Annals of Oncology 23: 435–441, 2012
doi:10.1093/annonc/mdr134
Published online 17 May 2011

T-cell therapy for EBV-associated nasopharyngeal carcinoma: preparative lymphodepleting chemotherapy does not improve clinical results

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Received 10 November 2010; revised 5 January 2011 & revised 21 January 2011; accepted 7 March 2011

Background: We and others have demonstrated that adoptive cell therapy with Epstein–Barr virus (EBV)-specific autologous cytotoxic T lymphocytes (CTLs) may control disease progression in patients with EBV-associated nasopharyngeal carcinoma (NPC). With the aim of favoring *in vivo* T-cell expansion, we optimized our cell therapy approach by administering higher doses of EBV-specific CTLs, following lymphodepleting chemotherapy.
Patients and methods: Eleven patients with EBV-related NPC in whom conventional treatment failed have been enrolled. Patients received nonmyeloablative lymphodepleting chemotherapy consisting of cyclophosphamide and fludarabine. Two doses of autologous EBV-specific CTLs were subsequently infused, 2 weeks apart. Study end points were feasibility and clinical outcome.
Results: All patients enrolled completed the treatment and were assessable for analysis. The median dose of CTLs per infusion was 3.7×10^8. Therapy was well tolerated, with no severe adverse events ascribable to either

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chemotherapy or cell therapy. Disease control (defined as either tumor regression or disease stabilization lasting >4 months) was obtained in 6 of 11 patients, in keeping with previously published results.
Conclusions: Our data confirm that EBV-specific CTL therapy is safe and associated with antitumor activity in patients with advanced NPC. The use of lymphodepleting chemotherapy before high-dose CTL infusion did not enhance the clinical benefit observed in our previous series.
Key words: cell therapy, cytotoxic T lymphocytes, Epstein–Barr virus, lymphodepletion, nasopharyngeal carcinoma

introduction

Nasopharyngeal carcinoma (NPC) is a squamous epithelium neoplasm, characterized by marked differences in geographic and population incidence. Although NPC is seen worldwide, it is most common throughout Southeast Asia, where the frequency is as high as 50 per 100 000 [1]. In Europe, it is rarely observed, with an incidence ranging between 0.1 and 22 per 100 000 [2]. State-of-the-art treatment strategies for locally advanced NPC consist mainly in radiotherapy alone or in combination with platinum-based chemotherapy [3] and yield an overall response rate of ∼90%, with substantial cure rates [4, 5]. Induction treatment is able to improve disease-free survival, while its impact on survival still needs to be demonstrated. In local–regional recurrent NPC not amenable to re-irradiation, combination platinum-based chemotherapy is the standard first-line treatment, with response rates reaching 40%–80%, mainly depending on previous treatment and duration of the disease-free interval [6, 7]. Combination platinum-based chemotherapy is standard therapy also in patients presenting with metastatic disease [6, 7]. Second-line therapies in patients refractory to platinum-based regimens may also lead to clinical benefit [8–10], which is generally short-lived, although long-term surviving patients with metastatic disease treated with chemotherapy and radiotherapy have been reported [11]. Overall, this finding supports the development of additional forms of treatment of NPC.

NPC is consistently associated with the Epstein–Barr virus (EBV). Within the tumor, viral infection is latent with expression of the EBV proteins latent membrane proteins (LMP)-1, LMP2, Epstein–Barr virus nuclear antigen-1 (EBNA1) and Epstein–Barr virus-encoded small RNA (EBER) and *Bam*HI A RNAs [12]. The continued expression of multiple viral proteins on malignant cells provides an opportunity to target viral proteins using virus-directed cellular therapy. In support of this concept, clinical studies have demonstrated how infusion of EBV-specific cytotoxic T lymphocytes (CTLs) expanded *in vitro* could safely and effectively either prevent or treat EBV-positive lymphoproliferative disease occurring in bone marrow or solid-organ transplant recipients [13–16].

