Clinical Cancer Research

Clinical and Antitumor Immune Responses in Relapsed/Refractory Follicular Lymphoma Patients after Intranodal Injections of IFNα-Dendritic Cells and Rituximab: a Phase I Clinical Trial

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Abstract

Purpose: This study was aimed at evaluating the feasibility, safety, immunologic and clinical responses in patients with follicular lymphoma treated with monocyte-derived dendritic cells generated in the presence of IFNα and GM-CSF (IFN-DC) in combination with low doses of rituximab.

Patients and Methods: Firstly, we analyzed *in vitro* and *in vivo* the immunologic properties of IFN-DC against follicular lymphoma. Thus, we performed a phase I trial in 8 patients with refractory and relapsed follicular lymphoma based on sequential intranodal injections of low-dose of rituximab and unloaded IFN-DC and report the safety, clinical, and immunologic results of the enrolled patients.

Results: Preclinical studies indicated that IFN-DC can synergize with rituximab leading to increased cytotoxicity and T-cell tumor infiltration. The clinical evaluation showed that the combined treatment was totally safe. The overall response rate was 50%, PET-negative complete response rate 37%, and remission is still ongoing in 2/4 of responding patients (median follow-up 26 months, range 11–47). Notably, following the combined therapy all patients showed induction/enhancement of T-cell responses by CD107 degranulation or IFNγ ELISPOT assay against patient-specific tumor IGHV sequences.

Conclusions: These results represent the proof-of-principle on the effectiveness of unloaded IFN-DC in inducing durable clinical responses and promoting induction of tumor-specific peripheral T cells, thus suggesting the occurrence of an effective endogenous antitumor vaccination. The overall findings indicate that some unique properties of IFN-DC can be successfully exploited to induce/enhance antitumor responses, thus representing a valuable antitumor strategy for novel and more effective combination therapies in patients with cancer.

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Translational Relevance

The development of protocols of *in situ* cancer immunotherapy aimed at inducing an endogenous vaccination is currently regarded as a practical and promising antitumor strategy, with potential advantages with respect to the use of defined tumor antigens for inducing a broader antitumor immunity, potentially targeting also neoantigens emerging during tumor progression and therapies. This study represents the first evidence on the safety and clinical effectiveness of IFN-DC, a unique type of DC rapidly generated from monocytes under simple GMP conditions and endowed with special immunostimulatory properties. Combined with low doses of intranodal injection of rituximab, IFN-DC induced clinical response in patients with cancer and promoted the induction of tumor-specific T cells. All this implies the occurrence of an endogenous antitumor vaccination possibly resulting in clinical response. The findings suggest that IFN-DC is a good candidate for a selective clinical use in combination therapies in patients with cancer.

Introduction

Follicular lymphoma is the most frequent indolent non-Hodgkin lymphoma. About a third of diagnosed follicular lymphoma s are asymptomatic patients in advanced stage. In this subset, treatment is deferred until development of symptoms or signs related to progression (1–3). Upon progression, patients are treated with rituximab combined with chemotherapy for 6–8 cycles, followed by rituximab-maintenance for 12 cycles. Approximately 80% of patients achieve sustained remission after rituximab-chemotherapy, which may last years (1). Notably, nearly all patients eventually relapse, thus indicating the clinical need for new treatments.

Follicular lymphoma is an immune-responsive malignancy for which therapeutic vaccination treatment has been explored, including trials based on dendritic cells (DC). In particular, some studies reported that DC-based therapies can elicit specific antilymphoma immunity and long-lasting remissions (4–6).

Over the past two decades, DC-based vaccines have been tested in patients with cancer showing variable responses and raising challenges for their development (7–9). While the absence of relevant toxicities represents a good starting point, several critical issues remain, including identification of the optimal DC to be used, and the source/loading of tumor antigens (10, 11). Today, we are facing a renovated interest in new generation DC-based vaccines, spearheaded also by the discovery of new immunomodulatory molecules expected to enforce cancer immunotherapies (7, 12, 13).

We developed a method for the *in vitro* generation of a unique DC population, differentiated from human monocytes in the presence of GM-CSF and IFNα (IFN-DC; ref. 14). IFN-DCs exhibit a partially mature phenotype and are endowed with a high migratory behavior and immunostimulatory ability. They have been shown to be more efficient than conventional control DC in internalizing tumor antigens and in the cross-priming of $CD8^+$ T cells, thus promoting antitumor immune responses (14–17). In particular, we have recently demonstrated that IFN-DC loaded with apoptotic lymphoma cells from patients with follicular lymphoma cultured for 2 weeks with autologous lymphocytes led to massive IFNγ production and Th1 response skewing, as well as to remarkable increase of both $CD8^+$ and NK-cell frequency (17) and enhanced cytotoxic effector function toward primary autologous lymphoma cells (17). Notably, we have recently tested IFN-DC in a phase I clinical trial in patients with advanced stage IV melanoma treated with dacarbazine, showing their safety and some preliminary evidence supporting therapeutic efficacy (18).

The vast majority of DC-based trials have used DC loaded *in vitro* with tumor antigens (8), while only few clinical studies have involved so far the intratumoral injection of unloaded DC with the major aim of inducing endogenous anticancer vaccination (5, 18, 19). Of interest, Kolstad and colleagues have recently reported that the combined treatments of local radiotherapy with low doses of rituximab, followed by the intranodal injections of antigen-unloaded immature conventional DC and GM-CSF, resulted in regression of disseminated follicular lymphoma, which correlated with the induction of antitumor immunity (5). These data, together with the unique features of IFN-DC, prompted us to define the background for the clinical use of IFN-DC in follicular lymphoma and perform a clinical trial in refractory and relapsed (R/R) patients based on the sequential intranodal injection of low-dose rituximab and unloaded IFN-DC.

Patients and Methods

In vitro preclinical studies

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors anonymously provided by the Immunohematology and Transfusional Center of Policlinico Umberto I, University of Rome (Rome, Italy). Informed consent was obtained from donors in accordance with the Declaration of Helsinki. PBMCs from patients with follicular lymphoma were obtained from Azienda Ospedaliera Sant'Andrea (Rome, Italy), under informed consent. Both informed consents and procedures were approved by the pertinent Ethics Committee. The follicular lymphoma Karpas-422 cell line (Interlab Cell Line Collection) was authenticated by morphology and growth *in vitro* and *in vivo* and routinely tested for *Mycoplasma*; master cell bank aliquots stored in liquid nitrogen were thawed and cells were used for experiments within three *in vitro* passages.

PBMCs were purified by Ficoll density gradient centrifugation (Seromed). IFN-DCs were obtained as described elsewhere (17). Patient's IFN-DC–mediated cytotoxicity against Karpas-422 lymphoma cells was determined by Calcein-AM assay. We resuspended target cells in 15 μmol/L Calcein-AM (Sigma-Aldrich) for 30 minutes at 37°C. IFN-DC was added at 5:1 ratio versus Karpas-422 and versus + Karpas-422+rituximab. Cells were incubated for 4 hours at 37°C. Spontaneous (only target cells in complete medium) and maximum release (only target cells in medium plus 2% Triton X-100) were also measured. Following incubation, the content of each well was mixed, plates were centrifuged, and 100 μL of the supernatant was transferred to a 96-well white culture plate. Fluorescence intensity was measured by means of a Microplate Reader (Perkin Elmer LS-50B) at 485 and 530 nm. The percentage of lysis was calculated as follows: lysis % = experimental release−spontaneous release/total release−spontaneous release

×100. Apoptotic cell bodies were generated by exposing lymphoma cell suspensions to heat shock, γ-irradiation, and UVC rays, as described previously (17). IFN-DC were pulsed with apoptotic tumor cells at a 1:2 ratio for 16 hours at 37°C and then cultured with autologous peripheral blood lymphocyte (PBL) at cell ratio of 1:4.

