The interplay between Epstein-Barr virus and the immune system: a rationale for adoptive cell therapy of EBV-related disorders

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ABSTRACT

The Epstein-Barr virus has evolved a plethora of strategies to evade immune system recognition and to establish latent infection in memory B cells, where the virus resides lifelong without any consequence in the majority of individuals. However, some imbalances in the equilibrium between the inherent virus transforming properties and the host immune system can lead to the development of different tumors, such as lymphoproliferative disorders, Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma. The expression of viral antigens in malignant cells makes them suitable targets for immunotherapeutic approaches, which are mainly based on the *ex vivo* expansion of EBV-specific T cells. Indeed, the infusion of virus-specific cytotoxic T lymphocytes has proved not only to be safe and effective, but also capable of restoring or inducing a protective anti-virus immunity, which is lacking, albeit to a different extent, in every EBV-driven malignancy. The purpose of this review is to summarize the results of adoptive immunotherapy approaches for EBV-related malignancies, with particular emphasis on the immunological and virological aspects linked to the clinical responses obtained. Data collected confirm the clinical relevance of the use of EBV-specific cytotoxic T lymphocytes in the field of adoptive immunotherapy and suggest the increasing importance of this approach also against other tumors, concurrent with the increasing knowledge of the intimate and continuous interplay between the virus and the host immune system.

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Introduction

Both in health and disease, the life cycle of the Epstein-Barr virus (EBV) is characterized by a continuous interplay with the host immune system. To survive successfully and establish latency in memory B cells of nearly the whole adult population worldwide, this virus has evolved different strategies to evade immune system recognition. These are the shutting down of the most immunogenic latent proteins, the expression of lytic proteins interfering with the antigen processing machinery and the MHC molecule expression in infected cells, and the production of viral homologs of human cytokines, as reported in depth and exhaustively reviewed by Ressing *et al.*[1] In fact, EBV is a highly immunogenic virus, as demonstrated by the strong response elicited at the time of primary contact, which successfully constrains the virus in a strictly latent, immunologically silent status. Infectious mononucleosis is a self limiting lymphoproliferative disease, whose symptoms are caused by the massive release of cytokines by activated $CD8^+$ T cells. Despite the self limiting nature of this disease, the interaction between the host and the virus leaves some traces lifelong, ranging from a different repertoire of virus-specific T cells with particular characteristics (e.g. low responsiveness to IL-15)[2] to a higher risk of developing Hodgkin's lymphoma (HL), although a clear causative association has not yet been disclosed.[3] Even when the infection is asymptomatic, the virus guarantees its own persistence through the activation of lytic replication in a small proportion of infected cells. Indeed, EBV can impair $CD4^+$ and $CD8^+$ T-cell recognition by a strong, albeit not complete, HLA I and HLA II downmodulation thanks to the activity of BNLF2a,[4] BILF1 and BGLF5,[1,5] which are expressed at different time points during the lytic cycle. Moreover, the virus actively interferes with the effector T-cell action through the viral IL-10 homolog encoded by the *BCRF-1* gene. After spreading during the lytic cycle, EBV establishes latency in memory B cells by shutting down the expression of the most immunogenic latent proteins. In memory B cells, the virus may be completely silent or may restrict the expression only to LMP-2 and/or EBNA1. This protein is essential for the maintenance of the viral episome in the dividing cells and is poorly recognized by $CD8^+$ T cells, as the presence of

