Original article

Annals of Oncology 15: 113–117, 2004
DOI: 10.1093/annonc/mdh027

Adoptive transfer of allogeneic Epstein–Barr virus (EBV)-specific cytotoxic T cells with *in vitro* antitumor activity boosts LMP2-specific immune response in a patient with EBV-related nasopharyngeal carcinoma

P. Comoli[1]*, R. De Palma[2], S. Siena[3], A. Nocera[4], S. Basso[1], F. Del Galdo[2], R. Schiavo[3], O. Carminati[3], A. Tagliamacco[4], G. F. Abbate[2], F. Locatelli[1], R. Maccario[1] & P. Pedrazzoli[3]

[1]*Laboratory of Transplant Immunology and Pediatric Hematology/Oncology, IRCCS Policlinico S. Matteo, Pavia;* [2]*Department of Clinical and Experimental Medicine, Second University of Napoli;* [3]*Divisione di Oncologia Medica Falck, Department of Oncology, Ospedale Niguarda Ca' Granda, Milano;* [4]*Transplant Immunology Unit, S. Martino Hospital, Genova, Italy*

Received 23 July 2003; revised 4 September 2003; accepted 17 September 2003

Background: The outcome of patients with nasopharyngeal carcinoma (NPC) presenting as advanced-stage disease or failing conventional radio-chemotherapy is poor. Thus, additional forms of effective, low-toxicity treatment are warranted to improve NPC prognosis. Since NPC is almost universally associated with Epstein–Barr virus (EBV), cellular immunotherapy with EBV-specific cytotoxic T lymphocytes (CTLs) may prove a successful treatment strategy.

Patient and methods: A patient with relapsed NPC, refractory to conventional treatments, received salvage adoptive immunotherapy with EBV-specific CTLs reactivated *ex vivo* from a human leukocyte antigen-identical sibling. EBV-specific immunity, as well as T-cell repertoire in the tumor, before and after immunotherapy, was evaluated.

Results: CTL transfer was well tolerated, and a temporary stabilization of disease was obtained. Moreover, notwithstanding the short *in-vivo* duration of allogeneic CTLs, immunotherapy induced a marked increase of endogenous tumor-infiltrating CD8+ T lymphocytes, and a long-term increase of latent membrane protein 2-specific immunity.

Conclusions: Preliminary data obtained in this patient indicate that EBV-specific CTLs are safe, may exert specific killing of NPC tumor cells *in vitro*, and induce antitumor effect *in vivo*.

Key words: cellular immunotherapy, cytotoxic T lymphocytes, Epstein–Barr virus, latent membrane protein 2, nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most important epithelial neoplasms in world health terms. Currently, the elective form of treatment for NPC is combined radiotherapy and chemotherapy, which cures 80–90% of cases presenting with early stage tumor [1, 2]. However, the outcome of patients with advanced stage disease at diagnosis or relapsing after first-line therapy is poor [1, 2]. Thus, additional forms of effective, low-toxicity treatment are warranted.

NPC is almost universally associated with Epstein–Barr virus (EBV), which is present in tumor cells in a latency II state, characterized by the expression of a restricted number of viral proteins, with latent membrane protein (LMP)1 and LMP2 as the only immunogenic antigens [3]. Recent work has shown that NPC cells are capable of immunological processing for cytotoxic T-lymphocyte (CTL) recognition [4, 5]. Based on these data, and on the encouraging results obtained by cellular immunotherapy with EBV-specific CTL lines in the prevention and treatment of the latency III, EBV-related post-transplant lymphoproliferative disease [6, 7], pilot trials of cellular immunotherapy for other EBV-associated cancers have been conducted [8, 9].

However, so far there is no evidence that EBV-specific CTLs reactivated from NPC patients are capable of lysing autologous tumor cells *in vitro* and mediating tumor killing *in vivo*. An increase in EBV-specific CTL precursor (CTLp) frequency was observed after infusion of autologous virus-specific CTLs in four patients with NPC, but no data on *in vitro* anti-tumor activity were available, and no clinical benefit could be observed [9].