In vivo preclinical studies

NOD/SCID female mice were purchased from Charles River Laboratories, used at 4–5 weeks of age, housed and maintained in accordance with the European Community. The experimental protocols were approved by the Ethical Committee for Animal Experimentation of the Istituto Superiore di Sanità (Rome, Italy). NOD/SCID mice were injected subcutaneously with 5×10^6 Karpas-422 cells resuspended in 0.2 mL RPMI1640 medium. NOD/SCID mice were reconstituted intraperitoneally with 30×10^6 PBL from HLA-A2+ healthy donors resuspended in 0.5 mL RPMI medium before tumors were 4 mm of diameter (day 10). After 2 days, hu-PBL-NOD/SCID mice received intratumoral injection of 100 μg of rituximab, followed 24 hours later by 2×10^6 IFN-DC. The treatment was repeated once every 7 days (3 injections) and, after 14 days, tumors were analyzed. Immunofluorescent labeling was performed on formalin-fixed paraffin-embedded (FFPE) 5-μm tissue sections. Slides were deparaffinized, hydrated through graded alcohols, and subjected to heat-induced epitope retrieval step by pH6 Citrate Buffer (Novus Biologicals) for 3 × 3 minutes in microwave. Sections were washed with PBS-T (0.01% Tween 20) and blocked in PBS-BSA 3% for 30 minutes at 37°C. Primary anti-CD8 antibody (Clone C8/144B, Dako) was added in PBS-BSA (0.5% BSA) and incubated 30 minutes at 37°C, followed by incubation with Alexa Fluor-488 F(ab)2 fragments of goat anti-mouse IgG. Primary anti–Granzyme-B antibody (AlexaFluor-647, Novus Biologicals) was added plus DAPI (Thermo Fisher Scientific). Slides were mounted in Vectashield (Vector Laboratories) and observed with a Leica TCS SP2 AOBS Confocal laser scanning microscopy.

Study design, patient selection, and treatment

The phase I clinical trial was approved by the Ethical Committee of the Sapienza University of Rome (Rome, Italy) and conducted according to Good Clinical Practice and Declaration of Helsinki principles. This trial was registered at https://eudract.ema.europa.eu as *2013-003158-25*. Subjects were included in the study after having given written, informed consent and having carried out all the procedures for complying inclusion/exclusion criteria. Main criteria for eligibility included biopsy-confirmed indolent $CD20^+$ follicular lymphoma, low tumor burden [Gelf-Sie], World Health Organization (WHO) performance status 0–1, adequate blood cell counts, and life expectancy >6 months. Patients were ineligible if they had progressive lymphoma in need of standard therapy according to Gelf-Sie criteria (20), known CNS involvement by lymphoma, HIV infection or other chronic infections, history of autoimmune disease, or were pregnant. Patients were rebiopsied before starting treatment, and $CD20^+$ positivity was confirmed for all patients. Tumor biopsies also showed PD1 expression. Enrolled patients underwent leukapheresis to collect PBMCs for IFN-DC preparation. Primary endpoints were safety and tolerability of the treatment as well as tumor-specific immune responses. Secondary endpoint was the evaluation of clinical response. Safety and tolerability of the treatment were assessed by evaluating frequency, type and intensity of adverse events (NCI/CTACE), as well as the patients' compliance. Adverse events were recorded following the nomenclature of the Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Tumor-specific immune responses were assessed by determining magnitude and quality of peripheral tumor-specific T-cell responses.

For staging, patients underwent standard laboratory and clinical work-up, including computed tomography (CT) of neck/thorax/abdomen, PET/CT, and bone marrow biopsy and aspirates. Patient clinical status was monitored at each visit during treatment, and restaging was performed by CT-PET between the 5th and the 6th cycle and after the last rituximab-IFN-DC cycle; and thereafter every 6 months by CT-scan and ultrasound. Molecular assessments: both lymph nodes or cutaneous biopsies and marrow were analyzed for full-length immunoglobulin heavy chain VDJ sequences by next-generation sequencing (NGS). Before, during, and after treatment, blood samples were analyzed to exclude the emergence of autoimmune markers.

Preparation and characterization of clinical IFN-DC

IFN-DCs were prepared as described previously (18). Patients underwent leukapheresis and monocytes enrichment was performed by the Elutra Cell Separation System Monocytes Enrichment Protocol. When purity of monocytes was below 60%, separation by density gradient with Lymphoprep (Axis-Shield) was performed. The enriched monocytes (2×10^6 cells/mL) were cultured for 3 days in bags (Afc/American Fluoroseal Corporation) in CellGro DC medium (CellGenix GmbH) containing GM-CSF (600 IU/mL; Leukine sargramostim, Bayer Healthcare Pharmaceuticals), and IFNα2b (10,000 IU/mL; Merck Sharp & Dohme Limited). IFN-DC were then harvested, counted, and resuspended in 5% human serum albumin (HSA; Baxter S.p.A.) containing 10% DMSO (WAK-Chemie Medical GmbH), cryopreserved in aliquots of 10^7 cells/cryovial and stored in liquid nitrogen vapor phase.

The recovery was evaluated as ratio of the number of thawed over frozen viable IFN-DC. Immunophenotype was analyzed by flow cytometry using antibodies specific for HLA-ABC, HLA-DR, CD45, CD11c, CD1a, CD86, CD83, CD80, and CD14 (all from BD Biosciences). Phagocytosis was assessed by flow cytometry using ovalbumin conjugated with fluorescein (OVA-FITC; Molecular Probes, Inc.). Flow cytometry was carried out with a FACSCan and data analyzed using FACSDiva Software (BD Biosciences). IFN-DC release criteria are listed in Supplementary Table S1 together with patient-observed values.

Patient immunomonitoring

Identification of patient's class I and class II HLA was performed by PCR sequencing based typing with specific primers (21, 22). Tumor IGHV sequence was determined on DNA from paraffin-embedded samples by NGS using the LymphoTrack Dx IGH FR2 Assay - PGM assay (InVivoScribe Technologies). The Fastq files generated were analyzed with the LymphoTrack-PGM software from Invivoscribe following the manufacturer's instructions. HLA class-I and class-II epitope prediction was performed *in silico* by SYFPEITHI (http://www.syfpeithi.de/), NetMHC (http://www.cbs.dtu.dk/services/NetMHC/), and NetCTL (http://www.cbs.dtu.dk/services/NetCTL/). For each patient, at the most two epitopes, with the highest score for the selected class-I or class-II alleles, were chosen as best candidates to detect T-cell responses

and are reported on Supplementary Table S2. The predicted peptides were obtained by fluorenylmethoxycarbonil synthesis (ProteoGenix) and were dissolved in DMSO and stored at −80°C. Spontaneous T-cell responses against IGHV-derived peptides were evaluated by analyzing CD107a degranulation through flow cytometry. Briefly, thawed PBMCs were stimulated overnight with 10 μg/mL IGHV-derived peptides, in the presence of fluorescein or phycoerythrin-labelled α–CD107a antibody (mouse IgG1, H4A3; BD Biosciences), Golgi-STOP Solution (BD Biosciences), and 10 μg/mL brefeldin (Sigma-Aldrich). Stimulation with HIV peptides was used as negative control. Nonspecific stimulation with 5 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) was employed as control. After stimulation, cells were stained for surface markers using APC-Cy7-α-CD4 (Mouse IgG1, RPA-T4) and BV421-α-CD8 (Mouse IgG1, RPA-T8). The Fixable Viability Dye eFluor 455UV (eBioscience, Affymetrix) was used to determine cell viability. At least 5×10^5 events were acquired. Flow cytometry analysis was performed with LSR-FortessaTM (Becton Dickinson), data analyzed with DIVA (BD), and FlowJo (Tree Star) software. The degranulation of CD107a was considered positive if at least doubled compared with the one observed after stimulation with the negative control (HIV peptides).