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a Gly/Ala repeat domain in its sequence reduces EBNA1 processing and presentation in the context of HLA class I molecules.[6,7] Using these strategies, the virus can persist lifelong in the host without causing any disease. The release of virions in saliva, which is a hallmark of EBV-positive individuals, is kept at basal levels by the action of high numbers of cytotoxic T lymphocytes (CTL) specific for lytic cycle antigens.[8] Conversely, in infectious mononucleosis, the relatively low number of these CTL allows for a higher viral shedding in the oropharyngeal cavity. At the systemic level, the reactivation of both lytic cycle and latent growth-transforming infection from more restricted forms of latency is under the control of EBV-specific CTL. The immune control exerted by these effector cells is so tight that the expression of the full oncogenic potential of the virus requires some deficits in the host immune system response. This is evident in the development of posttransplant lymphoproliferative disorders (PTLD), where the iatrogenic impairment of the cellular responses by the immune suppression regimen greatly favors the expansion of EBV-immortalized B cells. As proof, PTLD is the most immunogenic EBV-related tumor, characterized by the expression of all latency proteins, including the EBNA3 family proteins, which are immunodominant in eliciting $CD8^+$ T-cell responses. Even in the context of less immunogenic tumors (nasopharyngeal carcinoma-NPC, and HL) which usually arise in the immunocompetent host, some impairments in the antiviral immune response are, in any case, present at a local or systemic level.[9,10] Nonetheless, while some impairment of immune responses is required to allow the onset of virus-related tumors, the expression of antigenic viral proteins by malignant cells constitutes a good target for immunotherapeutic strategies. The feasibility and the effectiveness of EBV-specific CTL infusion was first proven in PTLD patients, since these tumors express the wide array of viral latent proteins and offer multiple targets to effector T cells.[11-13] The clinical transfer of these CTL approaches had been preceded by few pre-clinical studies, which nevertheless unequivocally demonstrated an improvement in survival in treated mice bearing a PTLD-like tumor compared to controls.[14-16] After the successes attained in PTLD management, which registered the largest number of treated patients, this immunotherapeutic approach was also extended to latency II malignancies, such as NPC and HL, even though with lower numbers of patients involved (Figure 1). In these settings, complete regression was achieved only in a few cases, mainly because of the reduced expression of viral antigens (namely LMP1, LMP2 and EBNA1), which limits the number of targets for effector cells, and because of a hostile tumor microenvironment which negatively impacts on EBV-specific T-cell activity. Nevertheless, several strategies to overcome these problems are currently being investigated to define the optimal conditions for adoptive cell therapy also in these cases.[17,20,21]

The aim of this review is to provide a survey of clinical, virological and immunological results of *in vivo* studies carried out so far in the context of EBV-related diseases, with a particular focus on the continuous and intimate interplay between the virus and the host immune system. We discuss the diseases that arise in both the immunocompromised and immunocompetent hosts, pointing out the particular features of these subsets of virus-related tumors. Moreover, we analyze the most recent protocols aimed at exploiting the optimal characteristics of EBV-specific T cells also in the context of different clinical entities.

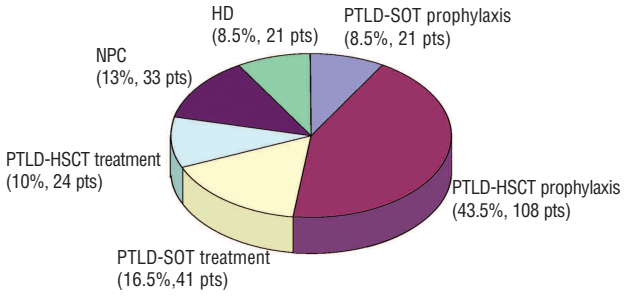

Figure 1. Total number of patients (248) treated with EBV-specific CTL, generated by repeated re-stimulations with autologous LCL (lymphoblastoid cell lines), grouped by the most relevant pathologies (PTLD after SOT, PTLD after HSCT, NPC, HD) and by purpose (treatment or prophylaxis). Results obtained with CTL enriched in LMP2 component[17] are omitted. Data are inferred by Merlo *et al.*[18] and updated on the basis of recent works.[13,19]

EBV-related malignancies in immunocompromised hosts

In contrast to healthy individuals, EBV-induced B-cell proliferation in immunocompromised hosts is no longer kept under control by the cell-mediated immunity elicited at the moment of primary infection, and this can result in a lymphoproliferative disease. Since PTLD arise in only 1.0 ± 0.3% of all transplant recipients,[22-24] it is likely that other conditions have to be present to allow the emergence of the disease (e.g. a particular cytokine milieu, as reviewed by Burns and Crawford[25]). However, the resolution of a high percentage of PTLD in response to the reduction of immunosuppression (RIS) and the success of donor lymphocyte infusion (DLI),[26] strongly suggest that the underlying state of immunosuppression is one of the most important licensing factors for PTLD development. In this context, immunotherapeutic strategies go straight to the heart of the problem, since they restore the lack of immunological control, as RIS and DLI do, but without the risk of allograft rejection or of graft-*versus*-host-disease (GvHD).

Despite clinical similarities, PTLD that arise in hematopoietic stem cell transplant (HSCT) or solid organ transplant (SOT) recipients display some differences that influence the immunotherapeutic approaches, as well as the treatment responses.