Based on these premises, two independent pilot studies have been conducted in the context of EBV-related NPC. The published results demonstrated that clinical and immunological responses could be obtained in some patients with radiotherapy- and chemotherapy-resistant, stage IV, EBV-related NPC through the administration of EBV-specific autologous polyclonal CTL therapy [17, 18]. In an attempt to further improve outcome in NPC patients treated with adoptive cell therapy, we have modified our previously published protocol [18] by administering higher doses of EBV-polyspecific CTLs following a lymphodepleting chemotherapeutic conditioning regimen.

patients and methods

patients

Eligible patients were <70 years of age with histologically confirmed, stage IV, EBV-LMP1- and/or EBER-positive NPC. For enrollment, patients were required to demonstrate documented disease progression by computed tomography (CT) and/or magnetic resonance imaging (MRI), not amenable to further systemic or local conventional treatments. Patients were required to have normal organ function, while they were excluded if undergoing immunosuppressive therapy or in the case of active brain metastases. None of the patients received cytotoxic therapies within 30 days of T-cell therapy.

Approval was obtained from the institutional review board. Patients gave written informed consent before enrollment.

generation and characterization of EBV-specific CTLs

EBV-specific CTLs were prepared according to a previously described procedure [19]. Peripheral blood mononuclear cells (PBMCs) and autologous plasma were collected from all patients through a single leukopheresis, collected at diagnosis or at disease relapse, before chemotherapy/radiotherapy administration. EBV-specific CTLs were expanded *in vitro* following good laboratory practice standard procedures. Before cryopreservation, T cells were examined for EBV specificity in a standard Cr 51-release assay against a panel of targets, including an autologous B-lymphoblastoid cell line (LCL) and autologous phytohemagglutinin (PHA) blasts pulsed with 2 μg/ml of a peptide mix containing 15-mer peptides spanning the EBV-LMP2 protein (Jerini, Berlin, Germany) or with control peptide. Lysis against allogeneic PHA blasts was also tested. In addition, CTLs were analyzed for sterility and for immunophenotype on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by direct cytofluorimetry employing the mAbs CD3 (anti-Leu-4) fluorescein isothiocyanate (FITC), anti-HLA-DR phycerythrin (PE), CD8 (anti-Leu-2a) FITC and PE, CD56 (anti-Leu-19) PE, CD4 (anti-Leu-3a) PE, CD19 (anti-Leu-12) FITC, and CD45 (anti-HLe-1) FITC (Becton Dickinson).

EBV-specific CTL infusion schedule and patient evaluation

The lymphodepletion treatment consisted in 4 days of consecutive nonmyeloablative chemotherapy: cyclophosphamide (30 mg/kg daily) on days 1–2 and fludarabine (30 mg/m^2 daily) for 4 days. The fludarabine dose was adjusted according to renal function. When profound lymphopenia was achieved (i.e. an absolute lymphocyte count $<0.2 \times 10^9$/l), cryopreserved CTLs were thawed and administered i.v.; a second infusion was carried out 2 weeks later. The median CTL dose was 3.7×10^8 (range 1.6–5); this infusion scheme was derived from our previous phase I study, in which administration of lower doses of autologous EBV-specific CTLs was demonstrated to be safe and feasible [18]. After the first infusion,

all patients also received low-dose recombinant interleukin 2 (IL-2); 1×10^6 U s.c. daily for 3 weeks) in order to prolong *in vivo* T-lymphocyte life span.

Patients were monitored for evidence of toxicity by physical examination, serum chemistry and daily complete and differential blood counts. Patient response was assessed using standard radiographic studies (CT scan, MRI of measurable lesions), physical examination and blood tests at baseline, 4 weeks following the second CTL administration and at regular 2-month intervals thereafter. Radiographic response was defined according to RECIST [20]. EBV DNA levels were monitored by PCR [21] on plasma samples at baseline, after each CTL infusion, and every 2 months thereafter. To evaluate the effects of CTL administration on the frequency of interferon-γ (IFN)-γ-secreting lymphocytes and on EBV-directed cytotoxic activity, patient peripheral blood samples were collected at baseline and at different times after CTL infusions.

