0.1 KE/ml) as measured by flow cytometry. Gating strategy of iDCs is the same as in Figure 1B. Quantitative data of iDC frequency from three healthy donors are presented. Values are means \pm SEM ($n = 3$). (B) Cell viability of iDCs with or without OK-432 (0.1 KE/ml). (C) CD83 expression on the surface of DCs cultured with or without OK-432 (0.1 KE/ml). Left panels show representative histograms (left) of three healthy donors. Frequency of CD83⁺ DCs cultured with or without OK-432 (right panel). All data are presented as means \pm SEM ($n = 3$). All P values were determined by Student's t test. n.s., not significant, $*P < .05$.

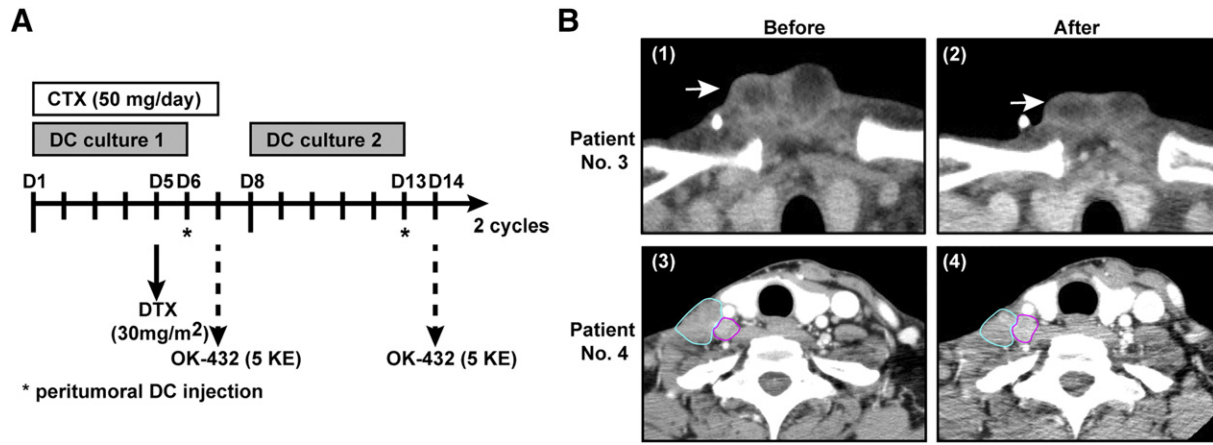


Figure 3. Combination therapy with peritumoral iDCs with OK-432 and low-dose CTX plus DTX for HNC. (A) Regimen for combination therapy with direct peritumoral iDC injection with OK-432 and low-dose CTX plus DTX. Asterisks denote iDC injection into peritumoral tissues. (B) CT scan images of targeted metastatic lesions before (1, 3) and after treatment (2, 4) in patients #3 and #4. White arrows on panels 1 and 2 and the area surrounded by blue lines on panels 3 and 4 show tumor lesions targeted by combination therapy. The purple lines indicate untargeted tumor lesions.

Treg population was significantly smaller in all patients (Figure 4D), whereas the number of MDSCs was reduced in two of the four patients (Figure 4F), indicating that the combination treatment also improved the immunosuppressive status in patients with HNSCC.

Discussion

Here we designed a clinical trial of combination therapy with direct peritumoral iDC injection with OK-432 and low-dose CTX plus DTX for HNC. Peritumoral iDC injection with standard therapy has been clinically demonstrated as effective in several cancers [3,23]. However, some reports suggested that iDCs have a negative impact on host antitumor immunity via T-cell tolerance even in the absence of stimuli [24]. This observation could render crucial functions of iDC controversial. Therefore, steady maturation of iDCs *in vivo* by systemic chemotherapy is essential to trigger the host immune response.

Our data indicate that treatment of low-dose DTX rather than high-dose DTX is sufficient to elicit iDC maturation via tumor apoptosis. A previous report showed that high-dose DTX treatment directly decreases the phagocytic function of iDC [25]. Additionally, DTX can initiate ICD in tumor cells and support secretion of calreticulin to differentiate and activate iDCs [13]. ICD in tumor cells treated with low-dose DTX might contribute to iDC maturation. OK-432 injection, which can activate iDCs, at the same location where iDCs were injected might reduce the proportion of iDCs with immune dysfunction.