First of all, PTLD in patients receiving HSCT usually arise from the donor B cells and, therefore, EBV-specific CTL have to be of donor origin.[27] The CTL infused find a favorable environment for their expansion, i.e. an immune system in active reconstitution, with no competitors for homeostatic cytokines and reduced numbers of immunosuppressive cells. Indeed, a long persistence is a prerequisite for a successful prophylaxis or treatment, and the presence of infused CTL was demonstrated directly *in vivo* or in *ex vivo* cultures up to 105 months by marker gene technique.[13] Conversely, more than 90% of PTLD arising after SOT derives from host B cells, thus requiring the generation of autologous EBV-specific T lymphocytes. Even though peripheral blood mononuclear cells (PBMC) from these patients are obtained from an immunosuppressed

environment, the establishment of virus-specific T-cell lines *in vitro* appears feasible. Moreover, a high-risk category of individuals ultimately developing PTLD is represented by EBV-seronegative patients, who lack EBV-specific precursors in their memory compartment. This is an obstacle to the generation of EBV-specific CTL, a tool which could have a crucial therapeutic role in this high-risk population. In the majority of reported cases, these limitations have been successfully overcome by the use of HLA-matched allogeneic CTL, mainly derived from the Edinburgh cryopreserved CTL bank.[11] These CTL can be readily infused in patients on the basis of the best HLA matching, although they are expected to present a short-term survival in the host, as an empty niche leading to their expansion is not present and because of the induction of anti-allogeneic responses. Nevertheless, the great amount of cells infused and their strong anti-EBV activity can bypass such limits and account for the good outcome reported so far.[11] Another problem is represented by the continuous administration of immunosuppressive agents in SOT recipients. In this regard, to increase survival of CTL to be employed in pharmacologically immunosuppressed hosts, T cells have been recently engineered to gain resistance to calcineurin inhibitors, the most commonly used immunosuppressants, while retaining their phenotypic and functional characteristics both *in vitro* and *in vivo* in an animal model.[28,29]

Unlike HSCT recipients, SOT recipients received unmarked CTL (CTL precursors); in this case, the fate and the permanence of the infused allogeneic CTL has been demonstrated only in few cases by clonotyping analysis or by tetramer staining;[11] in the remaining patients, such information could be inferred indirectly by the regression of tumor masses, the decrease in viral load and the rise in the number of EBV-specific CTLp. One notable case was the post-mortem demonstration of male donor CTL homing to the lesion of a female recipient.[30]

Despite existing limitations, EBV-specific CTL have been successfully infused not only in HSCT but also in SOT recipients, both with treatment and prophylactic purposes.

In a preemptive approach, virus-specific CTL were safely administered to 108 HSCT recipients[12,13,27,31-36] and 21 SOT recipients.[37-39] Such patients were considered at high risk to develop the disease on the basis of increased EBV DNA load, the EBV serology, and the type of transplant. No PTLD occurrence was reported at follow up, except for one patient who received cells lacking a well-defined EBV-specific component.[36] In particular, the only case-control study published to date[12] reported that none of the 39 patients treated preemptively with EBV-CTL developed PTLD in comparison to 11.5% in the control group. Furthermore, EBV DNA load showed a significant reduction in almost every patient after CTL infusion, concomitantly with an increase in the frequency of EBV-specific CTLp.

A smaller number of patients (n=24) receiving hematopoietic stem cell transplant was treated in therapy protocols with EBV-specific CTL after refractory PTLD diagnosis, and at least 10 complete remissions were registered (Figure 2).[11-13,27,31,32,34,35,40-44] According to Rooney *et al.*,[27] the resolution of the neoplastic lesions can be ascribed to the infused CTL and not only to a concurrent reactivation of the host EBV-specific immunity, which is still impaired up to one year after transplant. In the reported studies, only 2 patients did not respond to the therapy. Notably, in one of them,[43] the lack of efficacy was ascribed to the inactivity of CTL against one of the two viral isolates present in the host. Indeed, infused CTL were unable to recognize the predominant mutant virus bearing critical deletions in the regions coding for EBNA3B epitopes, which are the most represented specificities in EBV-specific T-cell lines. Therefore, despite the fact that the infused effectors derive from bulk cultures, the emergence of a mutant escape viral variant with low immunogenicity is still possible, in line with the concept of immunoediting.[45]