The IFNγ release enzyme-linked immunosorbent spot (ELISpot) assay was performed using the commercial kit "Human IFNγ Single-Color ELISPOT" (Cellular Technology Limited (CTL), Human IFNγ IMMUNOSPOT), according to the manufacturer instructions. Briefly, single peptides were seeded onto overnight precoated ELISPOT capture plates in triplicates and incubated 20 minutes at 37°C. Patient's PBMCs were plated (150,000 cells/well) and incubated at 37°C. Media and PBMC alone were used as negative controls, while 0.5 μg/mL of α-CD3 and α-CD28 (BD Biosciences) were used as unspecific stimuli. At day 3, spots were detected with anti-human IFNγ (biotin), streptavidin alkaline phosphatase, and blue developer solution. Spots were counted and analyzed by ImmunoSpot scanning and analysis service. The number of spots in negative control wells was subtracted from the number of spots in stimulated wells. Responses were considered significant if, at least, five IFNγ-producing cells were detected.

Statistical analysis

All experiments were conducted at least 3 independent times, and data are presented as the mean ± SD. The principal endpoint of the phase I clinical study was the safety and feasibility of rituximab/IFN-DC immunotherapy in patients with follicular lymphoma; therefore, a typical sample size calculation has not been made. For patients immunomonitoring, only tumor-specific immune responses higher than pretreatment basal levels were considered positive and were used to determine the response values range among patients for each test (CD107a and ELISPOT). Data were divided into tertiles, which were used to classify each patient's relative response level.

Results

Preclinical studies on IFN-DC features relevant for their use in the phase I trial

We have previously shown that IFN-DCs are fully competent for the uptake of follicular lymphoma apoptotic bodies and for the induction of antitumor $CD8^+$ T-cell response (17). However, clinical translation into GMP-compliant standards limits the use of autologous follicular lymphoma apoptotic bodies generated *ex vivo* for clinical use. Therefore, and stemming from the encouraging results gained in a previous clinical trial (18), we reasoned that intranodal injection of unloaded IFN-DC following rituximab would represent a more feasible approach to induce endogenous anticancer vaccination. Notably, the studies on follicular lymphoma immunotherapy are still hampered by the lack of validated syngeneic mouse models and by the impossibility to engraft immunodeficient mice with primary human follicular lymphoma cells (23–25). Follicular lymphoma cells resemble normal germinal center (GC) B cells, which depend on stromal cells for their survival and undergo rapid apoptosis *in vitro* (26).Thus, we utilized the Karpas-422 follicular lymphoma continuous cell line together with the humanized NOD-SCID mouse model for preclinical testing. We first tested direct cytotoxicity against follicular lymphoma cells and observed that follicular lymphoma patient–derived IFN-DCs have some basal cytotoxic effect against Karpas-422 cells that was strongly enhanced (4 folds) by target pretreatment with rituximab. No appreciable cytotoxicity was shown by incubating Karpas-422 cells with the sole rituximab (Fig. 1A). Then, to test the ability of IFN-DC to undergo complete activation/maturation step under *in vivo* conditions, we evaluated phenotype of antigen-loaded IFN-DC before and after 24-hour cocultivation with autologous PBL. As shown in Fig. 1B, despite already showing a partially mature phenotype (with high levels of CD80 and CD86 and minimal levels of CD83 and CD56), antigen-loaded IFN-DC promptly acquired a phenotype of fully mature DC upon encountering PBL. Next, we analyzed $CD8^+$ T-lymphocyte infiltration in Karpas-422 cell–induced lymphomas in hu-PBL-NOD/SCID mice following rituximab or rituximab + intratumoral injection of IFN-DC. As shown in Fig. 1C, both rituximab and rituximab/IFN-DC increased CD8/granzyme B^+ T-cell infiltration as compared with control mice, thus indicating that rituximab treatment alone is already capable of increasing $CD8^+$ lymphocyte infiltration and suggesting that intratumoral IFN-DC may direct polarization and strength of $CD8^+$ T-cell responses.

These preclinical data, together with previous results on the *in vitro* antitumor response induced by IFN-DC loaded with apoptotic cells from patients with follicular lymphoma (17), provided the rationale for the design of a phase I trial. In particular, we assumed that intranodal injection of unloaded IFN-DC in patients with follicular lymphoma could elicit the uptake of dead/apoptotic lymphoma cells and tumor-derived antigens shed from direct and indirect lymphoma cell killing by pretreatment with rituximab, ultimately resulting in activation of antitumor T-cell response.

Trial design, patient characteristics, and toxicity assessment

We then designed a phase I clinical study (EudraCT: *2013-003158-25)* aimed at evaluating safety and tolerability as well as the induction of immune and clinical response of an intranodal-based therapy with IFN-DC and low-dose rituximab in patients with advanced R/R follicular lymphoma.

From November 2014 to November 2017, 14 patients with confirmed diagnosis of follicular lymphoma were screened for their compliance to the study inclusion/exclusion criteria. Nine patients were enrolled, eight completed the treatment protocol,

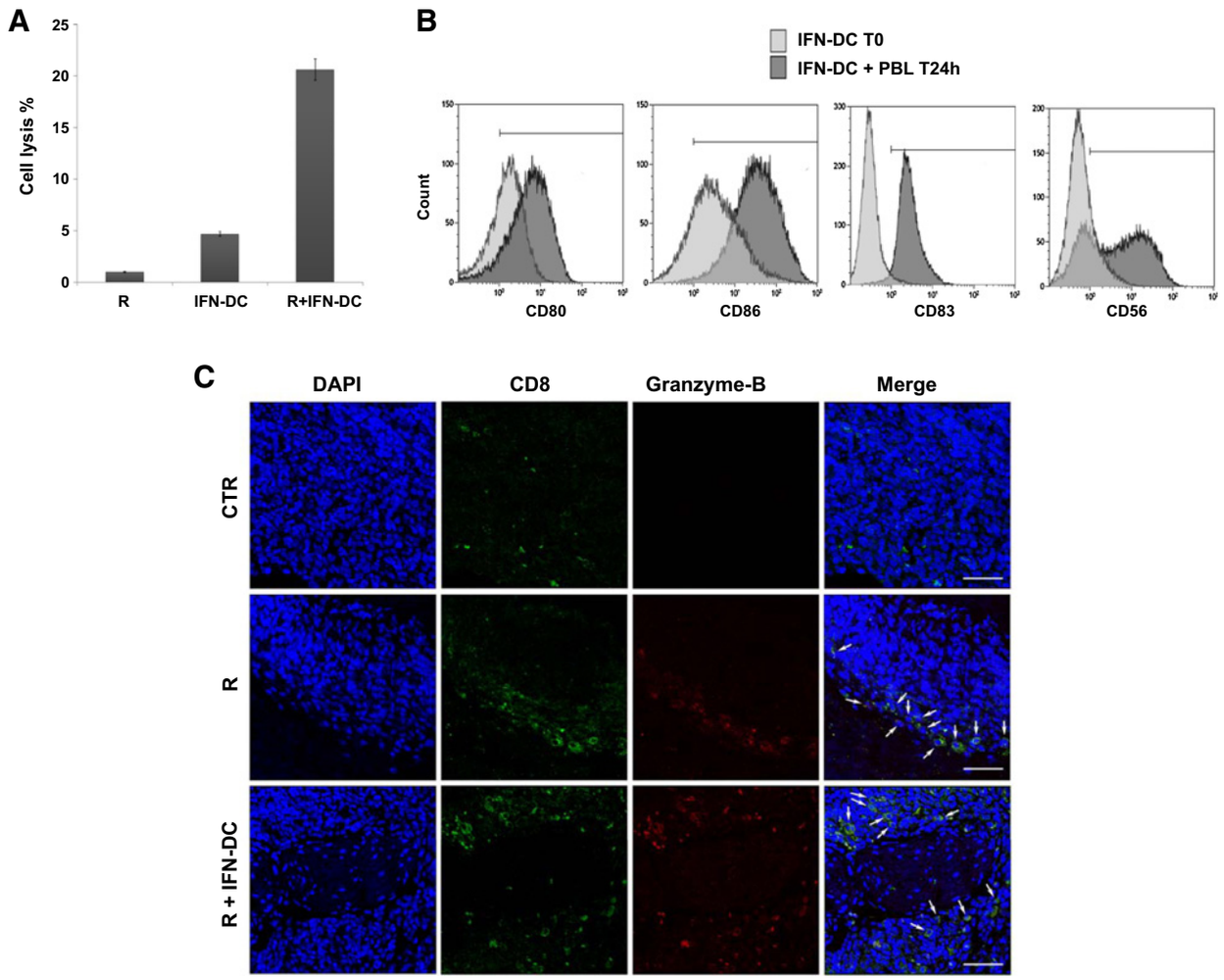

Figure 1.

