Adoptive T cell therapy as treatment for Epstein Barr Virus-associated malignancies : strategies to enhance potential and broaden application

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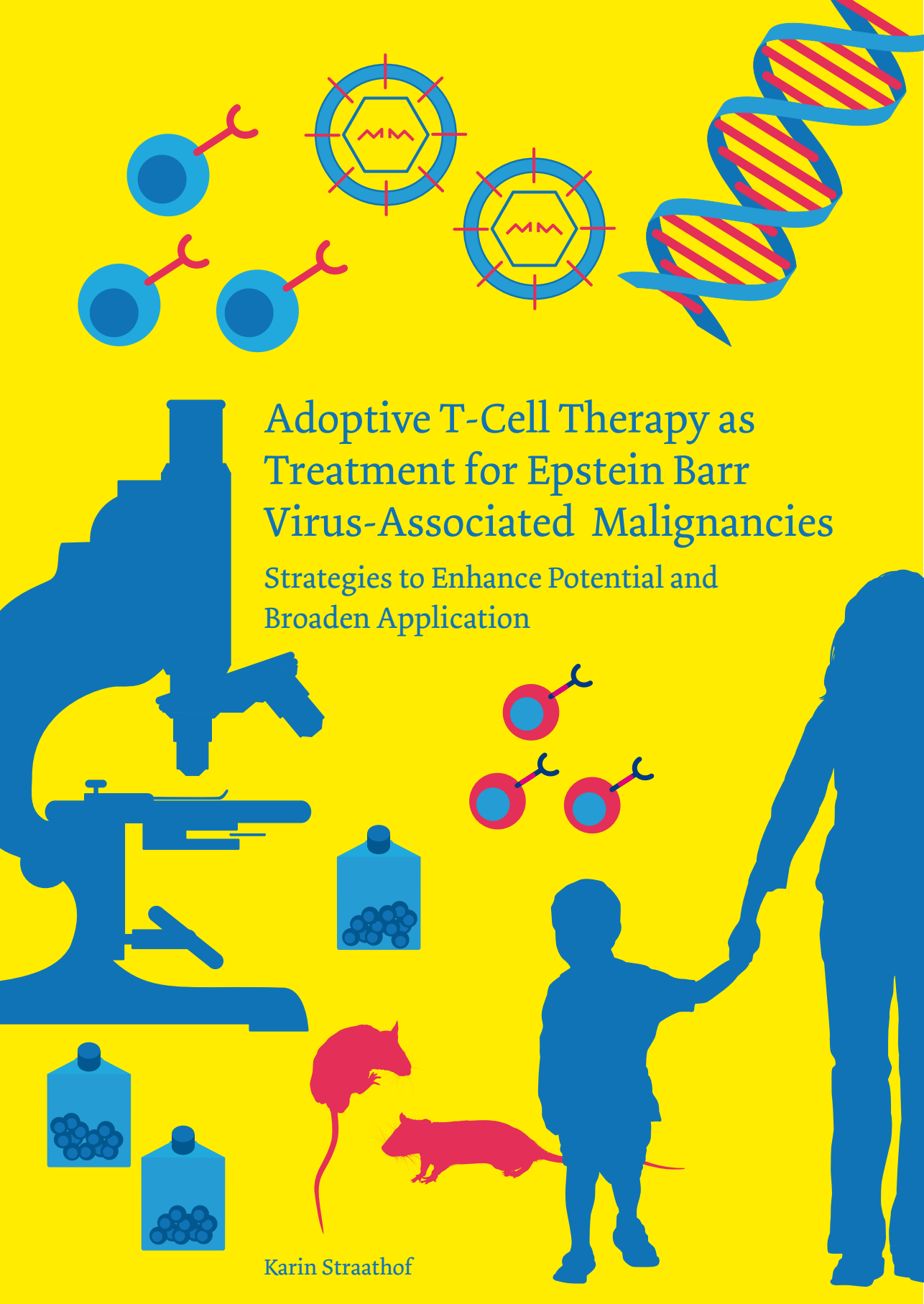

Adoptive T-Cell Therapy as Treatment for Epstein Barr Virus-Associated Malignancies

Strategies to Enhance Potential and Broaden Application

Karin Straathof

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"You've got to smell it,
and get your fingers burned,
and shed a few tears over it,
and everything else to get it right.
That's the way I look at it"