In SOT recipients, a consistent number of patients (n=41) received CTL therapeutically (Figure 2). Among all patients, only 8 were infused with autologous cells and, despite all previous treatment failure, they experienced reduction of neoplastic mass.[37,46-48] The remaining 33 patients received allogeneic CTL,[11,49] mainly derived from the Edinburgh CTL bank in the context of a phase II clinical trial.[11] This study, the only intent-to-treat trial reported so far in this field, registered 48% of clinical responses, either partial or complete, six months after infusion. The rate of response was comparable to that obtained in 46 RIS refractory patients undergoing treatment with anti-CD20 antibody.[50] Besides the demonstration of feasibility and success of the treatment, some significant considerations regarding the clinical outcome can be advanced. Among all the different factors, the EBV serological status, the time of onset, and the PTLD histopathology did not apparently influence the response to CTL. Furthermore, although it can be considered a hallmark of CTL activity, in this study the reduction of the viral load did not seem to reflect the clinical response to the therapy. Conversely, a higher HLA-matching between recipients and donors (which decreased the likelihood of anti-donor responses) and a greater relative percentage of infused $CD4^+$ T-helper cells did correlate with a better clinical outcome. This latter aspect is in line with the current strategies of immunotherapy, aimed at joining the "classical" tumor-specific $CD8^+$ T-cell response to a concurrent helper $CD4^+$ T-cell response.[51]

Both in treatment and prophylaxis, no relevant side-effects related to CTL infusion were observed, either at a systemic level or affecting the transplanted organ, thus excluding the presence of alloreactive cells in the preparations. Some considerations about treatment safety have nonetheless to be highlighted for patients with large tumor burden, since the strong immunological attack could be detrimental for the surrounding tissues. In the most impressive case described,[12] the wide inflammatory response associated with the T-cell infiltrate determined an air flow obstruction that required tracheotomy and intubation.

Apart from patients with transplant-related immunodeficiency, EBV-related B-cell lymphomas can also arise in different immunological deficit conditions, and are, therefore, susceptible to immunotherapeutic interventions. In particular, EBV-specific CTL have been administered in a patient with DiGeorge syndrome,[52] a patient with primary central nervous system (CNS) B-cell lymphoma with recurrent infections, peculiar anti-EBV antibody pattern and reduced response to mitogens,[53] and a patient with AIDS-related lymphoma.[49] Once again, the CTL infusion was devoid of side-effects and produced some clinical benefits in the first 2 cases, concomitantly with an increased *ex vivo* anti-EBV CTL activity and a reduction in viral load. Notably, the patient affected by primary CNS B-

cell lymphoma,[53] who had gone into coma despite all previous therapies, underwent steady neurological improvements and became fully conscious after the CTL infusion. Thus, clinical results demonstrated that activated CTL can cross the hematoencephalic barrier and could be considered a valid tool for the treatment of CNS EBV-related tumors, which account for 25% of AIDS-related lymphomas[54] and for 30% of PTLD.[55]

EBV-related diseases in immunocompetent hosts

Unlike posttransplant lymphoproliferative disorders, which constitute a highly immunogenic lymphoproliferation whose onset is greatly favored by the host immunodeficiency status, other EBV-related diseases can arise in immunocompetent patients. To develop successfully, these tumors have to hide themselves from immune recognition, primarily by shutting down the expression of the most immunogenic viral proteins, which belong to the EBNA3 family. As a consequence, despite the presence of a functional antigen processing and presentation machinery, NPC and HL cells are less susceptible to an immunotherapeutic attack with EBV-specific CTL. In fact, the *in vitro* expanded cultures reflect the immunodominance hierarchy of responses found in EBV-seropositive individuals, with only a minor fraction recognizing the typical proteins expressed by the tumors, like LMP1, LMP2, and EBNA1.[56]

Nevertheless, as healthy EBV-carriers present CTLp reactive to subdominant viral proteins, some impairments in the host immune response are likely present in patients developing HL or NPC. This can also be argued by the fact that there is a correlation between the peripheral response to tumor associated antigens, the presence of Tregs and the state of disease: in other words, the more severe the disease, the lower the frequency of tumor-specific CTLp and the higher the presence of Tregs, and *vice versa*. Moreover, the reported spontaneous resolution of a Hodgkin's lymphoma is highly suggestive of the involvement of immunological responses in the development and control of this tumor.[57] Indeed, in the case of Hodgkin's lymphoma, although tumor-associated antigen (TAA)-responsive T cells are present at the same frequency as in healthy controls, they are poorly responsive in functional tests;[58] this characteristic seems to correlate with the expression of Treg markers.[59-61] Moreover, tumor infiltrating lymphocytes (TIL) from EBV-unrelated but not from EBV$^+$ Hodgkin's lymphoma were shown to exert EBV-specific T-cell responses.[62,63] Similarly, TIL from NPC were shown to lack cytotoxic activity and IFNγ production,[9] despite the presence of an unaltered response in the blood to TAA with respect to healthy subjects. The defective host response against TAA appears to be linked to the hostile tumor microenvironment, in part orchestrated by tumor cells. In this respect, local production of the sup-

