A Dendritic Cell Vaccine Pulsed with Autologous Hypochlorous Acid-Oxidized Ovarian Cancer Lysate Primes Effective Broad Antitumor Immunity: From Bench to Bedside

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Abstract

Purpose: Whole tumor lysates are promising antigen sources for dendritic cell (DC) therapy as they contain many relevant immunogenic epitopes to help prevent tumor escape. Two common methods of tumor lysate preparations are freeze-thaw processing and UVB irradiation to induce necrosis and apoptosis, respectively. Hypochlorous acid (HOCl) oxidation is a new method for inducing primary necrosis and enhancing the immunogenicity of tumor cells.

Experimental Design: We compared the ability of DCs to engulf three different tumor lysate preparations, produce T-helper 1 (Th1)-priming cytokines and chemokines, stimulate mixed leukocyte reactions (MLR), and finally elicit T-cell responses capable of controlling tumor growth in vivo.

Results: We showed that DCs engulfed HOCl-oxidized lysate most efficiently stimulated robust MLRs, and elicited strong tumor-specific IFN-γ secretions in autologous T cells. These DCs produced the highest levels of Th1-priming cytokines and chemokines, including interleukin (IL)-12. Mice vaccinated with HOCl-oxidized ID8-ova lysate–pulsed DCs developed T-cell responses that effectively controlled tumor growth. Safety, immunogenicity of autologous DCs pulsed with HOCl-oxidized autologous tumor lysate (OCDC vaccine), clinical efficacy, and progression-free survival (PFS) were evaluated in a pilot study of five subjects with recurrent ovarian cancer. OCDC vaccination produced few grade 1 toxicities and elicited potent T-cell responses against known ovarian tumor antigens. Circulating regulatory T cells and serum IL-10 were also reduced. Two subjects experienced durable PFS of 24 months or more after OCDC.

Conclusions: This is the first study showing the potential efficacy of a DC vaccine pulsed with HOCl-oxidized tumor lysate, a novel approach in preparing DC vaccine that is potentially applicable to many cancers. Clin Cancer Res; 19(17); 4801–15. ©2013 AACR.
Translational Relevance

Whole tumor lysates can potentially elicit polyclonal antitumor responses via presentation of numerous immunogenic epitopes by DCs to CD4+ and CD8+ T cells, but their potency may vary. We developed a novel method of whole tumor lysate preparation with hypochlorous acid (HOCl) oxidation to induce rapid necrosis and increase the immunogenicity of tumor cells. Dendritic cells (DC) pulsed with HOCl-oxidized autologous whole tumor lysate (OCDC) elicited robust antitumor effects in mouse and human preclinical studies. In a pilot clinical study of five subjects with recurrent ovarian cancers, OCDC vaccine produced high interleukin (IL-12) ex vivo, and OCDC vaccination induced polyclonal antitumor IFN-γ-secreting cells and reduced peripheral blood regulatory T cells and serum IL-10 in vivo. Two subjects experienced durable progression-free survival intervals of 36 and 43 months after OCDC vaccinations. This is the first study in human clinical practice showing the immune effects of DC-HOCl oxidized tumor lysate therapy, and it is potentially applicable to many cancer types.

Human DC generation and maturation

DC generation was conducted as previously described (19). Monocytes of normal donors were cultured in CellGenix DC media (CellGenix), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Cellgro), 500 IU/mL human granulocyte-macrophage colony-stimulating factor (GM-CSF), and 250 IU/mL interleukin (IL)-4 (PeproTech) after obtaining a written informed consent from a tissue and blood procurement study approved by University of Pennsylvania (Philadelphia, PA) institutional review board (IRB). Eutritiated monocytes of the subjects were cultured with clinical grade GM-CSF (Leukine, Bayer Healthcare Pharmaceuticals) and animal-free research grade IL-4 (R&D Systems, Inc.). After 4 days, CD11c, CD14, and HLA-DR were more than 70% pure. After lysate-loading, DCs were matured with lipopolysaccharides [LPS; 60 EU/mL; Escherichia coli O113; gift from Dr. Suffredini at NIH (Bethesda, MD)] and IFN-γ (2,000 IU/mL; Intermune).

Human tumor lysate preparations and DC uptake

Methods for preparing autologous and allogeneic tumor lysates by UVB irradiation (UVB-L) and freeze-thaw cycles (FTL) have been previously described (20). The detailed methodology of preparing whole tumor lysates by HOCl oxidation was previously published (13) and is described in Supplementary Materials and Methods. After HOCl oxidation or UVB irradiation, tumor cells were subjected to 6 freeze-thaw cycles. For uptake experiment, HOCl-oxidized and UVB-irradiated SKOV-3 cells were not frozen and thawed before coculturing with DCs. Human DC uptake of tumor cells was conducted as previously described (13). SKOV-3 cells labeled with PKH26 (Sigma-Aldrich Corp.) were cocultured with normal donor DCs (1:1 ratio) for 4 hours at 37°C for active uptake or 4°C for passive transfer to DCs. Percentage uptake by DCs was determined by gating on HLA-DR+ PKH26+ cells in flow cytometry analysis conducted on BD FACS Canto (Becton Dickinson). Data were analyzed with Pro CellQuest software.

Human DC and T-cell cytokine and chemokine analysis

Normal donor DCs were pulsed with HOCl-L of 3 ovarian tumor lines—SKOV-3, OVCAR5, and A1847 (ratio of 1:1:1 in the lysate mixture), whereas subject DCs were

constantly observed in subjects vaccinated with DCs pulsed with whole tumor lysate compared with DCs pulsed with defined tumor-associated peptides or proteins (9). In fact, whole tumor lysates offer distinct advantages over defined antigens peptide vaccines such as all subjects are eligible for the therapy regardless of their human leukocyte antigen (HLA) type. Our increased understanding of private antigens expressed by most tumors favors the use of autologous over allogeneic lysate. Whole tumor lysates also provide a source of all potential antigens, eliminating the need to identify the most optimal antigen to target in a particular type of cancer. Importantly, multiple tumor antigens can be targeted at once, thereby bypassing issues of tumor antigen loss. Whole tumor lysates can be prepared with various methods such as freeze-thaw cycles or UVB irradiation to induce primary necrosis and apoptosis, respectively. Selection of the optimal and most immunogenic whole tumor lysate preparation is needed to enhance the efficacy of whole tumor lysate vaccines.