IFN-DC in combination with rituximab shows increased direct cytotoxicity and induction of antitumor response against Karpas-422 follicular lymphoma. **A,** Patient's IFN-DC–mediated cytotoxicity against lymphoma cells, determined by Calcein-AM assay. Karpas-422 cells were pretreated with rituximab (5 μg/mL) for 30 minutes; IFN-DC were added at 5:1 ratio and incubated for 4 hours at 37°C. The histogram shows the mean value ± SD for three replicates. **B,** IFN-DC profiles before and after cocultivation with autologous PBL. PBL isolated from healthy blood donors were cultured for 24 hours with IFN-DC (PBL/DC ratio 4:1) pulsed with apoptotic Karpas-422 cells (DC/Apo ratio of 1:2). Data are representative of three independent experiments. **C,** Evaluation of $CD8^+$ lymphocyte infiltration in hu-PBL-NOD/SCID mice bearing follicular lymphoma tumors. CLSM examinations (three-dimensional reconstruction images) of FFPE tissue sections from untreated (top), rituximab only (middle) or rituximab/IFN-DC–treated xenochimeric mice (bottom). Tumor-bearing hu-PBL-NOD/SCID mice were injected intratumorally with rituximab and 24 hours later with 2×10^6 IFN-DC every 7 days (3 injections). Fourteen days after the third injection, tumors were harvested and analyzed, and tumor sections were stained for CD8 (detected in green) and granzyme-B (red) expression. DAPI was used to stain nuclei (blue). Arrows depict $CD8^+$ T lymphocytes expressing granzyme-B in the fields displayed. Scale bars, 50 μm. Immunofluorescence slides were observed with a Leica TCS SP2 AOBS Confocal laser scanning microscopy. Image acquisition and processing were carried out using the Leica Confocal Software (Leica) and Adobe Photoshop CS5 Software Programs (Adobe Systems). At least ten fields for each condition were analyzed and representative results are shown.

whereas for 1 patient the IFN-DC production failed during initial monocyte separation. Median age was 57 years (range 27–72). All subjects had disseminated disease and enlarged lymph nodes or lesions greater than 1.5 cm at multiple sites available for local injections and monitoring for response. All patients were R/R after systemic therapy (median lines, 2; range, 1–4), 3/8 (37.5%) were R/R after autotransplantation. Patients' characteristics are listed in Table 1 and treatment regimen is illustrated in Fig. 2A and consisted in the subsequent administration of low-dose rituximab (5–10 mg/cycle) followed 24 hours later by IFN-DC (10×10^6 cells/cycle). Rituximab and IFN-DC were administered by direct intranodal injection in one affected superficial lymph node or lesion. The first 4 cycles were scheduled every 2 weeks and the remaining 4 were administered monthly. Treatment procedures were all carried out as an outpatient practice by a radiologist with the aid of an ultrasound probe. For patients showing multiple lesions, one single lymph node was selected as target for all injections.

The treatment was easy to perform and very well tolerated. All patients experienced a brief, localized pain after injections. Only 1 patient experienced mild swelling and redness localized at the site of rituximab injection classified as grade 2 adverse event.

Table 1. Patients' features at enrolment

Patient	Age/sex	Grade and type of follicular lymphoma	Time from 1st diagnosis	Previous lines of treatment	Stage
Patient 1	50/M	G1; nodular	52 m	R-CHOPx6 and Zevalin	III
Patient 2	61/M	G2; nodular	144 m	R-CHOP x 6; R-DHAP x3 and Zevalin; GMALL x4-Auto-BMT	III
Patient 3	54/M	G2; nodular	46 m	R-CHOP x6; R-DHAP x3 and Zevalin; R-bendamustine x5 and R-maintenance x8	III
Patient 4	27/F	G1–2; nodular	32 m	R-Bendamustine x6 and R- x2	III
Patient 5	72/F	G1, nodular	64 m	R-CVP x6	III
Patient 6	60/M	G2; nodular	117 m	CEOP x 12; R-DHAPx 3 and Auto-BMT; Radiotherapy	III
Patient 7	52/M	G1, nodular	47 m	R-CHOPX6 +2R R-MICMA X2 and Auto-BMT R-maintenance x12	III
Patient 8	64/F	G2; nodular	18 m	R x 6	IV

Abbreviations: Auto-BMT, autotransplantation with autologous hematopoietic stem cells; CEOP, cyclophosphamide, vincristine, methylprednisolone, etoposide; CHOP, cyclophosphamide, doxorubicine, vincristine, methylprednisolone; CVP, cyclophosphamide, vincristine, methylprednisolone; DHAP, dexamethasone, cisplatinum, aracytin; MICMA, mitoxantrone, carboplatinum, aracytin, methylprednisolone; R, rituximab.

No emergence of autoimmune markers was observed in the 8 patients after a median follow-up of 26 months.

Evaluation of clinical response

A median of 4 untreated nodal areas with lesions of more than 1.5 cm in largest diameter were utilized for monitoring of clinical responses (range 1–10). Responses were evaluated according to recently revised response criteria and recommendations (27, 28).

Figure 2B reports the swimmer plot analysis of the timelines of the clinical response in the 8 treated patients. After the first 5 cycles, 1/8 patients underwent ultrasound scan of superficial nodes and 7/8, who had deep lymphoma localization, underwent interim CT-PET. Overall 3/8 patients had a reduction of lymphoma lesions >50%, whereas remaining 5 subjects were in stable disease.

After the ending of treatment schedule, the overall response rate (ORR) was 50% (4/8), PET-negative complete response rate (CRR) 37% (3/8), and remission is still ongoing in 2/4 of respondents (median follow-up 26 months, range 11–47). In particular, patient 5 achieved partial response (PR) at +8, which converted to complete response (CR) at +13 month, still ongoing at +27 months (Fig. 2B). Notably, PET scans taken before and after therapy in patient 5 documented the disappearance of uptake signal by supraclavicular lymph node (Fig. 3C and D), selected to be target of treatment injections, but also by lumbar-aortic and iliac lymph nodes (Fig. 3E–H), not directly injected with rituximab/IFN-DC, thus indicating the induction of systemic response with abscopal effects.

Patient 2, a heavily pretreated patient, started rituximab/IFN-DC protocol 18 months after auto-BMT and achieved CR and molecular remission 6 months after rituximab/IFN-DC treatment start (Fig. 2B). At this time, lymph nodes directly receiving treatment injection as well as untreated lesions did not show residual uptake signal in PET scans (Supplementary Fig. S1). Patient 2 was still in lasting CR at +22 months, when he died for previous therapy-related AML.