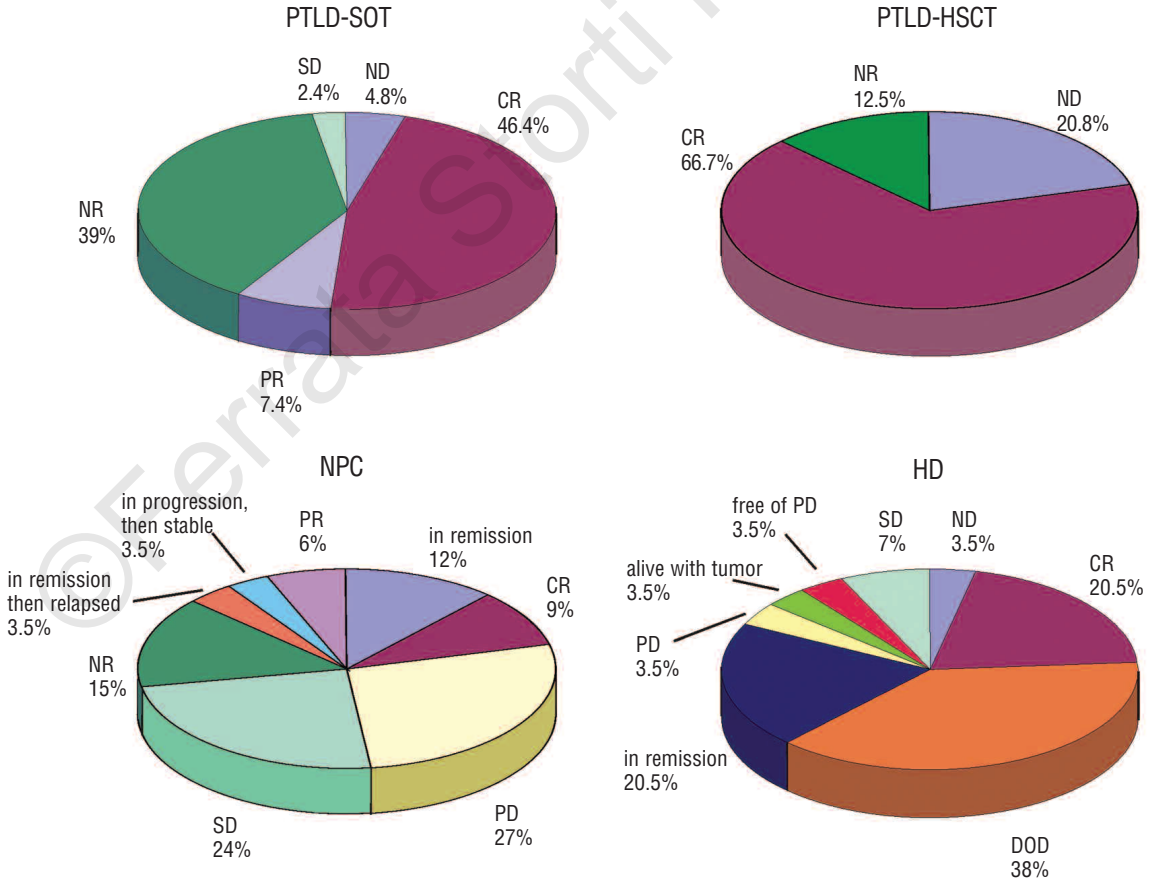

Figure 2. Diagrams show responses to treatment with EBV-specific CTL in patients with PTLD after SOT (total patients treated, 41), PTLD after HSCT (total patients treated, 24), NPC (total patients treated, 33), and HD (total patients treated, 21). CR: complete response; SD: stable disease; PD: progressive disease; NR: no response; PR: partial response; DOD: died of disease; ND: no data.