Hypochlorous acid (HOCl) is a strong bactericidal oxidant produced by activated neutrophils in acute inflammation. HOCl is also capable of potentiating the immunogenicity of tumor cells, but their potency may vary. We developed a novel method of whole tumor lysate preparation with hypochlorous acid (HOCl) oxidation to induce rapid necrosis and increase the immunogenicity of tumor cells. Dendritic cells (DC) pulsed with HOCl-oxidized autologous whole tumor lysate (OCDC) elicited robust antitumor effects in mouse and human preclinical studies. In a pilot clinical study of five subjects with recurrent ovarian cancers, OCDC vaccine produced high interleukin (IL-12) ex vivo, and OCDC vaccination induced polyclonal antitumor IFN-γ-secreting cells and reduced peripheral blood regulatory T cells and serum IL-10 in vivo. Two subjects experienced durable progression-free survival intervals of 36 and 43 months after OCDC vaccinations. This is the first study in human clinical practice showing the immune effects of DC-HOCl oxidized tumor lysate therapy, and it is potentially applicable to many cancer types.

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pulsed with HOCl-oxidized autologous tumor lysate in the presence of GM-CSF (500 IU/mL). After 8 hours of stimulation with LPS and IFN-γ, supernatants were analyzed. For T cells, subject peripheral blood mononuclear cells (PBMC) were cocultured with OCDC, unpulsed autologous DCs, or media only and the supernatant analyzed after 24 hours.

**Mouse bone marrow-derived DC preparation**

Bone marrow cells were isolated from hind-leg femurs and tibias of mice and plated at 1 × 10⁶ cells/mL in complete Iscove’s modified Dulbecco’s media (IMDM) containing 10% FBS, 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1,000 IU/mL GM-CSF. On day 3, floating cells representing granulocytes were removed and replenished with fresh complete IMDM media and 1,000 IU/mL GM-CSF. On day 5, IL-4 was added at 100 IU/mL. On day 6, ID8-ova tumor lysate was prepared by HOCl oxidation or UVB irradiation or 6 cycles of freeze-thaw were cocultured with DCs at 1:1 ratio for 20 to 24 hours. Then, DCs were stimulated with LPS (120 EU/mL) and IFN-γ (4,000 IU/mL) for 16 hours and used.

**Vaccination of mice and tumor challenge**

Mouse ID8-ova tumor line was generated to express surface SIINFEKL-H-2Kb complex by lentiviral transduction. Ovalbumin (OVA) plasmid vector pAC-Neo-OVA (ref. 21; plasmid 22533, deposited by Dr. Michael Bevan, Research Institute of Scripps Clinics, La Jolla, CA) was obtained from Addgene and the full-length OVA was amplified with PCR and inserted into the pELNS lentiviral vector (ref. 22; kind gift of Dr. Carl June, University of Pennsylvania). MOI 5 was used and cell surface SIINFEKL-H-2Kb complexes on transduced ID8 cells were detected by anti-mouse H-2Kb bound to SIINFEKL (Biolegend) after 24 hours of IFN-γ treatment. Positive cells were sorted by flow cytometry and SIINFEKL-H-2Kb expressions were confirmed after 1 week of culture and found to be 100% pure. Tumor lysates of ID8-ova were prepared (i.e., HOCl-L, UVB-L, or FTL), cocultured with mouse DCs, and matured as described above. Each mouse was inoculated with 5 × 10⁶ live ID8-ova cells intraperitoneally 3 weeks before the first tumor lysate–pulsed DC vaccination. Vaccine (1 × 10⁶ DCs/dose in 50 μL PBS) was given intradermally once weekly for 3 weeks. For IFN-γ ELISPOT, SIINFEKL pentamer analysis, sera IL-10, and regulatory T (Treg) cell phenotyping (see Supplementary Data), mice were sacrificed 2 weeks after the third vaccine. For tumor challenge, mice were sacrificed when they became distressed and moribund as per University of Pennsylvania guidelines.

**Clinical trial design and subjects**

We conducted a pilot study (UPCC-19809) in recurrent ovarian cancer subjects to determine the safety, immuno-genicity, and clinical efficacy of OCDC (i.e., HOCl-oxidized autologous whole tumor lysate–pulsed DC) vaccine. Eligible subjects were 18 years old or more; had undergone physician’s choice postoperative chemotherapy; and had a baseline Eastern Cooperative Oncology Group performance status ≤1. Enrollment was allowed 4 weeks or more after surgery. The study (NCT01132014) was approved by the Food and Drug Administration (BB-IND-14269) and University of Pennsylvania IRB. All patients gave written informed consent before initiation of any study procedures. Leukapheresis was conducted 15 to 30 days before harvesting PBMCs for OCDC vaccine manufacturing at Clinical Cell and Vaccine Production Facility (CVPF) at University of Pennsylvania as described in the Supplementary Material. Vaccines were cryopreserved at −140°C, thawed, and washed before administration. Subjects received 5 doses of vaccines (~5–10 × 10⁶ DCs/dose) intranodally every 2 weeks and continued on a monthly maintenance regimen until disease progression or exhaustion of vaccine supply. Subjects were evaluated every 2 weeks. Safety was determined using National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Subjects underwent computed tomography (CT) scan at enrollment (i.e., prevaccine) and approximately 4 weeks postvaccination on day 86 (i.e., end of study [EOS]). Clinical response was determined using Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. PBMCs were assessed at enrollment and at EOS with IFN-γ ELISPOT, HER-2/neu tetramer staining, tumor-specific T-cell cytokine assay, peripheral blood Treg cells) phenotyping, and sera IL-10 (described in Supplementary Data).

**Statistical analysis**

Comparisons among groups in Figs. 1 and 2 and Supplementary Fig. S1 were conducted on natural log-transformed data using ANOVA. For those comparisons found to be statistically significant by ANOVA, Tukey post hoc testing was used to conduct pairwise comparisons. The Kaplan–Meier method was used to estimate survival curves in Fig. 1D and was compared using the log-rank test. In Fig. 3, mean value was indicated by a line. In Figs. 5 and 6, statistical significance was assessed using Student paired t test. A significance level of 0.05 or less was considered statistically significant. Analyses were conducted in SPSS v19 (SPSS Inc).