We describe the case of a NPC patient who received EBV-specific CTLs reactivated from a human leukocyte antigen (HLA)-matched family donor, which showed *in vitro* cytotoxicity

**Correspondence to:* P. Comoli, Laboratorio Sperimentale di Trapianto di Midollo Osseo e Oncoematologia Pediatrica, IRCCS Policlinico S. Matteo, P.le Golgi 2, 27100 Pavia, Italia. Tel: +39-0382-502716; Fax: +39-0382-527976; E-mail: pcomoli@smatteo.pv.it

towards the patient's tumor cells, and, once transferred *in vivo*, induced temporary stabilization of disease and enhancement of the patient's EBV–LMP2-specific T-cell responses.

Study design

Patient

A 40-year-old Caucasian male was diagnosed to have stage IIB, EBV-positive NPC in 1989. He received cisplatin/5-fluorouracil chemotherapy and radiotherapy on the primary tumor and cervical nodes, achieving complete remission (CR). In 1997 local recurrence was documented, and the patient achieved a second CR after bleomycin, methotrexate and cisplatin chemotherapy plus brachytherapy. In August 1999, the disease relapsed locally and to the cervical nodes. He received cisplatin/5-fluorouracil therapy and subsequently paclitaxel, with no response. In October 2000 the patient presented with nasal obstruction and impairment of cranial nerves II and IV, due to bulky NPC progression infiltrating the skull base and the cavernous sinuses. Since no response was observed after fourth-line cisplatin–doxorubicin chemotherapy, we elected to give salvage adoptive immunotherapy with EBV-specific CTLs reactivated from a HLA-identical sibling.

Approval for this study was obtained from the Institutional Review Board, and both donor and patient gave written informed consent before enrolment.

EBV-specific CTL expansion and administration

EBV-specific CTLs were reactivated and expanded *in vitro* according to a method previously reported, following good laboratory practice standard procedures [7]. In brief, EBV-specific CTLs were prepared from fresh or frozen peripheral blood mononuclear cells (PBMCs), plated in 2 ml X-VIVO 20 medium (BioWhittaker, Walkersville, MD, USA) with 2% autologous plasma, at 2×10^6 cells per well and stimulated with irradiated autologous B-lymphoblastoid cell line (LCL) at a responder-to-stimulator (R:S) ratio of 40:1. After 10 days, cultures were restimulated with irradiated autologous B-LCL at a R:S ratio of 4:1. Starting on day 14, 20 U/ml recombinant interleukin-2 (rIL-2; Hoffman-La Roche, Basel, Switzerland) were added to the wells, and the cultures were subsequently restimulated weekly with irradiated autologous B-LCL in the presence of rIL-2.

Before cryopreservation, T cells were examined for immunophenotype, sterility and EBV specificity in a standard ^{51}Cr-release assay against a panel of targets including donor B-LCL, recipient EBER+ tumor cells, and recipient phytohemagglutinin (PHA) blasts. Tumor cells were obtained from a baseline tumor biopsy, after seeding tumor sections overnight in a culture flask in a small volume of IMDM (Gibco, Grand Island, NY, USA) supplemented with 20% fetal calf serum (FCS; Sigma, St Louis, MO, USA). Adherent cells were maintained with weekly passages in IMDM–FCS medium, until a substantial outgrowth of cells was observed, and then analyzed for immunophenotype and EBER expression. Early-passage neoplastic cells were used to minimize the possibility of loosing primary characteristics as a consequence of extensive *in vitro* re-culturing.

CTL infusion schedule included a first dose of 2×10^7 CTL/m^2, and 3-weekly subsequent doses of 4×10^7 CTL/m^2.

Analysis of CTL presence and persistence at the tumor site

Presence and persistence of infused CTLs of donor origin was monitored through T-cell receptor repertoire analysis by CDR3 spectratyping. In detail, CDR3 size length analysis was employed to characterize T-cell clonotypes on donor EBV-specific CTLs, lymphocytes derived from tumor site tissue before and after cell therapy, and on the peripheral blood of patients after CTL infusion, according to a previously described method [10].