Patient 3, another heavily pretreated subject, showed PR at interim restaging, but a month after the last IFN-DC cycle had marked enlargement of superficial lymph nodes (Fig. 2B). He was then re-treated with 5 more cycles (till the exhaustion of IFN-DC cells stocks) of intranodal injections, that were performed in lymph nodes not previously treated. Rather surprisingly, patient 3 had a prompt response and achieved PET-negative CR, with the complete regression of both treated and untreated tumor lesions (Supplementary Fig. S2). CR lasted 4 months (Supplementary Fig. S2), until the patient progressed. Worthy of note, the time to next treatment (TTN) was this time more than the double compared with the previous TTN recorded in his clinical history (data not shown). Of the remaining 5 patients, one is in stable PR (+11 months), two in SD (+28 and +11 months, respectively) and 2 patients have progressed (at +8 and +14 months, respectively).

No clinical features or type and number of previous treatments were apparently associated with the achievement of objective response (Table 1).

Evaluation of tumor-specific T-cell responses

At several time points after the beginning of the rituximab/IFN-DC treatment, patients' blood samples were collected and PBLs tested for the presence of tumor-specific $CD8^+$ and $CD4^+$ T cells. For each patient completing the treatment, IGHV epitopes were predicted on the basis of tumor and HLA sequence (Supplementary Table S2) and MHC-I and MHC-II restricted peptides were used to stimulate patients' lymphocytes. Figure 4 shows CD107a degranulation (A) and IFNγ ELISPOT (B) assays at different time points against class II and class I restricted IGHV peptides, respectively. These results were obtained with PBMCs of two different patients experiencing CR and PR, respectively (patient 5 and 7), and showed that the rituximab/IFN-DC treatment could induce the emergence of both CD4 and CD8 tumor-specific immune responses. Table 2 summarizes the level of tumor-specific T-cell response detected in all patients. The average level of immune response was calculated for each patient/assay among the time points and peptides showing a positive result. The enhancement/emergence of treatment-induced T-cell clones, showing functional reactivity against either MHC class I or MHC class II–restricted patient-specific IGHV epitopes, was detected in all patients. No significant correlations were found between clinical response and the extent of tumor-specific T immune response. Noteworthy, only a barely detectable induction of tumor-specific MHC-I–restricted T-cell response was observed in the clinically nonresponding patient 1, whereas considerably higher levels of tumor-specific T-cell responses were observed in the clinically responding patient 2, 3, and 5, even though the type of response (i.e., MHC class I vs. MHC class II restriction) differed remarkably. Analysis of NK-cell activity and T-cell phenotype (including Treg) were performed on a limited number of patients depending on sample availability without showing any meaningful trend in correlation with clinical response (data not shown).

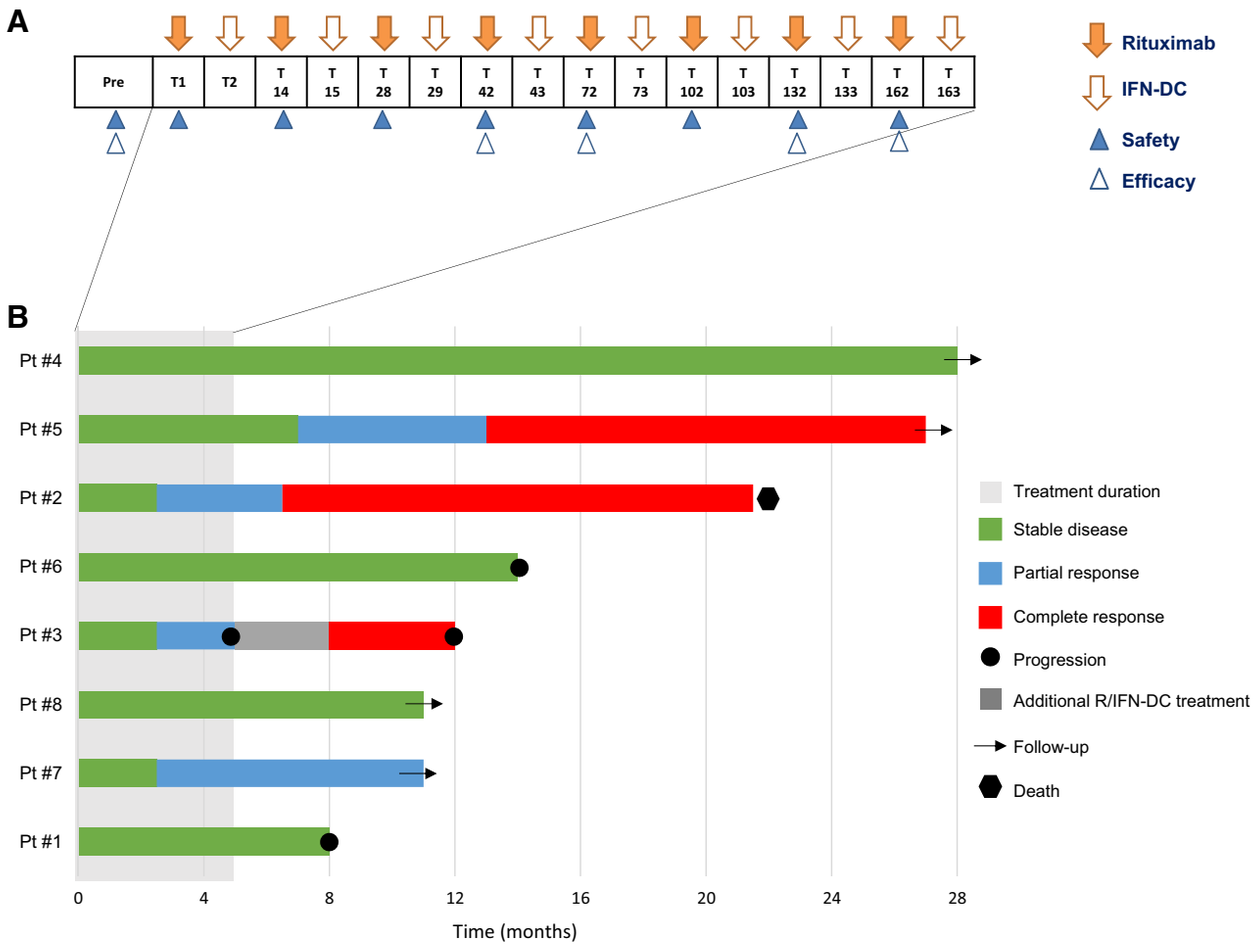

Figure 2.

Treatment schedule and clinical outcome of rituximab/IFN-DC clinical trial. **A,** Schedule of treatment regimen and blood samples for safety and efficacy endpoints evaluation. T, time point expressed as days; Pre, pretreatment time point. **B,** Swimmer plot of patients in the phase I clinical trial with rituximab/IFN-DC treatment. Progression-free survival of patients since date of first vaccination is represented by the filled bars. Periods of stable disease are shown by green bar, PR by blue bars, and CR by red ones. Circles indicate when patient showed progression; hexagon indicates death. Arrows indicate patients that are still in follow-up.

Discussion

In this study, we demonstrate that in heavily pretreated, advanced stage patients with follicular lymphoma the combined *in situ* treatment with low-dose rituximab followed by autologous antigen-unloaded IFN-DC is safe, clinically active, and associated with the generation of tumor-specific T-cell responses in the peripheral blood. This study represents the first proof-of-principle on the effectiveness of IFN-DC in inducing clinical response in a consistent fraction of patients and in promoting the induction of tumor-specific T cells in all of them, thus implying the occurrence of a successful endogenous antitumor vaccination. The findings suggest that some of the specific properties of IFN-DC, such as the high capability to induce a potentially protective T-cell immunity (14–17 and other refs included), can be successfully exploited for a selective clinical use in combination therapies.