**Results**

**Mouse DCs efficiently process HOCl-oxidized lysate**

ID8 murine ovarian tumor cell line transduced with ovalbumin (ID8-ova) was used to compare the efficacy of mouse DCs pulsed with whole tumor lysate prepared in 3 different ways: (i) HOCl oxidation followed by freeze-thaw (HOCl-L); (ii) UVB irradiation followed by freeze-thaw (UVB-L); and (iii) 6 cycles of freeze-thaw (FTL). Ovalbumin is chosen as the model antigen because it is a well-characterized research tool, which is readily available to assess immune responses. Mouse bone marrow-derived DCs were cocultured with these different lysates and matured with LPS and IFN-γ. DCs (gated on CD11c and MHC class II) were analyzed for crosspresentation of the MHC-I–restricted immunodominant epitope OVA257–264 (SIINFEKL)
using a specific antibody which recognized the SIINFEKL peptide bound to H-2Kb. Unpulsed DCs and DCs pulsed with the SIINFEKL peptides were used as controls. DCs pulsed with HOCl-L, UVB-L, or FTL lysate for 20 to 24 hours, matured with LPS and IFN-γ, and evaluated for the expression of ovalbumin immunodominant SIINFEKL on cell surface (n = 6 mice/group). B, evaluation of the number of IFN-γ-secreting tumor-reactive cells in the spleens of mice after DC-lysate vaccinations. Each vaccination group was represented by a different color bar. Data was from 2 independent experiments with 5 mice in each group. C, the percentage of CD3^+CD4^-CD25^-FOXP3^+ Treg cells was quantified in spleens 2 weeks after the third vaccine (left). Data was the mean of 3 independent experiments with 5 mice in each experiment. C, evaluation of IL-10 in sera 2 weeks following third vaccine (n = 15 mice/group; right). D, three weeks after live ID8-ova tumor cell inoculation in the peritoneum, mice were vaccinated with different DC-lysate vaccines and their percentage of survival was evaluated. PBS only (star) and unpulsed DC (open square) groups served as controls. Each curve represented data from 2 independent experiments of 5 mice per group per experiment (n = 10 mice/group). Data were presented as mean ± SEM. Double asterisks (**) indicate results that were highly significant at P < 0.001 and single asterisks (*) indicate results that were significant at P < 0.05. MFI, mean fluorescence intensity.

HOCl-oxidized lysates yield improved DC vaccines
To test the efficacy of DCs pulsed with the different lysates, C56Bl/6 mice were inoculated intraperitoneally with live ID8-ova tumor cells and allowed 3 weeks for tumor establishment. Mice were then vaccinated with DCs pulsed with HOCl-L, UVB-L, or SIINFEKL peptide, whereas control mice received unpulsed mDCs or PBS only. Some mice were sacrificed 2 weeks after vaccination (i.e. 56 days posttumor inoculation) for immune analysis, whereas groups of mice were followed for survival.

To determine whether DCs pulsed with whole tumor lysate were able to crosspresent and elicit T-cell responses against tumor-associated antigens, we cocultured splenocytes from vaccinated mice with live target ID8-ova cells at a 10:1 effector to target ratio. Mice vaccinated with HOCl-L DCs exhibited the highest mean number of IFN-γ-secreting T cells specific for ID8-ova cells (~300 spots/10^6)
splenocytes) compared with mice vaccinated with DCs pulsed with UVB-L (~125 spots/10^6 splenocytes; *, *P < 0.05) or FTL (~120 spots/10^6 splenocytes; **, *P < 0.001), unpulsed DCs (~7 spots/10^6 splenocytes; ***, *P < 0.0001), or PBS only (~18 spots/10^6 splenocytes; ****, *P < 0.0001) (Fig. 1B). To test whether these tumor-reactive T cells were directed towards OVA epitopes and also to additional tumor epitopes, we cocultured splenocytes from these mice with parent ID8 cells at the same effector to target ratio. We found that most of the IFN-γ tumor-reactive responses from mice in the HOCl-L–pulsed DC group seemed to be directed against OVA with very little reactivity against ID8 epitopes (Fig. 1B).

To verify the presence of T cells specific to OVA epitopes, we pulsed splenocytes with the SIINFEKL peptide. We found that HOCl-L–pulsed DCs elicited a relatively high frequency of SIINFEKL-reactive IFN-γ–secreting CD8^+ T cells, which was comparable with those elicited by SIINFEKL peptide-pulsed DCs (NS, not significant; Fig. 1B).

UVB-L–pulsed DCs elicited a similar number of SIINFEKL-reactive IFN-γ–secreting CD8^+ T cells as HOCl-L–pulsed DCs, but FTL-pulsed DCs yielded a smaller frequency of such cells (*, *P < 0.05 when compared with HOCl-L–pulsed DCs), in agreement with the relative ability of DCs to crosspresent the SIINFEKL peptide. The superior ability of HOCl-L–pulsed DCs to elicit SIINFEKL-specific CD8^+ T cells was confirmed by pentamer staining (Supplementary Fig. S1A).