pressive molecules galectin-1 or galectin-9 has been reported in Hodgkin's lymphoma and nasopharyngeal carcinoma (NPC), respectively,[10,64,65] and an increased presence of Treg cells has been found in both tumor types. Moreover, NPC cells are characterized by FasL or IL-10 expression, and the malignant Reed-Stenberg cells in HL lesions, by producing IL-10, TGFβ, and CCL17 (TARC), together with the surrounding inflammatory infiltrate (approximately 90-99% of the tumor mass), create an environment that promotes Th2 at the expense of Th1 responses.[66] Furthermore, T lymphocytes infiltrating Hodgkin's lymphoma show a suppressive activity against peripheral blood mononuclear cells that acts through several mechanisms, namely IL-10 release, cell-to-cell interaction, and CTLA-4 expression.[60]

As a consequence, therapy with EBV-specific CTL for HL and NPC has less chance of success than PTLD. First, EBV-specific CTL lines are dominated by reactivity against viral proteins not expressed by these tumors. Strategies aimed at increasing the T-cell fraction specific for subdominant proteins expressed by the tumors involved transduction of antigen presenting cells (dendritic cells and lymphoblastoid cell lines, LCL) with adenoviral constructs encoding LMP2. This permitted the preferential expansion of T cells recognizing the antigen,[17] and mediated in turn the successful resolution of bulky diseases in Hodgkin's lymphoma as well as NK/T non-Hodgkin's lymphoma and severe chronic active Epstein-Barr virus infection (SCAEBV) patients (a total of 15), all characterized by a latency II pattern expression.

Second, once CTL reach the tumor site (paradigmatic is the percentage of specific CTL in the pleural effusion of a Hodgkin's lymphoma patient compared to that of peripheral blood - 0.65% *vs.* 0.001%),[67] they have to overcome the inhibitory barriers to their action. This requirement should be achieved by genetically modifying T cells, with the aim of inducing expression of IL-12, a cytokine promoting the Th1 anti-tumor response, or TGFβ receptor dominant negative mutants, as described *in vitro*.[20,21]

Based on these considerations, the results reported so far appear even more impressive, despite the lower success rate, than those obtained in PTLD management. In detail, clinical responses, mainly in patients with limited disease burden, were obtained after infusion of polyclonal, polyspecific CTL lines in 33 NPC patients (3 complete remissions, 3 partial remissions, and 8 stable diseases; Figure 2)[19,68-71] as well as in Hodgkin's lymphoma patients (4 complete remissions and 2 stable diseases out of 20 reported outcomes; Figure 2).[67,72-75] Of note, these results were achieved in patients who failed previous standard treatments and by infusing cell cultures containing low percentages (< 5%) of LMP2-specific cells. Clinical responses were paralleled by a drop in the levels of circulating Epstein-Barr virus DNA, a reliable tumor marker of virus-related malignancies. Indeed, viral load fluctuation is a fundamental parameter in NPC clinical course, since it positively correlates with the disease stage and has prognostic relevance.[76] When measured, the EBV load decreased in the majority of treated patients and fell to undetectable levels in some cases, concomitantly with an increase in EBV- or LMP2-specific CTLp. However, these immunological and clinical responses proved to be short-lived, probably due to the lack of expansion of infused cells.

In contrast to HSC transplant recipients (where the regenerating hematopoietic system represents an optimal milieu for the persistence and expansion of infused cells), in immunocompetent patients the transferred CTL have to compete with endogenous lymphocytes for cytokines and biological niches. To create a proper immunological space, similar to that obtained in melanoma patients with chemotherapy or radiation,[77] a group of 8 NPC patients were pre-treated with anti-CD45 monoclonal antibodies,[19] achieving a more than 100-fold expansion and a persistence of infused cells (eight weeks) longer than that seen in previous approaches. While the achievement of a long-lasting persistence of infused cell (the purpose of this study) is a prerequisite for durable clinical effects and to avoid disease relapse, nonetheless, the outcome in this setting ultimately depends on the specificity of the infused CTL. Indeed, in the series reported by Louis and colleagues,[19] the patient experiencing a complete response received a bulk culture with a relevant LMP2-specific component, which was lacking in some other cases.

In the case of Hodgkin's lymphoma, persistence of infused cells could be demonstrated by a gene-marking technique for as long as 12 months.[67] This result was achieved without previous lymphodepletion, probably due to the concomitant lymphopenia related to the disease. Consistently, a better clinical response was generally observed.