enzyme-linked immunospot assay

For the enzyme-linked immunospot (ELISPOT) assay, a previously reported method was employed [18]. Briefly, 96-well multiscreen filter plates (MAIPS 4510; Millipore, Bedford, MA) were coated with 100 μl of primary antibody (IFN-γ; Mabtech, Nacka, Sweden) at 2.5 μg/ml and incubated overnight at 4°C. PBMCs were thawed and cultured overnight in RPMI medium with 10% fetal calf serum before use in the assay and were then seeded in the presence of EBV-LCL or 2 μg/ml of the EBV-LMP2 peptide mix. After incubating for 24 h at 37°C, 100 μl of the biotinylated secondary antibody (0.5 μg/ml; Mabtech) was added, and plates were then processed according to a standard procedure. IFN-γ-producing spots were counted using an Elispot reader (Bioline, Torino, Italy). The number of spots per well was calculated after subtracting assay background, quantified as an average of 24 wells containing only sterile complete medium, and specific background, quantified as the sum of cytokine spots associated with responders alone, LCL alone, or responders plated with dimethyl sulfoxide solvent control, as appropriate.

results

patients and EBV-specific CTL line characterization

Eleven consecutive patients received the treatment according to the schedule described; all had histologically confirmed, EBV-positive undifferentiated NPC. Relevant patient characteristics are reported in Table 1.

EBV-specific CTL lines were successfully generated *ex vivo* from all patients. Growth kinetics of the T-cell lines from this cohort of NPC patients with disease progression was comparable with those previously observed [18]. Phenotypic analysis indicated that the CTL lines generated from PBMCs of NPC patients were heterogeneous with respect to the percentages of CD3+/CD8+ and CD3+/CD4+ cells. In detail, CD8+ cells ranged from 31% to 91% (median 88%), CD4+ lymphocytes ranged from 3% to 69% (median 9%), and the CD3+/HLA-DR+ population was between 49% and 96% (median 76%). Moreover, the CTL lines contained a median of 26% cells that were CD3+/CD8+/CD56+ and 4% cells with the natural killer cell phenotype (CD56+/CD3−).

The CTL lines were specific for EBV since they exerted cytotoxicity toward autologous LCL (median percentage lysis at an effector-to-target ratio of 10 : 1 : 64). Activity of EBV-CTL lines against the LMP2 protein was present in 6 of the 11 patients (median percentage lysis for the six CTL lines at an effector-to-target ratio of 10 : 1, after subtracting control lysis: 16%, range 9–27). No lysis against allogeneic PHA blasts was observed.

toxicity profile of lymphodepletion followed by EBV-targeted CTL therapy

Patients enrolled in the study received the nonmyeloablative lymphodepleting chemotherapy and the first CTL infusion during an hospital stay, while the second CTL infusion was carried out on an outpatient basis. All patients were monitored for immediate adverse reactions. No grade IV hematological toxic effects were reported. Four patients developed grade III neutropenia and received antimicrobial prophylaxis until resolution. No episodes of grade III thrombocytopenia were observed, while grade II thrombocytopenia and grade II anemia were reported in two patients and one patient, respectively, not requiring blood transfusions. Regarding non-hematological side-effects of the lymphodepleting chemotherapy, no grade III or IV toxic effects were observed, while mild toxic effects such as fatigue and nausea occurred in six patients. Only one patient had a systemic reaction immediately after the second CTL infusion, with fever and tremors requiring antihistamine therapy.

Two patients (patient 2 and patient 7) developed swelling at a disease site a few days after CTL infusion. Patient 7 developed a severe inflammatory reaction at the orbital region, with visual field defects, which was treated with antiinflammatory therapy, including steroids. Patient 2 had a mild inflammatory reaction, consisting of orbital edema, which resolved spontaneously. Both side-effects are likely ascribable to tumor cell lysis. No other adverse events ascribable to the CTL therapy were observed in the remaining patients

lymphocyte kinetics after lymphodepletion followed by EBV-targeted CTL therapy

The lymphodepletion regimen employed in our patients profoundly decreased lymphocyte counts (Figure 1). At the first CTL infusion, all patients showed lymphopenia ($\leq 0.2 \times 10^9$/l) and lymphocyte counts returned to baseline levels only after administration of the second EBV-CTL infusion.