Given that whole tumor lysate potentially contains large number of potential epitopes for priming CD4^+ and CD8^+ T-cell responses, we investigated whether CD4^+ tumor-specific IFN-γ–secreting T cells were also being elicited by the DC–whole tumor lysate vaccines. Using intracellular staining, we found that mice that were vaccinated with DCs pulsed with HOCl-L developed a higher percentage of CD4^+ T cells that recognized and secreted IFN-γ in response to live ID8-ova cells (0.31%) compared with mice that were vaccinated with DCs pulsed with UVB-L (0.14%) or FTL.
Vaccination of mice with DCs pulsed with HOCl-L stimulated significantly higher percentage of CD4<sup>+</sup> T cells compared with vaccination with unpulsed DCs or PBS only (\( /C3 \), \( P < 0.05 \)). Interestingly, higher frequencies of SIINFEKL-specific CD8<sup>+</sup> and overall tumor-specific IFN-\( \gamma \) T-cell responses were also elicited in the mice that received DCs pulsed with HOCl-L (Fig. 1B and Supplementary Fig. S1A), suggesting that the development of stronger antitumor CD4<sup>+</sup> T-cell responses by the DCs pulsed with HOCl-L might help in stimulating stronger overall antitumor TH1 responses in this tumor model. Mice vaccinated with HOCl-L–pulsed DCs showed the lowest mean level of splenic Treg cells (9.61%), whereas mice that received UVB-L- or FTL-pulsed DCs had 14.45% (\( /C3 \), \( P < 0.05 \)) and 14.34% (\( /C3 \), \( P < 0.05 \)) splenic Treg cells, respectively (Fig. 1C, left). In addition, mice vaccinated with HOCl-L–pulsed DCs had no detectable IL-10 in sera, whereas mice receiving UVB-L- or FTL-pulsed DCs (mean of 173.22 pg/mL; \( ^* \), \( P < 0.05 \)) or FTL-pulsed DCs (mean of 54.67 pg/mL; \( ^* \), \( P < 0.05 \)) had detectable serum levels of IL-10 (Fig. 1C, right). Mice receiving only PBS exhibited a mean 15.33% of splenic Treg cells and had detectable serum IL-10 (mean of 97.3 pg/mL; \( ^* \), \( P < 0.05 \)).

To test the efficacy of vaccination in vivo, mice were followed for survival. Mice vaccinated with HOCl-L–pulsed DCs showed the longest overall survival, with median survival not reached at 180 days (60% alive at 180 days), whereas the median survival of all other groups was 78.5 days or less (\( P = 0.014 \); log-rank test; Fig. 1F). Interestingly, although strong responses against SIINFEKL were detected in mice vaccinated with DCs pulsed with SIINFEKL peptide or with UVB-L (Fig. 1B and C), this was insufficient for tumor control, and 80% and 100% of the mice from these groups, respectively, succumbed to ascites and tumor progression (Fig. 1D). Thus, HOCl-L–pulsed DCs were able to crosspresent tumor-associated immunodominant epitopes, elicited the highest frequency of IFN-\( \gamma \)-secreting tumor-reactive CD8<sup>+</sup> and CD4<sup>+</sup> T-cells, and achieved the best tumor response in vivo. This was also associated with a lower frequency of detectable splenic Treg cells and absence of serum IL-10. These results were encouraging and worthy of further clinical translation.

**HOCl oxidation enhances the uptake of tumor lysate by human DCs**

To define the optimal whole tumor lysate for clinical application, we compared the above three methods of whole tumor lysate preparation for pulsing human DCs—i.e., HOCl-L, UVB-L, and FTL. Because of the limited availability of PBMCs from ovarian cancer subjects, we evaluated PBMC-derived immature (i)DCs from normal donors to take up the different lysate preparations. We
cocultured iDCs with HOCl-L, UVB-L, or FTL of SKOV-3 cells. DCs took up more HOCl-L compared with UVB-L or FTL (\(^*/C_3\), \(P < 0.05\)) by 4 hours at 37°C (Fig. 2A). Passive transfer of tumor fragments to DC surfaces at 4°C was negligible, confirming that lysate uptake was an active process.

Following lysate uptake, DCs matured within 16 hours of stimulation with LPS and IFN-\(\gamma\), as assessed by upregulation of HLA-DR, CD40, CD80, CD86, ICAM-1, and CCR7 to levels similar to those of control unpulsed mature (m)DCs stimulated with LPS and IFN-\(\gamma\) (Supplementary Fig. S2), which were significantly higher than baseline expression in control unpulsed iDCs. There were no significant differences among DCs pulsed with the 3 different lysate types.

Human DCs pulsed with HOCl-L produce high levels of TH1-priming cytokines and chemokines and primed strong responses to ovarian tumor-associated antigen

We further characterized the cytokine and chemokine profiles of DCs from normal donors pulsed with tumor lysates of 3 ovarian tumor lines (i.e. SKOV-3, A1847, and OVCAR5; used at 1:1:1 ratio for DC loading). Culture supernatants were evaluated 8 hours after treatment with LPS and IFN-\(\gamma\). HOCl-L–pulsed DCs produced higher levels of IL-12, MIP-1\(\alpha\), and MIG (100-fold, 20-fold, and 15-fold higher, respectively) when compared with unpulsed mDCs (Fig. 2B). It was noted that HOCl-L–pulsed DCs produced significantly higher level of IL-12 compared with UVB-L or FTL (\(P < 0.05\)) pulsed DCs (Fig. 2B). More than 2-fold increases were also detected in IL-1Ra, IL-1\(\beta\), IL-15, IFN-\(\alpha\), IP-10, and RANTES in the HOCl-L–pulsed DC supernatants when compared with unpulsed mDCs (Fig. 2B). IL-10 secretion from HOCl-L–pulsed DCs was increased by about 2-fold but was very low compared with the 100-fold increase observed with IL-12, which is a TH1-polarizing cytokine (Fig. 2B). A 2-fold increase in IL-8 level was also observed from HOCl-L–pulsed DCs; however, it was very low compared with the 100-fold increase in IL-12. No increase was seen with IL-6 and TH2-priming cytokines such as IL-4, IL-5, and IL-13 from HOCl-L–pulsed DCs (data not shown). We did not assess TGF-\(\beta\) in our Luminex analysis. Comparable robust mixed leukocyte reactions (MLRs) were observed from DCs pulsed with HOCl-L, UVB-L, or unpulsed mDCs (Fig. 2C). Poor MLRs were observed from DCs pulsed with FTL or unpulsed iDCs. We further observed that DCs pulsed with HOCl-L made from SKOV-3 cells were more potent than DCs pulsed with UVB-L or FTL of SKOV-3 (\(P = 0.030\)) in priming IFN-\(\gamma\) production in naive autologous normal donor T cells after 10 days of co-culture. These primed T cells efficiently recognized live MDA-231 breast and OVCAR2 ovarian tumor lines that overexpressed HER-2/neu as well as HER-2/neu 369–377 peptide pulsed onto HLA-A2–expressing T2 lymphoblasts (compared with T cells primed with UVB-L or FTL-pulsed DCs: \(*/C_3\), \(P < 0.05\); Fig. 2D). Low IFN-\(\gamma\) production was observed in the presence of unpulsed T2. Unpulsed mDCs [i.e., media (no antigen)] were used as control. In summary, human DCs pulsed with HOCl-L exhibited favorable lysate uptake and cytokine/chemokine profiles.

