[haematologica reports]
2005;1(5):24-27

Treatment of Epstein-Barr virus-associated lymphomas: the role of Epstein-Barr virus-specific cytotoxic T cells

HELEN E HESLOP
CATHERINE BOLLARD
STEPHEN GOTTSCHALK
BARBARA SAVOLDO
MALCOLM K BRENNER
CLIONA M ROONEY

Center for Gene and Cell Therapy, Department of Pediatrics, Medicine, and Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, USA

Latent Epstein-Barr virus (EBV) infection is associated with a heterogeneous group of lymphoma, including Burkitt's lymphoma, Hodgkin's disease, NK-T lymphomas and lymphoproliferative disease (LPD).[1-3] All EBV-associated malignancies are associated with the virus' latent cycle, and three distinct types of EBV latency have been characterized.[4,5] All are EBER positive, but the EBV latent protein expression varies. Latency type III, is expressed in lymphoblastoid cell lines (LCL), which can be readily produced by infecting B cells *in vitro* with EBV and is characterized by expression of the entire array of nine EBV latency proteins: EBNAs 1, 2, 3A, 3B, 3C, LP, BARF0 and the two viral membrane proteins LMP1 and LMP2. This pattern of EBV gene expression characterizes the EBV-associated lymphoproliferative diseases (EBV-LPD) that occur in individuals severely immunocompromised by solid organ or stem cell transplantation, congenital immunodeficiency or human immunodeficiency virus (HIV) infection. Latency type II is the hallmark of EBV-positive Hodgkin's disease and peripheral T/NK-cell lymphomas where a more restricted array of proteins including EBNA-1, BARF0, LMP1 and LMP2 are expressed. In latency type I, found in EBV-positive Burkitt's lymphoma only EBNA-1 and BARF0 are expressed. As EBNA-1 is not processed by the Class I processing machinery,[6] lymphoma's expressing Type 1 latency are not a good target for immunotherapy approaches. However, immunotherapy approaches targeting EBV antigens does have potential for treating Type II and Type III latency EBV lymphomas.

EBV CTLs as therapy for Type III latency lymphomas

Post-transplant lymphoproliferative disorder post-hemopoietic stem cell transplantation

Post-transplant lymphoproliferative disorder (PTLD) is a serious, life-threatening disease and encompasses a heterogeneous group of lymphoproliferative disorders ranging from reactive, polyclonal hyperplasias to aggressive non-Hodgkin's lymphomas. EBV lymphoma arising after allogeneic hemopoietic stem cell transplantation (HSCT) is an excellent model to evaluate EBV specific CTLs, as the tumor cells express all 9 latent cycle EBV antigens (including the immunodominant EBNA3 antigens), most donors are seropositive, and the lymphoblastoid cell lines generated by infecting normal peripheral blood B cells with EBV function as excellent antigen presenting cells.

Donor LCL are generated by infection of donor lymphocytes with a laboratory strain of EBV and irradiated LCLs are then used to stimulate PBMC and expand EBV-specific CTL. After an initial primary and secondary stimulation with irradiated LCL, CTL are expanded by twice weekly addition of IL2 and once weekly stimulation with irradiated LCL.[7] The resulting CTL line is then characterized and if it meets release criteria for specificity and sterility can be adoptively transferred to the recipient. The resultant EBV-specific CTL are polyclonal and contain both CD4- and CD8- positive EBV-specific T cells. One limitation of this approach is that because of the prolonged *ex vivo* culture these CTL lines need to be grown in specialized facilities following Good Tissue Practices.[8]

Our group has used donor-derived EBV-specific T cell lines as prophylaxis for EBV-induced lymphoma in 58 patients who received a T cell depleted HSCT or who were transplanted for an EBV-associated malignancy. The first 26 patients received CTL, which were genetically modified with a retroviral vector encoding the neomycin resistance gene to enable tracking of infused cells and this gene marking component allowed us to show persistence of infused CTL for as long as seven years.[9] In patients with high EBV-DNA levels in peripheral blood prior to CTL infusion, which in this population is highly predictive for development of EBV-LPD,10 EBV-DNA

levels fell to undetectable levels coincident with an increase in EBV-specific CTL precursor frequency.[11,12] None of the patients treated prophylactically developed EBV lymphoma compared with an incidence of 11% in patients receiving the same transplant regimen who did not receive prophylactic CTLs.[12]