A total of five patients were included in our combination therapy trial. Recently, the combination therapy of CDDP+5FU+cetuximab [26] and immune checkpoint inhibitors [27] was identified as the first- or second-line therapy for locally recurrent/metastatic HNSCC. These therapies are currently preferred over DC-based immunotherapy, which is why the number of enrolled patients was so small in our clinical trial. Our clinical treatment was generally well tolerated, although we observed a variety of grade 3 toxicities (Table 2). Fever, which developed a few days after OK-432 injection but returned to normal within 1 day, was observed in all patients. This adverse effect is likely to be due to overproduction of inflammatory cytokines from the OK-432 injection site. Although patients who have multiple metastasized cervical lymph nodes depressing the internal jugular vein or branchial plexus experienced grade 2 facial edema or ulnar paresis, these toxicities were considered as unrelated to this therapy. One patient diagnosed with aspiration pneumonia and pneumomediastinum discontinued the trial therapy due to the risk of life-threatening infection. However, these episodes could have happened due to tumor progression. Taken together, we deemed this treatment to be acceptably safe.

The combination therapy was able to cause positive conversions of DTH and increased CD8+ effector T cells. Consistent with previous reports, Tregs were significantly decreased in the current patients. We also observed a strong tendency toward a reduction in the number of MDSCs after treatment. These data indicate that the combination therapy could change the immune cell profile in the host and improve

Table 2. Summary of Results in Patients Receiving Combination Therapy

Patient Number	Treatment Cycle	Average # of Cells/Injection	DTH (Before/After)	Side Effect	Response
1	2	(12.9 ± 9.7) x10 ⁶	(- / +)	Fever (grade 3), tumor hemorrhage (grade 2)	PD
2	2	(13.9 ± 6.0) x10 ⁶	(- / +)	Fever (grade 3), tumor hemorrhage (grade 2), Ulnar paresis (grade 2), facial edema (grade 2)	PD
3	1	(26.8 ± 21.5) x10 ⁶	(- / -)	Fever (grade 1), facial edema (grade 2), Aspiration pneumonia (grade 3), Spontaneous pneumomediastinum (grade 2)	-
4	2	(21.2 ± 8.3) x10 ⁶	(- / -)	Fever (grade 1)	PD
5	2	(40.3 ± 48.3) x10 ⁶	(- / +: weak)	Fever (grade 3), hypotension (grade 2)	PD

DTH, delayed-type hypersensitivity; PD, progressive disease.