To the best of our knowledge, only few studies have investigated so far DC-based therapies in follicular lymphoma (4–7), reporting objective and durable clinical responses. The rationale and treatment schedule followed by Kolstad and collaborators (5) was somehow similar to our protocol, because unloaded DCs were used, in combination with low-dose rituximab, given intratumorally with the aim of achieving a systemic response. However, in spite of these similarities, there are some major differences in our study worth to be underlined: (i) the use of IFN-DC versus the immature IL-4-DC; (ii) the omission of radiotherapy, which may be an additional burden for patients, further complicating the interpretation of the results, considering that rituximab alone may be sufficient to promote the release of tumor antigens; (iii) the higher number of cycles of rituximab/DC injection (8 vs. 3), which could be important for allowing persistence of antitumor response. Worthy of note, despite most of the subjects enrolled in our trial were heavily pretreated as compared with those enrolled by Kolstad and colleagues (5), who were mostly treatment-naïve, the ORR was 50%, PET-negative CRR 37%, and long-term remissions were

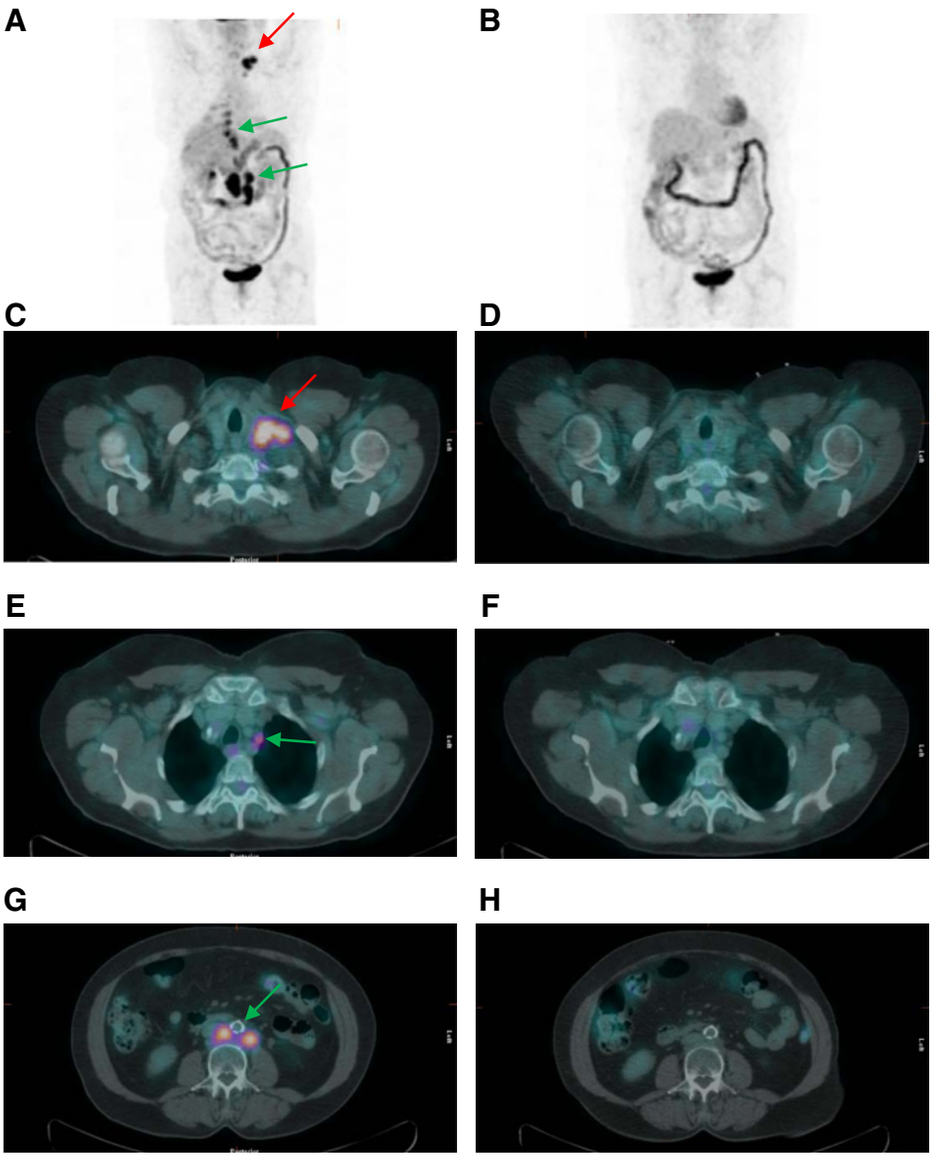

Figure 3.
Rituximab/IFN-DC treatment induces the regression of treated and untreated lymph nodes. CT-PET scan of patient 5 before (**A, C, E, G**) or 13 months after rituximab/IFN-DC treatment (**B, D, F, H**). **A,** Pretreatment frontal view PET scan showing pathologic lymph nodes, which were target of injections (red) or untreated lesions (green); all lesions disappeared in the posttreatment (13 months) scan (**B**). **C,** Pretreatment axial section of PET scan showing increased uptake by the left supraclavicular lymph node that was selected to be the target of rituximab+IFN-DC injections. The uptake completely disappeared in the posttreatment scan (**D**). **E** and **G,** pretreatment sections of lumbar-aortic and iliac untreated lymph nodes, respectively. Despite the fact that these lesions were not directly injected with rituximab/IFN-DC, the uptake signal completely disappeared in the posttreatment scan (**F** and **H**).

observed (median response duration = 13.5 months, range 4–20). Even though the limited number of patients evaluated in our trial do not allow drawing conclusions on the clinical effectiveness, the antitumor response observed in some patients with a poor prognosis is rather impressive. Of interest, the regression of the untreated lesions was frequently observed (abscopal effect), suggesting the occurrence of a systemic response after rituximab/IFN-DC intranodal injection.

The development of effective strategies to induce an endogenous vaccination by *in situ* cancer immunotherapy is currently considered an important research challenge (29–31). This approach may have therapeutically relevant advantages with respect to the use of defined tumor antigens, being able to induce a broader antitumor immunity, potentially targeting also neoantigens continuously generated during tumor progression and along the treatment. Of note, unlike other B-cell lymphomas, follicular lymphoma cells continuously undergo somatic mutations leading to the continuous generation of new tumor neoantigens (32, 33), thus representing the ideal setting for endogenous vaccination. The finding that all patients treated with our combination schedule showed induction of tumor-specific T-cell responses and the observation of the abscopal effect supports the original assumption and rationale that intratumoral injection of IFN-DC after rituximab exposure can very efficiently result in an endogenous antitumor vaccination. The use of a highly specific immunomonitoring platform for detecting tumor-specific responses directed toward clonal IGVH in patients with single follicular lymphoma did not disclose significant correlations between the clinical response and the generation of tumor-specific T-cell responses, probably due to the limited number of patients investigated. However, a strong induction of tumor-specific degranulating cytotoxic T cells, specific for either class-I–restricted CD8 or class-II–restricted CD4 idiotype epitopes, was detected in the