Evaluation of specific immunity

EBV- and LMP2-specific immunity before and after immunotherapy was evaluated by limiting dilution analysis of CTLp [7], by measuring the frequency of IFNγ-secreting cells in a ELISPOT assay [11], and by reactivation of specific CTLs.

In detail, to evaluate CTLp frequency to EBV, responder cells were stimulated with autologous irradiated B-LCL in a limiting dilution assay. Briefly, 24 replicates of decreasing numbers (8×10^4, 6×10^4, 4×10^4, 2×10^4, 10^4, 5×10^3, 2.5×10^3) of cryopreserved PBMCs, used as responder cells, were seeded in 96-well round-bottom microplates in a final volume of 200 µl RPMI (Gibco)–FCS medium in the presence of 10^4 autologous irradiated (7000 rads) B-LCL. On days 7 and 14, the cultures were restimulated with 10^4 autologous irradiated B-LCL. On day 21, the plates were split and tested against autologous or HLA-mismatched B-LCL in a standard cytotoxicity assay. For ELISPOT assay, 96-well multiscreen filter plates (MAIPS 4510; Millipore, Bedford, MA, USA) were coated with 100 µl of primary antibody (IFNγ; Mabtech, Nacka, Sweden) at 2.5 µg/ml, and incubated overnight at 4°C. PBMCs were thawed and cultured overnight in RPMI–FCS medium before use in the assay, and were then seeded in the absence or in the presence of peripheral blood monocyte-derived dendritic cells (DC) pulsed with vaccinia-LMP2. Controls included wells plated with vaccinia-βgal-pulsed DC. After incubation for 24 h at 37°C, 100 µl of biotinylated secondary antibody (Mabtech; 0.5 µg/ml) was added, and plates were then processed according to standard procedure. IFNγ-producing spots were counted using a Elispot reader (Bioline, Torino, Italy). The number of spots per well was calculated after subtraction of assay background, quantitated as the average of 24 wells containing only sterile complete medium, and specific background, quantitated as the sum of cytokine spots associated with responders alone, and responders plated with vaccinia-βgal-pulsed DC.

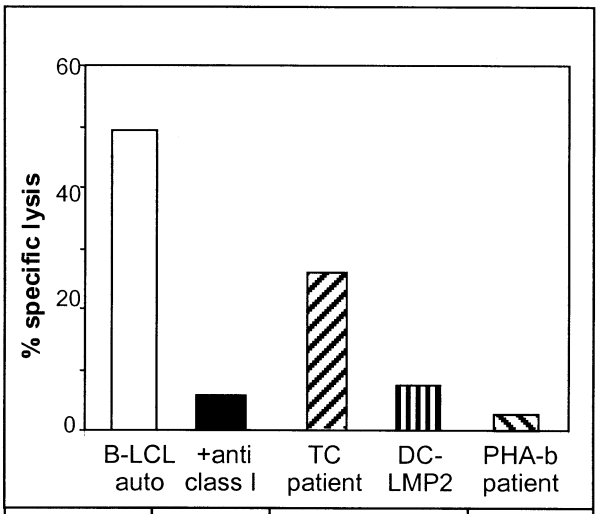
Tumor-infiltrating lymphocytes were obtained by culturing phosphate-buffered saline-washed tumor sections in RPMI–FCS medium containing IL-2 (100 IU/ml). Medium was replaced on day +6, and on day +10 cells were harvested, phenotyped and used fresh or cryopreserved.

Results and discussion

At the time of enrolment, expansion of the patient's EBV-specific CTLs was attempted, but due to poor CTL growth rate, total cell yield was insufficient for T-cell therapy. Thus, an EBV-specific CTL line was successfully expanded from the patient's HLA-matched sibling, which, in addition to strong lysis of autologous B-LCL, showed cytotoxicity against the patient's NPC cells in the absence of lysis toward the patient's PHA blasts. Phenotype and functional characteristics of donor CTLs are reported in Figure 1.