Figure 4. A, OCDC vaccine schedule of subjects. B, line graph showed regression or stabilization of 6 of 13 tumor metastatic deposits at 128 days (i.e. at EOS II) in subject S4 and CT imaging showed 3 additional nodules with central necrosis/cavitation (enlarged picture) at 128 days. C, taking into account the entire salvage treatment sequence of surgery, adjuvant therapy and OCDC, subject S2 experienced a second progression-free survival (PFS) of 36 versus an initial PFS of 26 months.
stimulated robust MLRs and ovarian tumor reactivity, and were therefore suitable for clinical translation.

**Autologous HOCl-L–pulsed matured DCs are suitable for therapy**

We tested HOCl-L–pulsed DCs for vaccine production in a pilot study for recurrent ovarian cancer. Elutriated monocytes were obtained from PBMCs of the subjects after leukapheresis and cultured in cell factories with GM-CSF and IL-4 as described in Materials and Methods and in Supplementary Fig. S3. On day 4 of culture, DCs were pulsed with HOCl-oxidized autologous tumor lysate for 16 to 20 hours, followed by treatment with LPS and IFN-γ for 6 to 8 hours. On day 5, matured OCDCs were harvested and cryopreserved until clinical use.

To test the functionality of OCDC, vaccine aliquots from 5 subjects (S1–S5; see Table 1 for demographics of the subjects) were rapidly thawed, washed, and cultured in fresh complete CellGenix DC media for 24 hours without GM-CSF and IL-4. Culture supernatants were then analyzed by Luminex cytokine and chemokine array. Similar to fresh HOCl–pulsed DCs of normal donors, thawed OCDC produced high levels of TH1-priming cytokines and chemokines such as IL-1Ra, IL-12, MIP-α, MIP-β, MCP-1, MIG, IP-10, RANTES, and TNF-α, and low levels of TH2 cytokines such as IL-4, IL-5, IL-10, and IL-13 (Fig. 3). These results also indicated that cryopreservation did not affect the ability of OCDC vaccine to produce strong TH1 proinflammatory cytokines and chemokines. A complete cytokine and chemokine analysis of each subject was presented in Supplementary Table S1.

**OCDC vaccine is safe and may offer clinical benefit in subjects with advanced recurrent ovarian cancer**

Five subjects (aged 48–63 years) were enrolled in UPCC-19809. The OCDC vaccine was tested and found to be negative for mycoplasma, bacteria, and fungi, contained <5 EU/mL endotoxin, and >70% CD86+ HLA-DR+ (see Supplementary Materials and Methods). The OCDC vaccine was also found to be free from residual HOCl solution.
The HOCl-oxidized whole tumor lysate was nontoxic to the DCs of the subjects as the DCs remained highly viable after lysate-pulsing, cryopreservation, and thawing (Supplementary Fig. S2, last column). Thus, the vaccine product met release criteria in all subjects (Table 1) and the yield of DCs was correlated with monocyte count derived from the leukapheresis (Supplementary Table S2). OCDCs were administered through direct injection into 1 to 2 groin lymph nodes bilaterally under ultrasound guidance. All subjects completed 5 vaccinations (Fig. 4A), except S1 who withdrew after 3 vaccinations due to disease progression. A total of 45 intranodal vaccinations were carried out. All vaccines were well tolerated and most toxicities were grade 2 (Supplementary Table S3). A common side effect was flu-like symptomatology (i.e. fatigue, fever, and chills), which was attributed to systemic cytokine activation induced by the vaccine (see below) and possibly by any residual LPS that remained in the OCDC vaccine.

Three subjects (S1, S4, and S5) entered the study with radiographically measurable disease, and 2 subjects (S1 and S5) who had progressing disease.

Table 1. Patient demographics and outcomes of OCDC vaccine treatment

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<th>Number of vaccines received</th>
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Figure 6. OCDC vaccines were Tc1 priming and primed HER-2/neu–specific T cells that could be expanded ex vivo. A, PBMCs from HLA-A2+ subjects S3 and S4 were further tested for recognition to various HLA-A2–restricted ovarian TAAs. B, five rounds of OCDC vaccinations were able to prime HER-2/neu 369–specific CD8+ T cells that could further be expanded in vitro after 10 days of stimulation with HER-2/neu369-377 peptide, IL-7, and IL-15 (10 ng/mL each). C, expression of various ovarian TAAs was confirmed in the primary tumor of subject S4 with immunohistochemistry. Asterisks indicate results that were significant at P < 0.05.
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S5) progressed, whereas one subject (S4) showed a mixed response by RECIST criteria at EOS. Subject S4 showed radiographic progression in 13 lesions at EOS (day 86) via CT (Fig. 4B). However, 6 weeks later, in the absence of additional therapy, she underwent repeat CT imaging (day 128; i.e. EOS II) and showed regression or stabilization of 6 of 13 tumor metastatic deposits. Interestingly, 3 additional nodules, which were measured as increased, at this time showed central necrosis (cavitation; Fig. 4B; see enlarged CT).

Two subjects (S2 and S3) entered the study with no evidence of disease after tumor recurrence, debulking surgery, and adjuvant chemotherapy. These subjects experienced durable progression-free intervals of 36 and 44 months. Both subjects achieved remission inversion [i.e., subjects experienced a longer second progression-free survival (PFS2) after vaccine therapy compared with the PFS after previous chemotherapy (PFS1)] in response to OCDC vaccination. PFS is important in assessing studies of novel chemotherapy or immunologic treatment strategies for patients with relapsed disease and was first used by Lee and colleagues to predict PFS2 in patients with relapsed acute myeloid leukemia (ref. 23; Fig. 4C).

As the historic remission inversion rate (PFS2 > PFS1) in ovarian cancer is 3% (24), these results are encouraging given the propensity of recurrent ovarian cancer to commonly recur in shorter intervals than the previous remission interval.