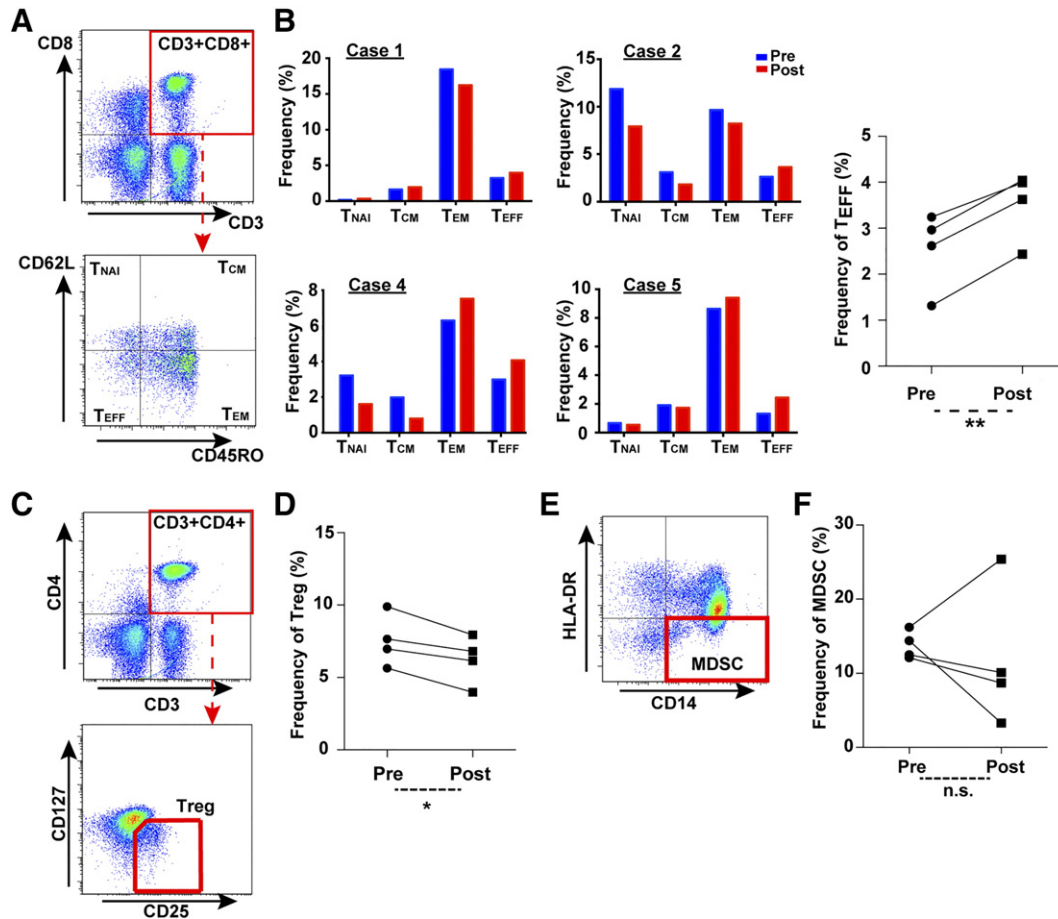


Figure 4. Combination therapy can increase the T_{EFF} subset and decrease Treg populations. (A) FACS plots showing that CD3⁺CD8⁺ T cells were gated and then subdivided into T_{NAI}, T_{CM}, T_{EM}, and T_{EFF} populations based on CD62L and CD45RO expression. (B) Frequencies of T_{NAI}, T_{CM}, T_{EM}, and T_{EFF} subsets in four patients before and after trial therapy are presented. Patient numbers correspond to those listed in Table 1. T_{EFF} frequency (as percentage) is shown in the right panel. (C & E) Gating strategy for Tregs, identified as CD3⁺, CD4⁺, CD25⁺, and CD127⁻ (C), or MDSCs, identified as CD14⁺ and HLA-DR⁻ (E). (D & F) Frequencies of Tregs (D) and MDSCs (F) from four patients. All *P* values were determined by two-tailed paired *t* test. n.s., not significant, **P* < .05, ***P* < .01.

the immunosuppressive status. However, we did not observe better clinical response in any of the subjects. These observations reveal two debatable points. First, combination immunotherapy might result in lower antitumor immunity due to exhaustion of T cells in the host immune system. Chronic antigen stimulation dampens T-cell activation and proliferation in the context of infection or cancer. Almost all the enrolled patients had received systemic chemotherapies or irradiation, which supplies abundant tumor antigens, prior to our combination therapy. Therefore, even though the combination therapy resulted in T_{EFF} proliferation and attenuation of immunosuppression via Tregs and MDSCs, the optimal therapeutic effect was not obtained.

Second, the tumor-specific cytotoxicity could not be monitored in this study. Antitumor immunity is evaluated by increased tumor antigen-specific IFN γ ⁺ CD8⁺ T-cell proliferation [1]. Because our combination therapy provides iDCs with good chances to encounter a large number of undefined tumor antigens, in theory, it is difficult to comprehend tumor antigen-specific antitumor immunity in patients with *in vivo* DC pulsing. All patients exhibited increases in the T_{EFF} population after two treatment cycles. Although we did analyze CD38 expression in T_{EFF}, which are T-cell activation markers, no change in CD38 expression was observed before and after treatment

(data not shown). A previous paper showed that circulating PD1⁺CD8⁺ T cells in peripheral blood correlate with tumor-infiltrating T cells with antitumor immunity [28]. Thus, PD1 expression in circulating CD8⁺ T cells might serve as a useful biomarker for gauging antitumor immunity evoked by our combination therapy.

In summary, our results suggest that combination therapy with direct peritumoral injection of iDCs with OK-432 and low-dose CTX plus DTX can be performed and shows no limitations, such as lack of tumor antigens with strong immunogenicity, HLA restriction, and severe adverse events. Moreover, the combination therapy may also induce a partial tumor regression with changing the immune profile of T-cell subsets and improving immunosuppression in patients with advanced HNC.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2017.11.006>.

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Conflict of Interest Statement

None declared.

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