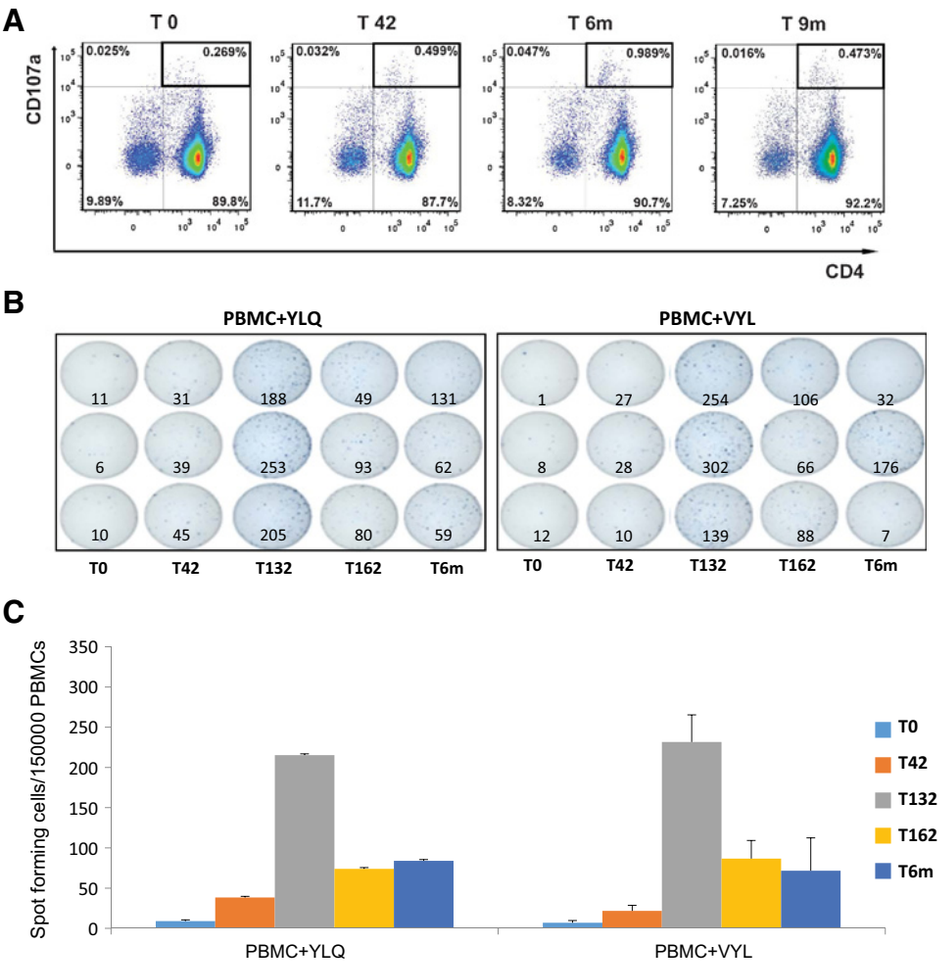

Figure 4.
Rituximab/IFN-DC treatment induces systemic antitumor CD4 and CD8 T-cell responses (**A**). A representative dot plot showing CD107a degranulation on $CD4^+$ T cells isolated from patient 5 following stimulation with class II–restricted IGHV peptide RFTISRDNSRNTLFL before, during, and after treatment. **B,** ELISPOT results showing patient 7 lymphocytes producing IFNγ upon stimulation with two different class I–restricted IGHV selected peptides YLQMNSLRV (YLQ) and VYLQMNSLRV (VYL) at the indicated time points. For each well, the number of spot is indicated (**C**). The histogram shows the mean value ± SD for the three ELISPOT replicates of patient 7 at the indicated time point.

peripheral blood of the 3 patients exhibiting a complete clinical response, showing a peak generally at 6–9 months after the beginning of the treatment, when objective clinical responses were observed. In this regard, it is worth mentioning that also tumor-specific $CD4^+$ T cells have recently been reconsidered as important effector cells in the immune control of B-cell lymphomas (34, 35) because IGVH-derived neoantigens are most frequently presented by MHC-II (34)

Table 2. Summary of tumor-specific immune response detected by Elispot or CD107 degranulation assay against IGHV-predicted peptides

		Immune response against IGHV peptides[a]			
		ELISPOT		CD107a degranulation	
Patient	Best response	MHC-I	MHC-II	MHC-I	MHC-II
Patient 1	PD	+[b]	ND	–	ND
Patient 2	CR	+++	ND	+++	ND
Patient 3	CR	–	+	++	–
Patient 4	SD	+	+	–	–
Patient 5	CR	+++	–	–	++
Patient 6	SD	ND	–	ND	++
Patient 7	PR	++	ND	–	ND
Patient 8	SD	+	–	+	–

Abbreviation: ND, not done.
[a]For each patient, IGHV epitopes were predicted on the basis of tumor and HLA sequence. MHC-I and MHC-II–restricted peptides were obtained and used to stimulate patients' lymphocytes collected at different time points during rituximab/IFN-DC immunotherapy. For each patient/assay, only responses higher than pretreatment basal levels were considered positive and were used to determine the immune response values range.
[b]+, ++, +++ indicate relative levels of immune response: the immune responses detected for each test were divided into tertiles. Immune response in the tertile with the lowest relative response level are indicated by a "+," in the mid-tertile by "++," and in the top tertile by "+++." –, negative.

Although our preclinical studies (Fig. 1) and previous evidence by us (14–17) and others, both in preclinical models (36) and patients (5), strongly support a critical role for DC, there is the remote hypothesis that rituximab can be also important for determining clinical and immune response. We consider unlikely that the low doses of rituximab administered in this protocol, about 7% of the dose delivered to low tumor burden–naïve follicular lymphoma subjects in the study by Ardeshna and colleagues (2), could have been sufficient to determine objective and sustained remission without the combination with IFN-DC remissions. However, we cannot completely rule out that rituximab may have contributed with a vaccination-like effect (37), even though such an abscopal effect mediated by low-dose intranodal rituximab in advanced stage follicular lymphoma has never been reported.

Patients with follicular lymphoma are presently treated with long-lasting and very expensive treatments, which allowed major improvement in life expectancy (1, 38). However, most patients experience subsequent relapses, increasing risk of transformation, and a growing burden of toxicities, thus effective long-term

follicular lymphoma management is a relevant unmet clinical need (1, 3, 38). In view of the safety and activity data shown in this clinical trial and taking into account the current challenges in performing clinical studies in patients with follicular lymphoma (39), we believe that this schedule deserves to be tested in a larger cohort. A third of patients with newly diagnosed follicular lymphoma have low tumor burden and most of them have superficial lymph nodes (1). Naïve low tumor burden follicular lymphoma, who is presently an orphan subset with no clear advantage to undergo early treatment (2), may be the ideal target for experimenting this approach.

Today, we are facing a great enthusiasm about cancer immunotherapy, mostly due to the clinical impact by immune checkpoint inhibitors, particularly those targeting programmed death (PD)-1 and PD-ligand (L)-1. However, most patients (70%–80%) remain resistant to this single therapy, underlining the interest in identifying novel and clinically effective combination protocols, including cancer vaccines (40). Of interest, a recent study has shown that the antitumor activity of anti-PD1 antibodies strictly depends on the occurrence of intratumoral DC producing IL12 (41). Notably, IFN-DCs, which undergo a rapid and complete maturation after PBL cocultivation (Fig. 1A), are high producers of IL12 (42) and therefore might strongly potentiate anti–PD1-based therapies.