To assess the immune response to EBV antigens, IFN-γ ELISPOT assays were carried out by stimulating PBMCs with autologous LCLs or with an LMP2 peptide pool. Lymphodepletion caused a decrease in the frequency of EBV-specific T cells, as indicated by a persistent lower virus-specific T-cell frequency also after the first CTL infusion (median and range IFN-γ spot-forming units/10^5 cells: 418, 19–541 versus 148, 11–397, respectively, $P < 0.05$) (Figure 2A). Only after the second dose of EBV-specific CTLs, did the median immune response to EBV increase again, returning to baseline levels or even to higher levels in some patients, indicating expansion of adoptively transferred T cells (Figure 2). In particular, patients 2 and 7, who achieved a partial clinical response, and patient 4, who maintained prolonged stable disease, showed an increase in the response to LMP2 antigen after the second CTL infusion (Figure 2B).

To investigate whether the slow kinetics of lymphocyte recovery after lymphodepleting treatment could be due to a hampered secretion of cytokines that promote homeostatic lymphocyte expansion in the lymphopenic host, we measured

Table 1. Main characteristics and clinical outcome of patients with NPC in progression after conventional therapy and treatment with EBV-specific autologous CTLs

Patients (UPN)	Age (years)	Sex	Stage at diagnosis	Site(s) of tumor involvement at the time of cell therapy	Prior therapies	ECOG PS	Adverse events	Best response (duration)
1	19	F	IV (T4N2M0)	Liver, spleen	RT, three lines of CT	0	None	SD (4 months)
2	65	M	III (T3N1M0)	Primary tumor, skull base	Two lines of CT, RT, surgery	0	Inflammatory reaction at the disease site; fever and tremors after second infusion	PR (8 months)
3	21	M	III (T3N1M0)	Primary tumor, skull base	Two lines of CT, RT	0	None	PD
4	40	F	III (T2N2M0)	Skull base, neck	Three lines of CT, RT	1	None	SD (8 months)
5	48	M	IV (T2N2M1)	Primary tumor, skull base	Two lines of CT, RT, surgery	1	None	PD
6	64	M	III (T3N0M0)	Primary tumor	Two lines of CT, RT	0	None	SD (16+ months)
7	49	M	Unknown	Skull base, lung, lymph nodes, orbital cavity	Three lines of CT, RT, surgery	1	Orbital edema and visual field defects	PR (5 months)
8	40	M	Unknown	Primary tumor, skull base	Three lines of CT, RT	0	None	MR (12 months)
9	66	M	IV (TXN2M1)	Primary tumor, neck	Three lines of CT	0	None	PD
10	50	M	IV (T4N2M0)	Liver	Two lines of CT	1	None	PD
11	46	M	II (T2N1M0)	Lung, lymph nodes, liver	Two lines of CT, RT, surgery	1	None	PD

NPC, nasopharyngeal carcinoma; EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocyte; ECOG PS, Eastern Cooperative Oncology Group performance status; F, female; M, male; RT, radiotherapy; ChT, chemotherapy; SD, stable disease; M, male; PR, partial response; PD, progressive disease; MR, minor response; UPN, unique patient number.

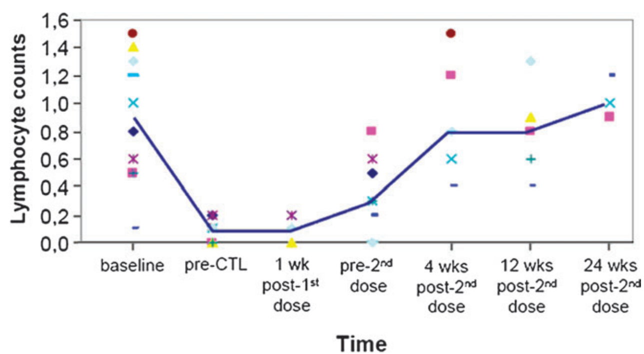

Figure 1. Kinetics of lymphocyte absolute numbers in treated patients. Lymphocyte counts per milliliter in the 11 patients at different times before and after lymphodepleting therapy and EBV-specific CTL infusion are shown. The median value for each time point is reported as a continuous blue line. EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocyte.