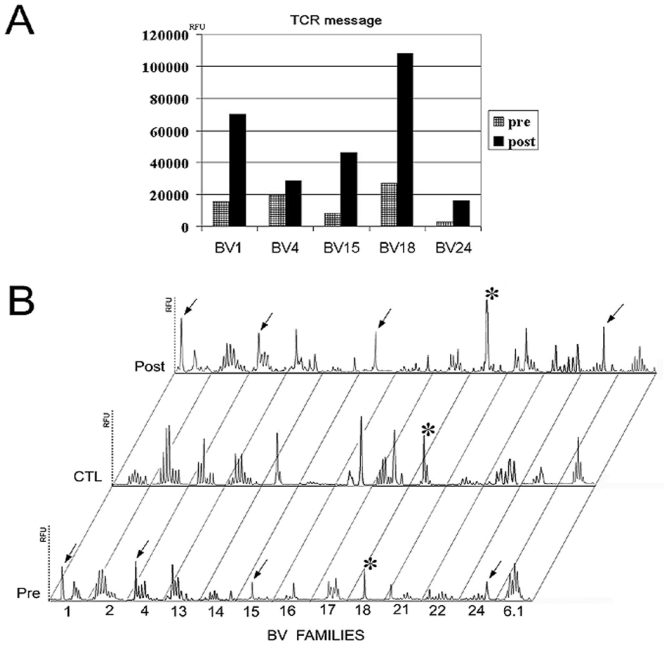
Allogeneic EBV-specific CTL infusions were well tolerated. A temporary appearance of symptoms due to cranial nerves II and IV impairment, likely due to an initial homing of infused CTLs in the tumor, was observed after the first infusion and resolved spontaneously. Notwithstanding the progressing, large tumor bulk at the time of immunotherapy, a CT scan performed 1 week after the fourth CTL infusion revealed a minimal reduction of the intracranial portion of the tumor, that after a 3-month stabilization, progressed.

Tumor biopsies were performed before and 2 weeks after completion of CTL infusion schedule. Immunohistochemical staining showed a marked increase of CD8+ lymphocytes in the tumor after CTL therapy (data not shown). We proceeded to analyze the T-cell repertoire, and found that a marked increase of the message



CD8	70%	HLA-DR	98%
CD4	26%	CD8/ CD56	8%
CD56	4%		

Figure 1. Characteristics of the transferred allogeneic Epstein–Barr virus (EBV)-specific cytotoxic T-lymphocytes (CTL) line. Cytotoxicity and phenotype features of donor EBV-specific CTLs are shown. EBV- and tumor-specificity of the cultured T cells is demonstrated by sizeable lysis of autologous EBV B-lymphoblastoid cell line (B-LCL; white bar) and patient's tumor cells (third bar), in the absence of cytotoxicity against patient's phytohemagglutinin (PHA) blasts (fifth bar). Human leukocyte antigen (HLA)-restriction is proved by prevention of specific lysis in the presence of anti-HLA class I monoclonal antibody (clone W6/32; Dako, Glostrup, Denmark) (black bar). Cytotoxicity against vaccinia-latent membrane protein (LMP)2-pulsed dendritic cells (DC) is also reported (fourth bar). Spontaneous chromium release was always <20% for all target cells. Data represent cytotoxicity at a 5:1 effector-to-target (E:T) ratio, and are the mean of triplicate wells.