**OCDC vaccine elicits a tangible Th1 antitumor immune response**

We quantified tumor-specific T cells in pre- and post-vaccine peripheral blood lymphocytes (PBL) by 40 hours IFN-γ ELISpot using aliquots of OCDC vaccine (DCs pulsed with autologous tumor lysate) as the antigen-presenting platform presenting whole tumor antigen. Unpulsed autologous DCs, developed exactly as OCDC (with GM-CSF/LPS/IFN-γ, except for lysate pulsing), were used as controls. We detected a significant increase in the frequency of tumor-reactive T cells after vaccination at EOS in subjects S1 (P = 0.044), S2 (P = 0.025 at EOS and P = 0.018 at 6 months post-EOS), S3 (P = 0.005), and S4 (P = 0.031), whereas all except subject S1 exhibited clinical benefit (Fig. 5A). We did not detect a significant increase in tumor-reactive T cells in subject S5 after vaccination (P = 0.312; NS).

In similar experiments as above, we characterized the cytokine profile of T cells at EOS by ex vivo stimulation with OCDC vaccine for 24 hours. OCDC vaccine induced strong Th1 responses as assessed by the high levels of IFN-γ secreted by EOS T cells over 24 hours (Fig. 5B, first panel). Importantly, these EOS T-cells did not exhibit features of Th1 2, Th3, or Th17 cells, as judged by the almost undetectable IL-4, IL-5, and IL-10, and very low levels of IL-17, respectively. Very low or undetectable levels of IFN-γ, IL-4, IL-5, IL-10, and IL-17 were observed from OCDC vaccines alone that served as controls (Fig. 5B; diagonal bar). The above results also indicated that the OCDC vaccine generated ex vivo were robust antigen-presenting cells as shown by their ability to stimulate strong tumor-specific IFN-γ secretions from T cells of subjects at EOS.

Treg cells have been associated with poor outcome in ovarian cancer (25) and experimentally depleting them has shown to be critical for tumor suppression (26). We measured the effects of vaccination on peripheral Treg cells. We observed a reduction in peripheral blood Treg cells at EOS in subjects S3, S4, and S5 (Supplementary Fig. 4A). We also determined the tumor-reactive IFN-γ–secreting CD3+ cells to CD3+CD4+CD25+FOXP3+ Treg cells ratio in the peripheral blood and observed a 1.5- to 3.6-fold increase in the ratio in subjects S2, S3, S4, and S5 at EOS. Subject S1 showed no change in the ratio (Fig. 5C, left). For subject S2, we followed T cells beyond EOS during maintenance vaccination and observed that peripheral Treg cells were decreased to approximately 2% at 6 months after vaccination (Supplementary Fig. 4B). At the same time, the number of IFN-γ–secreting tumor-reactive T cells in subject S2 was increased by 1.7-fold (Fig. 5A), resulting in a 7.5-fold increase in the tumor-reactive IFN-γ–secreting CD3+ cells to total peripheral Treg cell ratio (Fig. 5C, right). In summary, peripheral Treg cells were reduced in subjects S2, S3, S4, and S5 after OCDC vaccinations and during maintenance.

Finally, we characterized the serum cytokine changes after intranodal administration of OCDC vaccine. We detected a potent systemic inflammatory activation with 0.5-fold or more increase in serum IL-1β, IL-2, IL-15, TNF-α, MCP-1, MIP-1α, MIG, and eotaxin in at least 2 of 5 subjects (Supplementary Fig. S4C). There was no increase in IL-12-associated cytokines, including IL-4, IL-5, and IL-13 in the sera of all the subjects (data not shown). In particular, we observed decreases in serum IL-10 levels ranging from 0.24 to 2.28 pg/mL in subjects S1–S4 at EOS (Fig. 5D) who also showed tumor-reactive IFN-γ responses after OCDC vaccinations.

**OCDC vaccine efficiently crosspresents and elicits polyclonal CD8+ responses to common ovarian cancer antigens**

Ovarian cancers express a variety of well-characterized tumor-associated antigens (TAAs; refs. 27). To test whether such response was directed towards any known TAAs, we took advantage of the finding that subjects S3 and S4 were HLA-A02-positive. We detected a postvaccine increase in T cells directed against several HLA-A2–restricted epitopes of known ovarian-specific antigens, including a MHC class II-restricted HER-2/neu epitope (S4; P = 0.045), as well as MHC class I-restricted epitopes of MUC-1 (S4; P = 0.047), NY-ESO-1 (S3; P = 0.033 and S4; P = 0.02), WT1 (S4; P = 0.001), mesothelin (S4; P = 0.008), survivin (S4; P = 0.041), and hTERT (S4; P = 0.016; Fig. 6A). Detection of these polyclonal tumor-specific CD8+ T-cell responses thus indicated that the OCDC vaccine was highly efficient in crosspresentation of the ovarian TAAs. We confirmed the increase in HER-2/neu 369-specific CD8+ T cells in subjects S3 and S4 after vaccination by tetramer analysis, and we expanded these cells when PBMCs taken at EOS were pulsed.
with HER-2/neu369–377 peptide (Fig. 6B). Expressions of HER-2/neu, mesothelin, and WT1 were further confirmed by immunohistochemistry on prevaccine tumor sample for subject S4 (Fig. 6C).

Discussion

Whole tumor lysates are a promising source of antigen for DC-based vaccine therapy as they contain many relevant immunogenic epitopes for priming the immune system against tumors (28). Autologous whole tumor lysates that carry private tumor-associated antigens might be especially important in stimulating long-lasting antitumor responses in cancer subjects. However, whole tumor lysates can be prepared in several ways, and the methods of inducing cell death or the chemical modification of proteins could impact the immunogenicity and efficacy of the therapy (28). We sought to address which type of whole tumor lysate preparation, if any, is optimal for use with DCs.