plasma levels of IL-15 before lymphodepletion, before CTL infusion and 1 week after CTL administration. We observed a significant increase ($P < 0.05$) in the plasma levels of IL-15 at the time of the CTL infusion, which returned to baseline within 1 week (Figure 3). Therefore, failure to show a prompt lymphoid expansion after CTL transfer does not seem to depend on an altered cytokine milieu at the time of CTL infusion.

clinical benefit of EBV-targeted autologous CTL therapy

At the first evaluation, 4 weeks after the second CTL infusion, two patients (patients 2 and 7) showed a partial response (PR) defined according to RECIST, which lasted 8 and 5 months, respectively; one patient (patient 8) showed a minor response (i.e. 20% reduction in the size of target lesions), lasting 12 months. Three patients had stable disease, lasting a median of 8 months (range 4–22 months). The patients who showed clinical responses received maintenance doses of EBV-CTLs (median number of additional infusions: 5, range 4–8). Five patients had progressive disease; 10 patients died at a median of 14.5 months (range 5–23 months) after CTL infusion, and 1 patient is alive with active disease at 38 months from CTL treatment. The outcome of each patient is summarized in Table 1.

Plasma EBV DNA is a marker of disease in NPC patients. Therefore, we evaluated clinical response also on the basis of this parameter. Before lymphodepletion, the patients showed

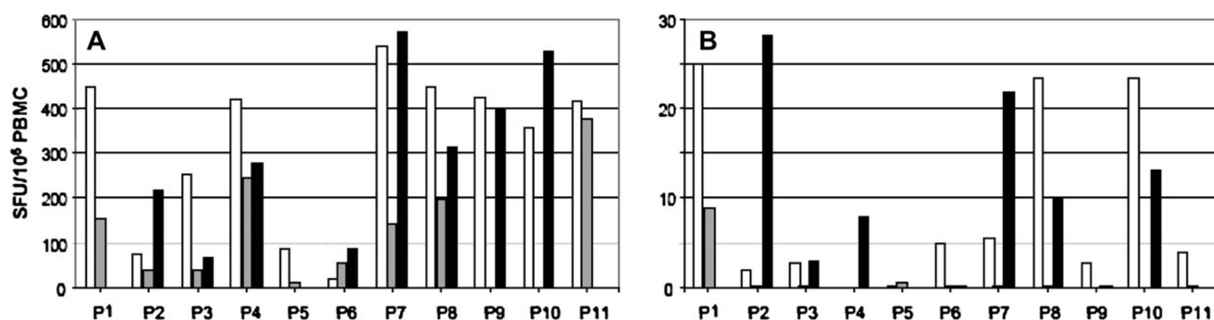

Figure 2. Immune response to EBV-LCL and to LMP2 antigen in treated patients. Data on the frequency of IFN-γ-secreting lymphocytes, measured in patient PBMCs obtained before (white bars), 2 weeks (gray bars), and 8 weeks (black bars) after CTL therapy, in response to EBV-LCL (panel A) and EBV-LMP2 protein peptide mix (panel B) are reported. On the horizontal axis, the 11 consecutive patients are reported. IFN-γ-secreting cells are represented as number of spots/10^5 PBMCs (mean spots of triplicate experiments). EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; LMP2, latent membrane proteins 2; IFN, interferon; PBMC, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocyte.

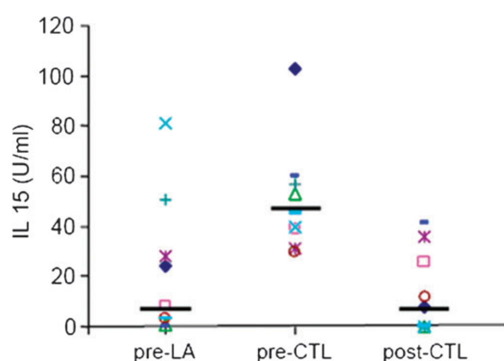

Figure 3. Levels of IL-15 in the plasma of patients before and after lymphodepletion. Data on the plasma levels of IL-15, measured in patient samples obtained before lymphoablation (pre-LA), before CTL infusion (pre-CTL), and 1 week after CTL infusion (post-CTL) are reported. IL-15 concentrations were quantified by ELISA using mAb pairs (Pierce Endogen, Rockford, IL). Plates were coated with purified antibodies at the appropriate concentrations. Standard curves were prepared with recombinant human cytokine (Pierce Endogen). Biotin-labeled antibodies (Pierce Endogen) were added and HRP-conjugated streptavidin (Pierce Endogen) was used to develop the reactions. Plates were read at 450 nm (Titertek Plus MS 212M). IL, interleukin; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase.