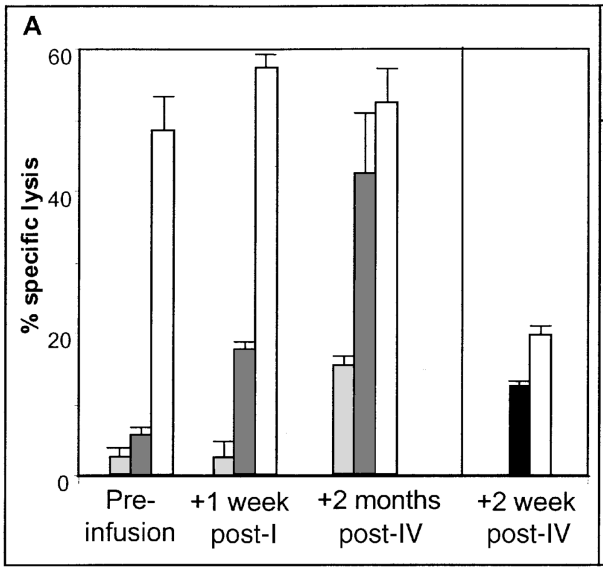
C

CDR-3			
	BV 18		BJ 2.3
Tumor pre IT	A A Y F C A S S P	A G G D T D T	Q Y F G P G T
	gca gct tat ttc tgt gcc agc tca ccc	gcc ggg gga gac aca gat acg	cag tat ttt ggc cca ggc acc
CTL	A A Y F C A S S P	Q G Y Y H P	Q Y F G P G T
	gca gct tat ttt tgc gcc agc tcc cca	cag gga tac tat cat ccc	cag tat ttt ggt cca ggc acc
Tumor post IT	A A Y F C A S S P	A G G D T D T	Q Y F G P G T
	gca gct tat ttc tgt gcc agc tca ccc	gcc ggg gga gac aca gat acg	cag tat ttt ggc cca ggc acc

Figure 2. T-cell repertoire (TCR) analysis on tumor tissue before and after T-cell therapy. (**A**) The analysis for five representative BV families (BV1, BV4, BV15, BV18, BV 24), before (empty bar) and after (black bar) cytotoxic T-lymphocyte (CTL) infusion. (**B**) The TCR analysis for 13 BV families. Families BV19 and BV10 were not shown because these are pseudogenes, the TCR-BV families 3, 5.1, 5.3, 7, 8, 12, 20, 25 showed a normal, Gaussian-like, distribution. The arrow indicates the BV families found to have a prominent peak before and after CTL infusion. The sequence of these peaks showed that they had the same sequence at both nucleotidic and amino acid level (data not shown). The asterisks indicate the TCR-BV18 family whose sequence is shown in (**C**). BV and BJ identify the gene families.

for T-cell repertoire was present after the CTL infusion (Figure 2). Moreover, the repertoire was found to be identical, as for CDR3 length, before and after CTL infusion, but different to the infused CTLs (Figure 2B). To better characterize these T cells, we sequenced some of the BV families found to be more expanded. The results concerning BV18 (Figure 2C) show the same prominent peak before and after infusion, and a 3bp shorter peak in the CTLs. The sequencing data revealed that the same clone was found before and after infusion, and no sequence motif was found in the CDR 3 region of the same family in the CTLs, suggesting that donor CTLs were not present at the time of the analysis.

We then assessed whether infusion of allogeneic EBV-specific CTLs had any effect on the patient's specific immunity. In particular, we analyzed the frequency of EBV LCL-specific and LMP2-specific CTLp and IFNγ-secreting cells, together with specific cytotoxic activity of CTL lines reactivated from the patient before and after CTL infusion (Figure 3A and B). The frequency of CTLp and IFNγ-secreting cells directed towards EBV LCL, which was in the range reported for EBV-positive healthy individuals [11] before infusion, was found to be only slightly increased after CTL treatment, likely reflecting an initial increase followed by rapid reduction upon development of reactivity towards the allogeneic T cells (Figure 3B). However, the frequency of LMP2-specific CTLp and IFNγ-secreting cells, which was very low or undetectable before CTL administration, increased after cell therapy, reaching values observed in healthy EBV-seropositive controls [11]. Thus, whereas no substantial long-term difference could be observed in specific immunity toward EBV LCL, a measurable increase of LMP2-directed responses was detected after CTL infusions, which translated into a stronger LMP2-specific component in EBV-specific CTLs reactivated from the patient after allogeneic CTL infusion (Figure 3A). Since CDR3 length analysis performed



B	Pre-infusion	Post-IV infusion
CTLp		
EBV LCL	100	111
LMP2	3	6
β-gal	<1	<1
IFNγ-producing cells		
EBV LCL	950 (10.5)	960 (2.5)
LMP2	0 (0)	25 (3.3)