Freeze-thaw cycles and UVB irradiation are the two most commonly used methods of whole tumor lysate preparations in the clinic. HOCl oxidation induces primary necrotic tumor cell death (13). HOCl is also unique in its ability to enhance protein immunogenicity allowing for improved tumor protein antigen processing and presentation by DCs (13, 14). In our mouse study, we found that DCs pulsed with HOCl-oxidized ID8-ova lysate were able to cross-present SIINFEKL efficiently. In comparison, DCs pulsed with FTL were inefficient in presenting SIINFEKL. Accordingly, DCs pulsed with HOCl-oxidized ID8-ova lysate produced the best results with respect to tumor suppression in vivo and led to 60% survival in mice. We found that most of the tumor-reactive T cells elicited by the vaccine were directed against OVA epitopes (directly confirmed by CD8\(^+\) T cells recognizing SIINFEKL), as vaccine-primed T cells directly recognized ID8-ova cells but not the parental ID8 cells. This finding was interpreted as encouraging, as it proved that DCs could crosspresent an immunodominant epitope admixed with the whole tumor lysate as efficiently as DCs pulsed only with the purified peptide. The fact that DCs did not elicit a potent response against other ID8 antigens was not considered disappointing, as immunodominant epitopes of xenotransplant can easily skew immune responses at the expense of less immunogenic tumor antigens. We detected a higher frequency of tumor-specific IFN-\(\gamma\)-secreting T cells by producing strong TH1-polarizing cytokines and chemokines, efficiently crosspresenting relevant tumor-specific T cells to MHC class I and II molecules, high levels of costimulatory surface molecules of CD80, CD86, and CD40, and high levels of CCR7, and secreting high amounts of IL-12 and other T\(_{H1}\)-polarizing cytokines and chemokines and low amounts of T\(_{H2}\)-priming cytokines (such as IL-4, IL-5, IL-10, and IL-13); and (ii) the ability to elicit a significant increase in tumor-specific IFN-\(\gamma\)-secreting T cells in peripheral blood assays, including ELISPOT. Our DC vaccine met all these criteria in the subjects S2, S3, and S4 who experienced clinical benefit or mixed response.

We tested the above DC vaccine in 5 subjects with stage II/IV recurrent ovarian cancer in a pilot clinical study, using a short DC manufacturing protocol optimized for generating OCDC as previously reported (19). DCs of subjects behaved similar to normal donor DCs, producing high levels of IL-12 and other important T\(_{H1}\) cytokines and chemokines such as IL-1Ra, IL-12, MIP-1\(\alpha\), MIP-1\(\beta\), MCP-1, MIG, IP-10, and RANTES. Following vaccination, we confirmed a potent systemic inflammatory activation in these subjects, as judged by increased serum IL-1\(\beta\), IL-2, IL-15, TNF-\(\alpha\), MCP-1, MIP-1\(\alpha\), MIG, and eotaxin in at least 2 of 5 subjects. Serum IL-10 was decreased in 4 of 5 patients at EOS. These were associated with induction of a significant antitumor T-cell response in 4 of 5 subjects. Tumor-reactive T cells exhibited a strong T\(_{H1}\) polarization and made little or no IL-17, IL-10, IL-4, or IL-5. As documented in subjects S3 and S4, the vaccine-elicited IFN-\(\gamma\)-secreting tumor-specific T cells were polyclonal and were directed against several known HLA-A2–restricted epitopes of ovarian cancer-specific antigens including HER-2/neu, mesothelin, NY-ESO-1, MUC1, and WT1. Thus, the results showed that OCDC vaccine was highly efficient in crosspresentation to CD8\(^+\) T cells. OCDC vaccine also efficiently primed tumor-specific CD4\(^+\) T-cell response as we detected a significant IFN-\(\gamma\) response in the postvaccine PBMCs of subject S4 to a HER-2/neu class I-derived peptide. These observations were further supported by two previous studies that showed that HOCl oxidation promoted crosspresentation and elicited tumor-specific CD4\(^+\) and CD8\(^+\) T cells (13, 14). These studies showed that DCs efficiently crosspresented HOCl-oxidized SKOV-3 tumor cells (which are HLA-A2 negative and allogeneic to the HLA-A2 DC and T cells that were used in these studies), and elicited IFN-\(\gamma\) responses from CD8\(^+\) T cells to HLA-A2–restricted epitopes of HER-2/neu and MUC1, as well as CD4\(^+\) T cells to MHC class II-derived HER-2/neu peptides. Thus, we showed that the OCDC vaccine generated ex vivo were robust antigen-presenting cells by producing strong T\(_{H1}\)-polarizing cytokines and chemokines, efficiently crosspresenting relevant tumor-associated antigens, and eliciting a sharply T\(_{H1}\)-polarized T-cell response.

The ability of HOCl to increase the immunogenicity of whole tumor lysate and subsequently enhancing DC ability to produce strong IL-12 following LPS and IFN-\(\gamma\) could be explained by different potential mechanisms—(i) as HOCl induces necrotic tumor cell death (13), numerous danger signals such as DNA, RNA, ATP, uric acid, high mobility
group box 1, and HSPs could be released to activate and mature DCs. One study showed that SKOV-3 tumor cells that were treated with 40 to 60 μmol/L HOCl induced upregulation of human DC maturation markers including CD40, CD83, CD86, and HLA-DR [13]. Thus, HOCl-oxidized tumor cells partially activate DCs and could promote stronger IL-12 production following stimulation with LPS and IFN-γ; (ii) the danger signals released from the HOCl-L might help increase the uptake of HOCl-L by the DCs. We conducted 6 cycles of freeze and thaw following HOCl oxidation of tumor cells, which could release more danger signals to activate DCs. We showed in this study that DC uptake of HOCl-L was significantly better compared with the uptake of UVB-L or FTL; (iii) as tumor cells contain a whole array of biomolecules including proteins, lipids, and glycoproteins that are targets of HOCl oxidation, these oxidized products could simultaneously engage and activate numerous types of scavenger receptors such as LOX-1 (12), CD36, and MARCO, and possibly Toll-like receptors on DCs. The simultaneous activation of these receptors might help enhance the IL-12 production from DCs loaded with HOCl-L followed by LPS and IFN-γ stimulation; (iv) ovarian tumor cells frequently produce suppressive molecules that could inhibit DC activation, including prostaglandin E2, TGF-β, and IL-10 [29, 30]. Treatment of whole tumor cell lysate with HOCl oxidation and not UVB irradiation or freeze-thaw might inactivate these suppressive molecules, leading to improved DC maturation by LPS and IFN-γ stimulation and hence IL-12 production.