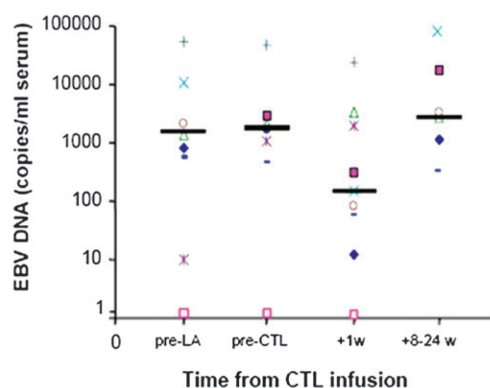

Figure 4. Kinetics of EBV DNA levels in treated patients. Data on EBV DNA levels in the plasma of patients before lymphoablation (pre-LA), immediately before (pre-CTL), and 1 week (+1 week) and 2–6 months (+8–24 weeks) after CTL infusion are reported. EBV DNA analysis was carried out according to a previously described method [22]. EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocyte.

a median plasma EBV DNA load of 1360 copies/ml (range 0–52 980), which slightly increased to 1740 copies/ml (range 0–47 240) before CTL administration. After T-cell therapy, an overall 1-log decrease was observed (median of 154 copies/ml, range 0–23 680), reaching a 2-log reduction in the patients showing clinical responses (Figure 4). Further evaluations showed a new increase in the median levels of plasma EBV DNA, corresponding to disease progression (Figure 4). The patients that had PRs or long duration of stabilized disease, and received additional CTL doses, showed fluctuations in EBV DNA levels according to the administration of maintenance EBV-CTL infusions.

discussion

We previously documented the feasibility of generating *in vitro* autologous EBV-specific CTLs from NPC patients, which possessed *in vitro* antitumor activity and were able to induce disease control once administered *in vivo* [18]. In particular, repeated infusions of up to 1×10^6 CTLs/kg body weight provided a clinical benefit in 6 of 10 heavily pretreated patients. In an attempt to improve the efficacy of our adoptive EBV-specific cell therapy, we chose to modify our approach according to recent reports that suggested a role for a high cell dose preceded by lymphodepletion in T-cell therapy for solid tumors [23]. We reasoned that, in the context of a solid tumor such as EBV-related NPC with large tumor burden, a higher number of EBV-specific CTLs would enhance clinical responses, as observed in melanoma patients treated with tumor-infiltrating lymphocytes [21]. Moreover, in the latter setting, delivery of lymphoablative chemotherapy, consisting of fludarabine and cyclophosphamide, before T-cell administration, favored the spontaneous expansion of infused T cells, contributing to CTL persistence [24]. Based on this study and others, it has been suggested that lymphodepleting chemotherapy could play a role in favorably modifying the tumor microenvironment, by reducing levels of both regulatory T cells and regulatory cytokines, which suppress T-cell effector function and, consequently, tumor surveillance [25–28].