Epstein-Barr virus is also involved in other diseases, such as NK/T-cell lymphoma and SCAEBV. These are rare conditions, more common in Japan and East Asia,[78] whose immunopathogenesis is not completely understood. In both conditions, NK and T cells are the target of EBV infection. Although the reduced antigen expression and the limited immunogenicity intrinsic to these cells allow them to escape immunological control, on the other hand, these characteristics mean they are not an ideal target for immunotherapeutic strategies. Nevertheless, the observations that in chronic active Epstein-Barr virus (CAEBV) patients the frequency of LMP2 CTLp and EBV-specific CTL activity are commonly found to be impaired,[79,80] and that HSC transplant from virus seropositive donors is one of the most successful treatments,[81] suggest a pathogenetic role for some form of immune impairment, which could be reverted by the infusion of EBV-specific T cells. This was indeed demonstrated by the successful treatment of a mild form of the disease, commonly observed in patients in the US, with EBV CTL administration.[82] In detail, 4 of 5 patients with mild/moderate CAEBV experienced not only normalization of anti-EBV antibody titres, but also improvement of symptoms and disease stabilization for the subsequent three years after the treatment, in contrast with the progressive worsening of the quality of life commonly observed during the course of the disease. Conversely, in the case of severe CAEBV, no clinical improvement was observed in 2 of 3 treated patients, despite some transient viral and immunological responses.[83,84] In this case, although the feasibility of generating and expanding functional EBV CTL *in vitro* is maintained, it is likely that an imbalanced immunological environment may ultimately hamper their *in vivo* activity.

Encouraging results, although not conclusive due to the low number and the heterogeneity of patients, were obtained in the unique study focusing on natural killer/T-cell lymphoma:[85] in this setting, CTL infusion proved to be safe and in 2 of 3 cases a clinical response has been reported, thus indicating that adoptive immunotherapy could

represent an alternative therapeutic choice in relapsed disease, which usually has a very poor prognosis. As previously reported for NPC and Hodgkin's lymphoma, new protocols are currently under investigation to generate CTL lines enriched in specificities for the antigens expressed by these diseases, namely LMP1, LMP2 and EBNA1. The attention has been focused on cytotoxic CD4$^+$ T cells, which proved to be effective, at least *in vitro*, not only against EBV LCL but also, more importantly, against infected NK or T cells, the natural target in NK/T-cell lymphoma and CAEBV.[86,87] Such observations differ from the previous reports related to these diseases[82-85] which only demonstrated the activity against LCL that express a larger pattern of viral antigen and costimulatory molecules than EBV-infected NK/T cells.