We presented here the clinical and immunologic data of the first cohort of an ambitious phase I study which will treat 35 subjects with ovarian cancer. The results were highly encouraging as we observed a close correlation in this cohort between clinical benefit and immune response to vaccine. The two subjects (S2 and S3), who were in clinical remission when enrolled in the study, remained in remission for a period of time much longer than expected based on historic observations of recurrent ovarian cancer treated with available second or third line chemotherapeutic drugs. Both subjects showed a PFS2 > PFS1 in response to OCDC vaccination as second-line therapy following relapse from first-line therapy. The comparison of second versus first PFS is important in assessing studies of novel chemotherapy or immunologic treatment strategies for patients with relapsed disease including ovarian cancers, and is used to model second response based on first-response duration (31) and interpret the clinical efficacy of second-line therapies (32). Especially interesting is the durable remission in S3, who, after 2 recurrences, remains in remission to date for a total of 44 months, of which 24 months on no additional therapy since the last vaccine. Furthermore, we were encouraged by the response observed in subject S4, who, in spite of progression of disease by RECIST, showed stabilization or initial regression in half of the tumor deposits and cavitation in 3 additional tumor nodules. It is interesting to note that in the 3 subjects (S2, S3, and S4) with clinical benefit or mixed response, we noticed favorable immune parameters, including high levels of IL-12 (>40 ng/mL in 24 hours) produced by OCDCs ex vivo, induction of IFN-γ-secreting tumor-reactive cells, induction of polyclonal tumor-reactive T cells directed against known tumor antigens, and similar to the mouse, reduction in peripheral blood Treg cells and serum IL-10. High levels of serum IL-10 have been shown to correlate with poor prognosis in subjects with hepatocellular carcinoma [33, 34] and hindered the proper maturation of circulating DCs in these subjects [35]. Given that Treg cells are known to produce IL-10, it is reasonable to postulate that the significant reduction in periphery Treg cells observed in our subjects following repeat OCDC vaccination might have contributed to the reduction of serum IL-10 level in the same subject. Furthermore, the overall strong polarization to Th1 responses after OCDC vaccinations could impact myeloid cells to produce less IL-10. A surprising finding of our analyses is that DCs from S2, S3, and S4 secreted low IFN-α (<0.02 ng/mL), which contrasted with the high IFN-α produced by OCDC from S1 and S5 who progressed. This finding will be confirmed in a larger phase II study, which is ongoing.

In this trial, we chose the intranodal route of administration because it allows us to administer a defined quantity of DCs directly to the site of T-cell sensitization. This approach also allows the peak IL-12 secretion to be synchronized with their proximity to T cells, where IL-12 can exert its full effects during antigen presentation [36]. In a murine tumor model, DC pulsed with tumor lysate and injected intranodally resulted in greater sensitization of T cells and improved antitumor responses [37]. In a randomized, phase I dose-escalation trial, Lambert and colleagues [37] compared different administration routes (intravenous, intranodal, and intradermal) in metastatic melanoma receiving 4 autologous peptide-pulsed DC vaccinations. The results showed that intranodal administration led to superior T-cell sensitization as measured by de novo target cell recognition and delayed type hypersensitivity priming, indicating that intranodal injection may be the preferred route of administration for matured DC vaccines [38]. We used LPS to mature the DCs of cancer subjects because it was available in clinical grade from the NIH, and together with IFN-γ had consistently induced strong IL-12 production from the HOCL-L-pulsed DCs. We found that MPLA, a detoxified version of LPS widely used in human therapy, was less efficient than LPS when used at the same concentration alone or in combination with IFN-γ in stimulating IL-12p70 production from DCs (Chiang L-L.C, Unpublished Data). DCs stimulated with MPLA produced approximately 2-fold less IL-12p70 than DCs stimulated with LPS. We could, however, obtain similar IL-12p70 levels as achieved with LPS stimulation by doubling the concentration of MPLA (Chiang L-L.C, Unpublished Data). One study compared the differential stimulatory effects of various lipid A derivatives (including LPS from E. coli and MPLA from S. minnesota) on mouse DCs and macrophages and found that LPS had stronger proinflammatory effects than MPLA [39–41]. The main reason could lie with the structural differences of the lipids and the signal transduction pathways.
and the type of proinflammatory cytokines and chemokines that they activate.

Two recent clinical trial studies described the use of a similar DC vaccine platform. Although there were differences in the DC preparation, antigen used for loading DCs, and maturation stimuli for activating DCs, these studies described the positive correlation of IL-12 production to antitumor responses and length of progression-free survival of the subjects. In a clinical trial conducted by Okada and colleagues (42), of 22 subjects with recurrent malignant glioma, it was showed that increased IL-12 production by DCs was positively correlated to time to progression. In a clinical trial conducted by Alfado and colleagues (43) on 28 subjects with metastatic cancers, it was shown that DC vaccination induced increased IL-12p70 levels in sera. The type of maturation stimuli for activating DCs may affect the production of different T-cell polarizing cytokines and chemokines. Hardwick and colleagues (44) observed that the use of poly I:C alone to mature DCs loaded with HOCl-oxidized tumor cells led to the induction of significant TGF-β production from DCs (15). Our study showed that LPS and IFN-γ induce strong production of IL-12 by HOCl-oxidized whole tumor lysate-pulsed DCs. Thus, robust IL-12 production by the DC vaccine might be a key criterion for successful antitumor vaccination.

In conclusion, through methodical preclinical experimentation, we developed an immunogenic whole tumor lysate preparation for DC-based immunotherapy. This vaccine platform proved encouraging in mice and in human preclinical experiments and was translated to the clinic where it showed early encouraging results in subjects with recurrent ovarian cancer. The data showed are part of cohort 1 of a planned phase I clinical study, where OCDC vaccine is subsequently tested in combinations (NCT01132014). We reported the results from this first cohort to show the preclinical development of OCDC as monotherapy and its promising biologic effects in the treated cancer subjects. Further improvements can be developed in the clinic to increase the vaccine efficacy. For example, we showed that angiogenesis blockade can enhance the homing of T cells in tumors and increase the potency of vaccine (42, 43). The addition of antiangiogenesis therapy, such as bevacizumab, could prove useful. Furthermore, although our vaccine was associated with a reduction of Treg cells in blood, further therapy aiming at attenuating Treg cells, for example with low-dose cyclophosphamide (44–46), could prove beneficial. These hypotheses are tested in the ongoing phase 1 study. Finally, checkpoint blockade could significantly enhance the efficacy of vaccine, and this can be tested as these agents become available.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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