Six patients have also been treated for established EBV lymphoma with complete responses seen in 5 patients accompanied by accumulation of genemarked CTL at sites of disease in two patients who had follow up biopsies. In one patient with extremely bulky disease significant inflammation was seen at sites of disease after CTL administration illustrating the benefits of treating patients with early rather than advanced disease.[12] The patient who failed treatment was found to have an mutation resulting in deletion of the two immunodominant HLA 11 restricted epitopes in EBNA 3B, recognized by the donor CTL line.[13] HLA11 is a dominant restricting allele so that lines with this HLA type have restricted specificity for EBNA 3B. In other EBV CTL lines generated in situations where the HLA type results in immunodominance of particular EBV derived peptides, there may be target antigen restriction after only one week of culture[14] and a restricted pattern of TCR usage on spectratyping has been observed.[15] Although mutations in immunodominant EBV antigens are not common, the risk of tumor escape mutants remains a concern even when polyclonal lines rather than clones are infused. However, overall these studies showed that adoptively transferred EBV-CTL persist long term and can prevent as well as effectively treat EBV PTLD.

The activity of donor derived EBV specific CTL in allogeneic transplant recipients has been confirmed by a group from Sweden who treated six T cell depleted allogeneic BMT recipients prophylactically with EBV-specific CTLs. One patient, who received a T cell line lacking a major EBV-specific component, progressed to fatal EBV-positive lymphoma but in the other five patients infusion of EBV specific CTLs treatment resulted in reduction of the viral load thereby confirming the utility of approaches to reconstitute T cell immunity.[16]

Post-transplant lymphoproliferative disorder post Solid Organ Transplant

The success of this approach in treating hemopoietic stem cell transplant recipients led to evaluation of this strategy in solid organ transplant recipients who are also at risk of developing EBV-associated PTLD. However, generation of EBV-specific CTL in this patient population presents some differences from HSCT recipients when the normal transplant donor is available. Solid organ recipients and donor are not HLA-matched and PTLD occurring after solid organ transplant is often of recipient origin, so that the use of donor-derived CTL is not appropriate. The options are therefore to use closely matched allogeneic CTLs or autologous CTLs.

a) Autologous EBV specific CTLs

Several groups have evaluated autologous EBV CTLs in solid organ transplant recipients. An initial concern was whether it was possible to generate autologous CTL in patients receiving immunosuppression but this proved to be feasible.[18-20] The first prophylaxis study was reported by Haque *et al* who administered three injections of autologous CTLs at monthly intervals to three recipients of solid organ transplant.[21] The numbers of CTL precursor cells increased following the infusions reaching their highest level after the third infusion then gradually declined. EBV genome copy number became undetectable and remained lower than the pretransplant level in all patients for up to 3 months. Comoli *et al*. [20] reported 7 patients where an increase of the EBV-specific cytotoxicity was observed after infusion coincident with a decrease in EBV DNA levels in 5 patients. Our group has seen similar results with an increase in EBV-precursor frequency after each infusion that persisted for around 4 weeks.[22] We also saw indirect evidence of CTL accumulation in two patients who had biopsies post CTL confirming the *in vivo* function of adoptively transferred T cells.[22] However, the modest and temporary increase observed in the EBV-precursor frequency suggests that the massive *in vivo* expansions seen in T cell-depleted SCT recipients do not occur in SOT patients perhaps because of ongoing immunosuppression.

There have also been reports EBV CTL use in patients with established disease. Khanna *et al*.[18] used autologous EBV-specific CTL to treat a renal transplant recipient with EBV-LPD and observed significant regression following two infusions of CTLs. However after this initial response new lymphoma lesions developed 10 weeks after the second CTL infusion.[18] This contrasts with the experience post HSCT transplant where the infused CTL persisted long term and there were no recurrences in patients successfully treated for PTLD and raises the possibility that transferred CTL may not function long term in solid organ recipients who receive continuous immunosuppressive therapy. An additional issue is that after retreatment with CTLs the patient died, with evidence of necrosis and hemorrhage found in a pulmonary vein at autopsy. This reinforces experience in stem cell transplant recipients and emphasizes that patients with bulky disease may have inflammatory reactions after receiving EBV specific CTLs and that debulking treatment with Rituximab should be considered prior to CTL infusion. In a second report from this group a cardiac transplant patient who developed multiple subcutaneous nod-

ules required six doses of CTLs to achieve remission and response was coincident with reconstitution of T cell reactivity to latent epitopes.[23]

These studies using autologous CTLs have allayed concerns that EBV-specific CTL might have alloreactivity and cause graft rejection and have shown that EBV-specific immunity can at least be temporally restored in these patients. However, the persistence of CTL is much less than after HSCT transplant and the optimum dosing schedule in SOT patients who remain on long term immunosuppression requires further investigation.

b) Matched allogeneic EBV specific CTLs

One solution to the issues discussed above is to develop a bank of allogeneic EBV specific CTLs so an *off the shelf* product is immediately available. A recent report describes eight patients with PTLD who received partly matched allogeneic EBV specific CTL from a frozen bank.[24] Three of the five patients who completed treatment had a complete response although the patients had also had reduction of immunosuppression and the authors did not show persistence of the adoptively transferred allogeneic CTLs. Another study reported two SOT patients who both responded to allogeneic CTLs with regression of PTLD.[25] While these results are encouraging the patients who responded also has their immunosuppressive treatment reduced so it is difficult to definitively ascribe benefit to the allogeneic CTLs especially as neither group showed persistence of the allogeneic cells.