Conclusions

Immunotherapy of EBV-related malignancies, together with melanoma, can be regarded as a paradigmatic example of the potential use of adoptive T-cell therapy.[88] The success of EBV-specific adoptive cell therapy (ACT) relies on numerous, lucky circumstances. First of all, in this case tumor-associated antigens are viral proteins, in particular those associated with the latent cycle, and, more importantly, they are non-self so that there is no need to break the host immune tolerance. Moreover, EBV-specific CTL can be easily reactivated from almost all donors, since 95% of the adult population worldwide is EBV-seropositive and harbors in the memory compartment a relatively high frequency of specific precursors (0.05-1% of the circulating memory CD8$^+$ T cells in healthy carriers without previous history of infectious mononucleosis are specific for latent EBV epitopes).[89] Moreover, the need for a suitable antigen presenting cell is satisfied by the availability of LCL, B-cell lines carrying the virus and displaying the complete array of latency-associated proteins (latency III phenotype). This model, which closely resembles PTLD, is easy to handle *in vitro* and to translate to a pre-clinical setting.[14] All these characteristics contributed to the success of EBV-specific CTL, especially in the case of PTLD. However, these results were obtained in a relatively uncommon disease. Moreover, this methodology requires highly specialized facilities and trained personnel, thus limiting the wide diffusion of this approach. Efforts are currently being made to overcome the technical limitations intrinsic to the protocol (namely, the time required to generate CTL cultures) and to broaden the field of action of CTL. The creation of the CTL bank in Edinburgh fulfilled the first requirement, providing CTL lines as an off-the-shelf product ready to be promptly infused into patients on the basis of the best HLA-matching. Furthermore, an increasing number of new protocols appears to shorten the time required for CTL generation, not exclusively in the field of EBV.[90-93] Such approaches allow not only the time lag between diagnosis and treatment to be shortened, but also CTL to be produced at a less differentiated stage for infusion, thus allowing a longer *in vivo* persistence of EBV-specific effectors.[94] Another strategy to improve EBV immunotherapy could rely on "new" virus-associated antigens to be exploited as targets. Bulk cultures infused so far are constituted primarily by CD8$^+$ T cells, which are mainly directed against the immunodominant latent proteins belonging to the EBNA3 family. Responses directed against subdominant antigens, like LMP2, have been achieved by genetically modifying the APC. Furthermore, attention has been mainly focused on latent antigens, and, in some cases, the generation of responses against late lytic antigens has been prevented by the use of acyclovir-cultured LCL as APC.[67] However, the evidence that a fraction of tumor cells undergoes lytic cycle in the context of PTLD and NPC implies that lytic antigens could also be regarded as potential targets. In particular, targeting of lytic antigens could effectively block *de novo* infection and thus could be important in a prophylactic setting in patients at high risk to develop PTLD. Moreover, despite the fact that cells undergoing lytic cycle could escape immune recognition through partial HLA class I and II downregulation and are in any case committed to die, viral proteins released by this minor fraction of cells could be taken up by neighboring tumor cells and therefore sensitize them to the immune attack. This could resemble what was seen *in vitro* with LCL cultures by Adhikary and colleagues.[95] Among lytic antigens, a suitable target might be represented by the BARF1 protein, which has been demonstrated to be secreted by infected cells,[96] and to induce CD8$^+$ and CD4$^+$ T-cell responses in NPC patients.[97] Moreover, to underline the importance of lytic antigens, they have been recently demonstrated to be immunodominant in the CD4$^+$ T-cell response.[95,98] In this regard, CD4$^+$ T cells are now emerging as something more than "simple" helper cells. Indeed, they can be endowed with cytotoxic activity, as demonstrated not only *in vitro* but also directly *ex vivo*, especially in the context of viral diseases.[99] Moreover, tumor regression by CD4$^+$ T cells alone was demonstrated in mouse models of Burkitt's lymphoma,[100] and gamma-herpesvirus induced lymphoma,[101] and, more recently, in the context of ACT against human melanoma. In particular, the importance of CD4$^+$ T cells has been recently underlined by the correlation of clinical responses with the percentage of CD4$^+$ T cells infused in patients with EBV-related PTLD,[11] and by the complete regression of metastatic lesions in a refractory melanoma patient following the infusion of an NY-ESO-1-specific CD4$^+$ T-cell clone.[102] In both cases, the activity was merely supposed to depend on the helper function of CD4$^+$ T cells, without considering a possible direct, cytotoxic effect of the infused effectors.

Exquisitely in the context of EBV-associated malignancies, CD4$^+$ T-cell responses against latent proteins also seem to acquire more importance. The immunological control exerted by EBNA1-specific CD4$^+$ is crucial, since a loss of this response was recently demonstrated in patients with EBV-associated lymphomas,[103] and children with Burkitt's lymphoma.[104] These findings, together with the fact that EBNA1 expression is associated with all types of EBV latency and generally does not induce an important CD8$^+$ T-cell response, suggest, in principle, a primary role for EBNA1-specific CD4$^+$ T-cell lines in adoptive T-cell therapy for all EBV-related tumors, irrespective of the latency phenotype.

In any case, the success of EBV-specific T cells, the relatively non-problematic generation and, primarily, the long-lasting persistence of the infused cells (mainly in PTLD developing after HSCT) prompted researchers to exploit this system in other clinical settings. By modification of the APC, Leen *et al.* simultaneously generated CTL specific for viruses implicated in the morbidity of transplanted patients, namely EBV, CMV and adenovirus.[105,106]

EBV-specific CTL were exploited also against different malignancies, by introducing new specificity through TCR or chimeric antigen receptor (CAR) transfer.[107] In particular, successful treatment was achieved in glioma patients with the infusion of EBV-specific CTL transduced with anti-GD2 CAR.[108,109] These CTL, along with the newly acquired specificity for glioma, retained the capacity to recognize viral antigens. Thus, the antigenic stimulation exerted by EBV-infected B cells *in vivo* provided EBV-specific CTL a survival advantage with respect to autologous CAR-transduced activated T cells. Therefore, the peculiar characteristics of the interaction between EBV and the host immune system could transform the virus from a simple target of CTL action to an effective means of sustaining EBV-specific CTL endowed with new specificities.

Overall, these recent results look forward to a broader use of this approach, which may result clinically relevant also in diseases which are not virus-related. As we learn more about the intimate host-virus interplay, the wider potential of EBV-specific CTL is becoming clearer and future successes of the clinical use of adoptive cell therapy are, therefore, expected.

Authorship and Disclosures

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