Figure 3. Immunological effects of allogeneic Epstein–Barr virus (EBV)-specific cytotoxic T-lymphocyte (CTL) transfer on patient's specific immunity. (**A**) Bulk-culture cytotoxicity of patient's peripheral blood mononuclear cells (PBMCs), collected at different times from CTL infusion, stimulated with autologous lymphoblastoid cell line (LCL), and tested against autologous LCL (white bars) or vaccinia-latent membrane protein (LMP)2-pulsed dendritic cells (DC) (dark grey bars). Cytotoxicity against vaccinia-βgal-pulsed DC is also reported (light grey bars). (Vaccinia constructs were kindly provided by Dr C. Rooney, Houston, TX, USA.) To the extreme right of the graph, data regarding cytotoxicity of tumor-infiltrating lymphocytes against EBV LC L (white bars) and patient tumor cells (black bars) are also reported. Data represent cytotoxicity at a 5:1 effector-to-target (E:T) ratio + standard error (SE). (**B**) Data on the frequency of CTL precursors (CTLp) and IFNγ-secreting lymphocytes, measured in patient's PBMCs obtained before and after CTL therapy, in response to EBV LCL, vaccinia-LMP2-pulsed DC and vaccinia-βgal-pulsed DC are reported. CTLp are represented as number/10^6 PBMCs. IFNγ-secreting cells are represented as number of spots/10^6 PBMCs (mean spots of triplicate experiments); the values reported for LMP2 stimulation (SE in parentheses) were obtained after subtraction of mean spots of responders plated with vaccinia-βgal-pulsed DC.

on the patient's PBMCs obtained 2 months after the fourth CTL infusion (data not shown) did not reveal the presence of donor-derived expanded clones in the peripheral blood, and, more importantly, no donor cells were found at the tumor site 2 weeks after the fourth infusion, we assume that donor T cells were absent from the patient's peripheral blood at the time of immunological analysis. Thus, the increase in LMP2-specific responses is likely ascribable to a patient's T cells. The boosting effect of donor T-cell infusions on patient's immune response may have been due to both a bystander activation and a direct cytotoxic effect of the CTLs, which induced a release of tumor antigens. Since EBNA3 antigens, the dominant target of EBV LCL-induced CTL responses, are not present in NPC tumor cells, no boosting of the patient's EBV LCL-directed response could be observed.

Since donor CTL infusion was almost immediately followed by the appearance of symptoms suggestive of CTL trafficking to tumor site, it is reasonable to hypothesize that infused CTLs were responsible for the tumor's reduction, but were no longer present when the second biopsy was taken. Indeed, as recently demonstrated by Yee et al., autologous CD8+ T-cell clones infused in patients with melanoma persisted for a median of 7 days after transfer, in the absence of systemically delivered IL-2 [12]. In our case, although CTLs included CD4+ T cells, known to provide 'help' for antigen-specific CD8+ T-cell persistence, the half-life was likely reduced due to the allogeneic setting. Notwithstanding the increased endogenous anti-LMP2 immunity, and the augmented number of CD8+ cells in the tumor, the clinical response in our patient was transient. This might be due to reduced donor CTL persistence, associated to a low activity towards autologous tumor cells and EBV LCL exerted by tumor-infiltrating lymphocytes of patient origin (Figure 3A), in comparison with donor CTLs. Moreover, treatment-induced selection for antigen-loss variants may also have played a role.

Preliminary data obtained in this patient indicate that EBV-specific CTLs are able to exert specific killing of patients' NPC cells *in vitro*, can be used safely, and may show antitumor effect *in vivo*. Given the short *in vivo* duration of allogeneic CTLs, immunotherapy of NPC may be implemented by the use of autologous EBV-specific CTLs [13, 14]. Recently, it has been reported that immunization with LMP2 peptide-pulsed autologous DCs boosted epitope-specific T-cell responses in NPC patients, inducing partial clinical responses in selected patients [15]. Thus, to improve antitumor response, LMP2 and/or LMP1-specific CTLs [16, 17] could be employed, supported by low-dose IL-2 administration [12].