EBV CTLs as therapy for Type II latency lymphomas

In EBV-associated lymphomas which express Class II latency, the tumor cells may be less susceptible to immunotherapeutic approaches because they express a more restricted array of subdominant EBV-encoded antigens.[26] For example, Reed Sternberg cells, in patients with EBV genome positive Hodgkin's disease, express only LMP-1, LMP-2 and EBNA-1. In polyclonal CTL lines, the majority of clones recognize the more immunodominant EBNA-3 family of antigens and only a few clones, if any, will recognize subdominant antigens.[27] However, we hypothesized that any clones recognizing tumor associated antigens would expand *in vivo*[28] and undertook a study to evaluate the activity of autologous LCL-activated, EBV-specific cytotoxic T lymphocytes (EBV-CTL) to treat patients with multiply relapsed Hodgkin Disease (HD).[29,30] We treated 14 patients and showed that it is possible to generate from these patients, polyclonal EBV specific CTL lines with an effector memory phenotype and containing clones specific for the subdominant tumor antigen LMP2 expressed by the malignant Reed Sternberg cells. The gene-marking component of the study showed that infused effector cells could further expand by several logs *in vivo*, contribute to the memory pool (persisting up to twelve months), and traffic to tumor sites.[30] Tetramer and functional analyses showed that T cells reactive with the tumor-associated antigen LMP2 were present in the infused lines, expanded in peripheral blood following infusion, and also entered tumor.[30] Viral load decreased, demonstrating the biologic activity of the infused CTLs. Clinically, EBV-CTLs were well tolerated, could control type B symptoms (fever, night sweats, weight-loss), and had anti-tumor activity. Following CTL infusion, five patients are in complete remission at up to 40 months, two of whom had clearly measurable tumor at the time of treatment. One additional patient had a partial response, and 5 had stable disease. The performance and fate of these human tumor antigen-specific T cells *in vivo* suggests they may be of value for the treatment of EBV-positive Hodgkin lymphoma.30 Another group has used HLA matched allogeneic CTLs in patients with relapsed EBV+ve Hodgkin's disease and also seen clinical responses.[31]

Future directions

EBV specific CTLs have shown efficacy for the prophylaxis and therapy of Type III latency PTLD after both HSCT and solid organ transplantation and provide "proof of principle" for immunotherapy approaches. Responses have also been seen in the Type II latency tumor EBV+ve Hodgkin's disease although the response rate is lower and in several patients response was limited and transient. In follow up studies we are attempting to improve CTL therapy by generating LMP2 CTLs that will target the subdominant LMP2 antigen expressed on these tumors and by pretreating patients with a lymphodepleting antibody to improve CTL expansion.[32] In preclinical studies we are exploring strategies for overcoming tumor evasion mechanisms.[33,34]

This work was supported by NIH grants PO1 CA94237, CA61384, the GCRC at Baylor College of Medicine (RR00188), a Specialized Center of Research Award from the Leukemia Lymphoma Society, and a Doris Duke Distinguished Clinical Scientist Award to HEH.

Refences

1. Cohen JI. Benign and malignant Epstein-Barr virus-associated B-cell lymphoproliferative diseases. Semin Hematol. 2003;40:116-123.
2. Ambinder RF, Lemas MV, Moore S et al. Epstein-Barr virus and lymphoma. Cancer Treat Res. 1999;99:27-45.
3. Gottschalk S, Rooney CM, Heslop HE. Post-Transplant Lymphoproliferative Disorders. Annu Rev Med. 2005;56:29-44.
4. Young LS, Dawson CW, Eliopoulos AG. The expression and function of Epstein-Barr virus encoded latent genes. Mol Pathol. 2000;53:238-247.