Despite having employed both a lymphodepleting regimen and higher doses of EBV-specific autologous CTLs, the results obtained in the present cohort are substantially comparable with our previous experience, with clinical benefit observed in about half the study population. These data indicate that either the two variables did not provide additional benefit or had competing effects. We favor the hypothesis that the lymphodepleting chemotherapy counteracted the possible benefits derived from a higher CTL dose. It is possible that during the 5-week interval before resolution of peripheral lymphopenia, the new environment was not able to fully support expansion and activation of transferred CTLs. In addition, with regard to its impact on T-regulatory lymphocytes, the profound and prolonged lymphopenia may have also depleted factors able to promote T-cell effector function or expansion. Indeed, the transient IL-15 production peak observed after lymphodepletion might not have been adequate to favor cell expansion in the absence of helper T cells producing other homeostatic factors. In support of this hypothesis, studies using highly selected tumor-reactive CD8+ clones, administered following lymphodepletion, did not provide evidence of tumor regression, suggesting that the presence of CD4+ cells is necessary to mediate an antitumor response [23, 29]. In line with these observations, it has been recently reported that autologous antigen-specific CD4+ cells, given in the absence of lymphodepletion, persisted *in vivo* for at least 3 months and induced a long lasting complete remission in a patient with metastatic and refractory melanoma [30]. This report suggests that CD4+ T cells play a central role in antitumor immunity and supports the hypothesis that, at least in the NPC setting, an environment with a subverted cytokine profile, as observed following lymphodepletion, may impair antitumor surveillance by hampering CTL function. Whether a more intensive, myeloablative, approach [31] may provide a more favorable environment for CTL expansion remains to be tested in a cohort of less compromised patients at an earlier stage of disease. Alternatively, the use of a less toxic agent, such as the CD45 lymphodepleting antibody, may be a better choice in NPC patients. Louis et al. [32] reported an increase in peripheral blood frequency of EBV-specific T cells after CTL infusion preceded by CD45 antibody treatment, compared with their previous experience without lymphodepletion, although the clinical effects did not seem to be strikingly improved. The lymphodepleting effect of the mAb treatment was of shorter duration than our chemotherapy-based regimen, and the more prolonged lymphopenia we observed may account for failure to detect early T-cell expansion after CTL therapy.

The kinetics of EBV DNA load in our patients indicates that also in the setting of cell therapy, this parameter correlates with tumor burden [22] and may be employed to monitor response in the course of treatment and follow-up.

Our study confirms that EBV-specific CTL therapy is associated with antitumor activity in patients with advanced NPC. The use of lymphodepleting chemotherapy before T-cell administration did not improve clinical results but possibly dampened preexisting EBV-specific T cells. Importantly, compared with our previous experience, we have shown that CTLs can be given at higher doses without additional toxicity. Thus, the priority for future studies will be to ameliorate the quality of the cell product rather than act on the tumor environment.

In this perspective, efforts are being made toward augmenting the pool of T cells specific for the subdominant antigens expressed on EBV latency II tumor cells within the infused product, with the aim of increasing T-cell therapy efficacy. In detail, the subdominant component of EBV-specific immune response directed toward LMPs LMP1 and LMP2 has been shown to expand, by stimulation with dendritic cells or EBV-LCL genetically modified to express the antigens [33–35]. In a pilot study enrolling EBV-positive Hodgkin or non-Hodgkin lymphoma, five of six patients with active relapsed disease showed a tumor response after infusion of autologous LMP2-specific CTLs [36].

Based on these preliminary data, and on the results of the immunological monitoring of the treated patients in our cohorts, which confirm a correlation between the emergence of LMP2-specific T cells and a clinical response, we are now working on methods to obtain enrichment of CTLs specific for the subdominant antigen EBV-LMP2 and other antigens potentially present on NPC tumor cells, such as LMP1 and EBNA1. These cell products will likely exert an optimal antitumor effect if employed in earlier phases of the disease. In particular, we intend to conduct future studies in patients relapsing, or not achieving remission, after conventional first-line treatment. In this setting, we would use CTLs as a consolidation treatment after achieving response to second-line therapy. Likewise, we could consider T-cell therapy for consolidating maximal response in patients treated for metastatic disease.

funding

Associazione Italiana per la Ricerca sul Cancro to P.C., F.L., P.P. and S.S.; Ministero della Salute: Progetti Ricerca Oncologica (RFPS-2006-4-341763 to F.L., RFPS-2006-2-340145 to F.L., RFPS-2006-Regione Umbria to P.C. and F.L.); Progetti Ricerca Finalizzata; Regione Lombardia to M.Z.; Fondazione IRCCS Policlinico San Matteo, Progetti di Ricerca Corrente to M.Z., R.M. and P.P.; Oncologia Ca' Granda Onlus Fondazione to P.P. and S.S.

disclosure

The authors declare no conflict of interest.

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