Acknowledgements

Supported in part by grants from the Associazione Italiana Ricerca sul Cancro (AIRC) to P.C., F.L. and R.M., grants RFM/02 from IRCCS Policlinico S. Matteo to P.C., F.L. and R.M., grant RFM/02 from IRCCS Ospedale S. Raffaele to F.L., and unrestricted Grant from ONLUS Oncologia Ca' Granda, Milan, Italy. S.B. is a recipient of a grant from 'Associazione Morgan di

Gianvittorio'. The authors wish to thank Massimo Labirio for outstanding technical support. P.C. and R.D.P. equally contributed to this work.

References

1. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med 2001; 345: 1890–1900.
2. Chan ATC, Teo PML, Johnson PJ. Nasopharyngeal carcinoma. Ann Oncol 2002; 13: 1007–1015.
3. Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein–Barr virus infection. Annu Rev Immunol 1997; 15: 405–431.
4. Khanna R, Busson P, Burrows S et al. Molecular characterisation of antigen-processing function in nasopharyngeal carcinoma (NPC): evidence for efficient presentation of Epstein–Barr virus cytotoxic T cell epitopes by NPC cells. Cancer Res 1998; 58: 310–314.
5. Lee SP, Chan ATC, Cheung S et al. CTL control of EBV in nasopharyngeal carcinoma (NPC): EBV-specific CTL responses in the blood and tumors of NPC patients and the antigen-processing function of the tumor cells. J Immunol 2000; 165: 573–582.
6. Rooney CM, Smith CA, Ng CYC et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein–Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 1998; 92: 1549–1555.
7. Comoli P, Labirio M, Basso S et al. Infusion of autologous Epstein–Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. Blood 2002; 99: 2592–2598.
8. Roskrow MA, Suzuki N, Gan Y et al. Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's Disease. Blood 1998; 91: 2925–2934.
9. Chua D, Huang J, Zheng B et al. Adoptive transfer of autologous Epstein–Barr virus-specific cytotoxic T cells for nasopharygeal carcinoma. Int J Cancer 2001; 94: 73–80.
10. De Palma R, Gorski J. Restricted and conserved T cell repertoires involved in allorecognition of class II major histocompatibility complex. Proc Natl Acad Sci USA 1995; 92: 8836–8840.
11. Yang J, Lemas VM, Flinn IW et al. Application of the ELISPOT assay to the characterization of CD8+ responses to Epstein–Barr virus antigens. Blood 2000; 95: 241–248.
12. Yee C, Thompson JA, Byrd D et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci USA 2002; 99: 16168–16173.
13. Huls H, Popat U, Gee AP et al. Cytotoxic T lymphocyte therapy for Epstein–Barr virus-positive nasopharyngeal carcinoma. Blood 2002; 100: 121a (Abstr).
14. Comoli P, Basso S, Schiavo R et al. Autologous Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes (CTL) for the treatment of patients with EBV-positive relapsed nasopharingeal carcinoma. Bone Marrow Transplant 2003; 31: S207 (Abstr).
15. Lin C, Lo W, Lee T et al. Immunization with Epstein–Barr virus (EBV) peptide-pulsed dendritic cells induces functional CD8+ T-cell immunity and may lead to tumor regression in patients with EBV-positive nasopharyngeal carcinoma. Cancer Res 2002; 62: 6952–6958.
16. Gahn B, Siller-Lopez F, Pirooz AD et al. Adenoviral gene transfer into dendritic cells efficiently amplifies the immune response to LMP2A antigen: a potential tratment strategy for Epstein–Barr Virus-positive Hodgkin's lymphoma. Int J Cancer 2001; 93: 706–713.
17. Gottschalk S, Edwards OL, Sili U et al. Generating CTLs against the subdominant Epstein–Barr virus LMP1 antigen for the adoptive immunotherapy of EBV-associated malignancies. Blood 2003; 101: 1905–1912.