5. Hsu JL, Glaser SL. Epstein-barr virus-associated malignancies: epidemiologic patterns and etiologic implications. Crit Rev Oncol Hematol. 2000;34:27-53.
6. Levitskaya J, Coram M, Levitsky V et al. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. Nature. 1995;375:685-688.
7. Smith CA, Ng CYC, Heslop HE et al. Production of genetically modified EBV-specific cytotoxic T cells for adoptive transfer to patients at high risk of EBV-associated lymphoproliferative disease. J Hematother. 1995;4:73-79.
8. Gee AP. Regulatory issues in cellular therapies. Expert Opin Biol Ther. 2003;3:537-540.
9. Bollard CM, Kuehnle I, Leen A, Rooney CM, Heslop HE. Adoptive Immunotherapy For Viral Infections Post Transplant. Biology of Blood and Marrow Transplantation. 2004;10:143-155.
10. Rooney CM, Loftin SK, Holladay MS et al. Early identification of Epstein-Barr virus-associated post-transplant lymphoproliferative disease. Br J Haematol. 1995;89:98-103.
11. Heslop HE, Ng CYC, Li C et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nature Medicine 1996;2:551-555.
12. 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: 1549-55.
13. Gottschalk S, Ng CYC, Smith CA et al. An Epstein-Barr virus deletion mutant that causes fatal lymphoproliferative disease unresponsive to virus-specific T cell therapy. Blood. 2001;97:835-843.
14. Koehne G, Gallardo HF, Sadelain M, O'Reilly RJ. Rapid selection of antigen-specific T lymphocytes by retroviral transduction. Blood. 2000;96:109-17.
15. Musk P, Szmania S, Galloway AT et al. *In vitro* generation of Epstein-Barr virus-specific cytotoxic T cells in patients receiving haplo-identical allogeneic stem cell transplantation. J Immunother 2001;24:312-22.
16. Gustafsson A, Levitsky V, Zou JZ et al. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop post-transplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. Blood. 2000;95:807-14.
17. Lucas KG, Burton RL, Zimmerman SE et al. Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. Blood. 1998;91:3654-61.
18. Khanna R, Bell S, Sherritt M et al. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. Proc Natl Acad Sci U S A 1999;96:10391-6.
19. Savoldo B, Goss J, Liu Z et al. Generation of autologous Epstein Barr virus (EBV)-specific cytotoxic T cells (CTL) for adoptive immunotherapy in solid organ transplant recipients. Transplantation 2001; 72:1078-86.
20. 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; 99:2592-8.
21. Haque T, Amlot PL, Helling N et al. Reconstitution of EBV-specific T cell immunity in solid organ transplant recipients. J Immunol 1998;160:6204-9.
22. Savoldo B, Huls MH, Lopez T et al. Autologous EBV-Specific Cytotoxic T Lymphocyte (CTL) Infusions as Early Intervention for Liver Transplant Recipients with Lymphoproliferative Disease. [abstract]. Blood. 2002;100:363a.
23. Sherritt MA, Bharadwaj M, Burrows JM et al. Reconstitution of the latent T-lymphocyte response to Epstein-Barr virus is coincident with long-term recovery from posttransplant lymphoma after adoptive immunotherapy. Transplantation 2003;75:1556-60.
24. Haque T, Wilkie GM, Taylor C et al. Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. Lancet 2002; 360:436-42.
25. Sun Q, Burton R, Reddy V, Lucas KG. Safety of allogeneic Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for patients with refractory EBV-related lymphoma. Br J Haematol 2002;118:799-808.
26. Gottschalk S, Heslop HE, Rooney CM. Treatment of Epstein-Barr virus-associated malignancies with specific T cells. Adv Cancer Res. 2002;84:175-201.
27. Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases [In Process Citation]. Annu Rev Microbiol 2000;54:19-48.
28. Qu L, Rowe DT. Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J Virol 1992;66:3715-24.
29. Roskrow MA, Suzuki N, Gan Y-J et al. EBV-specific cytotoxic T lymphocytes for the treatment of patients with EBV positive relapsed Hodgkin's disease. Blood 1998;91:2925-34.
30. Bollard CM, Aguilar L, Straathof KC et al. Cytotoxic T Lymphocyte Therapy for Epstein-Barr Virus+ Hodgkin's Disease. J Exp Med 2004; 200:1623-33.
31. Lucas KG, Salzman D, Garcia A, Sun Q. Adoptive immunotherapy with allogeneic Epstein-Barr virus (EBV)-specific cytotoxic T-lymphocytes for recurrent, EBV-positive Hodgkin disease. Cancer 2004;100:1892-901.
32. Bollard CM, Straathof KC, Huls MH et al. The generation and characterization of LMP2-specific CTLs for use as adoptive transfer from patients with relapsed EBV-positive Hodgkin disease. J Immunother 2004;27:317-27.
33. Bollard CM, Rossig C, Calonge MJ et al. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. Blood 2002;99:3179-87.
34. Wagner HJ, Bollard CM, Vigouroux S et al. A strategy for treatment of Epstein-Barr virus-positive Hodgkin's disease by targeting interleukin 12 to the tumor environment using tumor antigen-specific T cells. Cancer Gene Ther 2004;11:81-91.