Thus, we conclude by stating that, in today's era of cancer immunotherapy, where DC-based therapies have begun showing promising results, IFN-DC may represent a new and highly valuable DC type, endowed with antitumor activities and suitable for the development of more effective combination therapies including checkpoint inhibitors (9, 12).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Casulo C, Nastoupil L, Fowler NH, Friedberg JW, Flowers CR. Unmet needs in the first-line treatment of follicular lymphoma. Ann Oncol 2017;28: 2094–106.
2. Ardeshna KM, Qian W, Smith P, Braganca N, Lowry L, Patrick P, et al. Rituximab versus a watch-and-wait approach in patients with advanced-stage, asymptomatic, non-bulky follicular lymphoma: an open-label randomised phase 3 trial. Lancet Oncol 2014;15:424–35.
3. Freedman A. Follicular lymphoma: 2018 update on diagnosis and management. Am J Hematol 2018;93:296–305.
4. Di Nicola M, Zappasodi R, Carlo-Stella C, Mortarini R, Pupa SM, Magni M, et al. Vaccination with autologous tumor-loaded dendritic cells induces clinical and immunologic responses in indolent B-cell lymphoma patients with relapsed and measurable disease: a pilot study. Blood 2009;113: 18–27.
5. Kolstad A, Kumari S, Walczak M, Madsbu U, Hagtvedt T, Bogsrud TV, et al. Sequential intranodal immunotherapy induces antitumor immunity and correlated regression of disseminated follicular lymphoma. Blood 2015; 125:82–9.
6. Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. Blood 2002;99:1517–26.
7. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. Lancet Oncol 2014;15:e257–67.
8. Garg AD, Vara Perez M, Schaaf M, Agostinis P, Zitvogel L, Kroemer G, et al. Trial watch: dendritic cell-based anticancer immunotherapy. Oncoimmunology 2017;6:e1328341.
9. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. Immunity 2013;39:38–48.
10. Bol KF, Schreibelt G, Gerritsen WR, de Vries IJM, Figdor CG. Dendritic cell-based immunotherapy: state of the art and beyond. Clin Cancer Res 2016; 22:1897–906.
11. Santos PM, Butterfield LH. Dendritic cell–based cancer vaccines. J Immunol 2018;200:443–9.
12. Garg AD, Coulie PG, Van den Eynde BJ, Agostinis P. Integrating next-generation dendritic cell vaccines into the current cancer immunotherapy landscape. Trends Immunol 2017;38:577–93.
13. Anguille S, Van De Velde AL, Smits EL, Van Tendeloo VF, Juliusson G, Cools N, et al. Dendritic cell vaccination as postremission treatment to prevent or delay relapse in acute myeloid leukemia. Blood 2017;130:1713–21.
14. Santini SM, Lapenta C, Logozzi M, Parlato S, Spada M, Di Pucchio T, et al. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. J Exp Med 2000;191:1777–88.
15. Lapenta C, Santini SM, Logozzi M, Spada M, Andreotti M, Di Pucchio T, et al. Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-α. J Exp Med 2003;198: 361–7.
16. Spadaro F, Lapenta C, Donati S, Abalsamo L, Barnaba V, Belardelli F, et al. IFN-α enhances cross-presentation in human dendritic cells by modulating antigen survival, endocytic routing, and processing. Blood 2012;119: 1407–17.
17. Lapenta C, Donati S, Spadaro F, Castaldo P, Belardelli F, Cox MC, et al. NK cell activation in the antitumor response induced by IFN-α dendritic cells loaded with apoptotic cells from follicular lymphoma patients. J Immunol 2016;197:795–806.

18. Rozera C, Cappellini GA, D'Agostino G, Santodonato L, Castiello L, Urbani F, et al. Intratumoral injection of IFN-alpha dendritic cells after dacarbazine activates anti-tumor immunity: results from a phase I trial in advanced melanoma. J Transl Med 2015;13:139.
19. Subbiah V, Murthy R, Hong DS, Prins RM, Hosing C, Hendricks K, et al. Cytokines produced by dendritic cells administered intratumorally correlate with clinical outcome in patients with diverse cancers. Clin Cancer Res 2018;24:3845–56.
20. Ghielmini M, Vitolo U, Kimby E, Montoto S, Walewski J, Pfreundschuh M, et al. ESMO consensus guidelines: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL) | ESMO. Ann Oncol 2013;24:561–76.
21. Tonks S, Marsh SG, Bunce M, Bodmer JG. Molecular typing for HLA class I using ARMS-PCR: further developments following the 12th International Histocompatibility Workshop. Tissue Antigens 1999;53:175–83.
22. Entz P, Toliat MR, Hampe J, Valentonyte R, Jenisch S, Nürnberg P, et al. New strategies for efficient typing of HLA class-II loci DQB1 and DRB1 by using Pyrosequencing™. Tissue Antigens 2005;65:67–80.
23. Epron G, Ame-Thomas P, Le Priol J, Pangault C, Dulong J, Lamy T, et al. Monocytes and T cells cooperate to favor normal and follicular lymphoma B-cell growth: role of IL-15 and CD40L signaling. Leukemia 2012;26:139–48.
24. Mourcin F, Pangault C, Amin-Ali R, Amé-Thomas P, Tarte K. Stromal cell contribution to human follicular lymphoma pathogenesis. Front Immunol 2012;3:280.
25. Amé-Thomas P, Tarte K. The yin and the yang of follicular lymphoma cell niches: role of microenvironment heterogeneity and plasticity. Semin Cancer Biol 2014;24:23–32.
26. Kridel R, Sehn LH, Gascoyne RD. Pathogenesis of follicular lymphoma. J Clin Invest 2012;122:3424–31.
27. Cheson BD, Pfistner B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. J Clin Oncol 2007;25:579–86.
28. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol 2014;32:3059–68.
29. Sagiv-Barfi I, Czerwinski DK, Levy S, Alam IS, Mayer AT, Gambhir SS, et al. Eradication of spontaneous malignancy by local immunotherapy. Sci Transl Med 2018;10:eaan4488.
30. Frank MJ, Reagan PM, Bartlett NL, Gordon LI, Friedberg JW, Czerwinski DK, et al. *In situ* vaccination with a TLR9 agonist and local low-dose radiation induces systemic responses in untreated indolent lymphoma. Cancer Discov 2018;8:1258–69.
31. Aznar MA, Tinari N, Rullán AJ, Sánchez-Paulete AR, Rodriguez-Ruiz ME, Melero I. Intratumoral delivery of immunotherapy—act locally, think globally. J Immunol 2017;198:31–9.
32. Papaioannou D, Strothmeyer A-M, Dühren-von Minden M, Keppler-Hafkemeyer A, Zirlik K, Mikesch K, et al. Evidence for idiotype-directed immunosurveillance is restricted to follicular lymphoma and attributable to somatic hypermutation. Haematologica 2015;100:e143–6.
33. Aarts WM, Bende RJ, Steenbergen EJ, Kluin PM, Ooms EC, Pals ST, et al. Variable heavy chain gene analysis of follicular lymphomas: correlation between heavy chain isotype expression and somatic mutation load. Blood 2000;95:2922–9.
34. Khodadoust MS, Olsson N, Wagar LE, Haabeth OAW, Chen B, Swaminathan K, et al. Antigen presentation profiling reveals recognition of lymphoma immunoglobulin neoantigens. Nature 2017;543:723–7.
35. Weng J, Baio FE, Moriarty KE, Torikai H, Wang H, Liu Z, et al. Targeting B-cell malignancies through human B-cell receptor specific $CD4^+$ T cells. Oncoimmunology 2016;5:e1232220.
36. Manzur S, Cohen S, Haimovich J, Hollander N. Enhanced therapeutic effect of B cell-depleting anti-CD20 antibodies upon combination with in-situ dendritic cell vaccination in advanced lymphoma. Clin Exp Immunol 2012;170:291–9.
37. Hilchey SP, Hyrien O, Mosmann TR, Livingstone AM, Friedberg JW, Young F, et al. Rituximab immunotherapy results in the induction of a lymphoma idiotype-specific T-cell response in patients with follicular lymphoma: support for a "vaccinal effect" of rituximab. Blood 2009;113:3809–12.
38. Danese MD, Reyes CM, Gleeson ML, Halperin M, Skettino SL, Mikhael J. Estimating the population benefits and costs of rituximab therapy in the United States from 1998 to 2013 using real-world data. Med Care 2016;54:343–9.
39. Maddocks K, Barr PM, Cheson BD, Little RF, Baizer L, Kahl BS, et al. Recommendations for clinical trial development in follicular lymphoma. J Natl Cancer Inst 2017;109:djw255.
40. Karaki S, Anson M, Tran T, Giusti D, Blanc C, Oudard S, et al. Is there still room for cancer vaccines at the era of checkpoint inhibitors. Vaccines 2016;4:37.
41. Garris CS, Arlauckas SP, Kohler RH, Trefny MP, Garren S, Piot C, et al. Successful anti-PD-1 cancer immunotherapy requires T cell-dendritic cell crosstalk involving the cytokines IFN-γ and IL-12. Immunity 2018;49:1148–61.
42. Santini SM, Lapenta C, Donati S, Spadaro F, Belardelli F, Ferrantini M. Interferon-α-conditioned human monocytes combine a Th1-orienting attitude with the induction of autologous Th17 responses: role of IL-23 and IL-12. PLoS One 2011;6:e17364.