CB Stubblefield, famed Texas barbeque competitor talking about Texan BBQ

Contents

Chapter 1

Introduction

Over the past 50 years the number of cancer deaths, with exception of some rare tumors, has not decreased[1] despite decades of research and investment of tremendous resources into improving conventional therapies such as chemotherapy, radiotherapy and surgery. Furthermore, the largely non-specific nature of these treatments results in severe acute and long-term side effects including secondary malignancies. New approaches are needed and immunotherapy held much promise to provide an alternative means of cancer therapy with minimal toxicity. The fundamental principle of immunotherapy is to utilize the immune system with its highly specialized capacity to distinguish between self and non-self to eradicate tumors. It therefore has the potential of being highly tumor specific. However, despite many clinical studies, success has been limited[2] resulting in a current wave of skepticism. The mainly disappointing results so far may have been caused by a limited knowledge of the tumor-directed immune response and by a lack of identified tumor antigens. More recently, new insights into the interaction between immune system and tumor environment and new techniques for the identification of tumor antigens allow for the design of improved strategies to induce or augment a tumor-specific immune response.

Epstein-Barr Virus (EBV)-associated tumors provide an excellent model system to develop and study immunotherapeutic strategies and the prevention and treatment of EBV-associated lymphoma occurring after stem cell transplantation (SCT) has been a resounding success.[3-5] The work in the first part of this thesis builds on this expertise and investigates the feasibility and efficacy of adoptive immunotherapy as treatment for EBV-positive nasopharyngeal carcinoma (NPC). A broader application to other inherently less immunogenic malignancies, however, requires alternative strategies to obtain tumor-specific T-cells. The work in the second part of this thesis therefore explores retroviral transfer of native and engineered T-cell receptors (TCR) as a method to generate T-cells with desired tumor antigen specificities. The clinical use of such genetically modified T-cells will be facilitated by the implementation of a safety switch that can be activated in the event of toxicity *in vivo*. So, in the final part of this thesis we detail the development of a suicide gene suitable for use in T-cell-based therapy. This first chapter provides a general introduction of these areas of immunotherapy research and describes the scientific questions addressed in this thesis.

1 Use of the immune system for treatment of cancer

1.1 Why does the immune system fail to reject tumors?

The immune system is equipped with innate and adaptive arms to provide a defense mechanism against foreign pathogenic invaders. It is capable of eliciting a specific, sustained and highly potent immune response, which engenders rejection of the offending pathogen and infected cells.[6] First, effector cells of the innate immune system recognize generic structures on infected cells. These respond in part by the secretion of cytokines that have direct lytic or immunomodulatory functions. This innate response initiates and directs the adaptive immune response and uses germline receptors with generic specificities. In contrast, the adaptive immune system uses receptors generated by gene rearrangements with narrow specificity. B-lymphocytes recognize pathogen-derived proteins, polysaccharides and lipids. B-cell activation, which requires T-helper cell involvement in the case of protein-derived antigens, induces B-cell proliferation and antibody production. The secreted antibodies bind to (virus or pathogen) antigen resulting in either neutralization of the pathogen or lysis by effectors of the innate immune system. T-lymphocytes recognize immunogenic peptides derived from intracellularly degraded and processed foreign antigens in the context of major histocompatibility (MHC) molecules. Naïve T-cells encounter antigen presented on professional antigen presenting cells (APC) in the lymph nodes. Activated effector T-cells enter the circulation and locate the antigen in peripheral tissues where they secrete cytokines and lyse infected cells by perforin and/or fas-mediated mechanisms. Together, these branches of the immune system can reject a virtually unlimited assortment of pathogens ranging from viruses and bacteria to multi-cellular parasitic organisms.

The immune system should be capable of distinguishing tumor cells from their normal counterparts in an analogous fashion. A number of antigens have been identified that are expressed by tumor cells but are absent from normal tissues.[7] These include viral antigens (EBV, human papilloma virus (HPV)), highly expressed or mutated oncogenes (p53, Ras) and fusion proteins (BCR-ABL). In addition, cell-lineage specific proteins that are over-expressed on tumor cells (MART-1, gp100), or proteins aberrantly expressed on tumor cells that are normally only present in testis or fetal tissues (NY-ESO-1) may provide the immune system with a means of discrimination between normal and malignant cells. However, it is clear that, despite this opportunity, the immune system fails to reject tumors. There are many potential explanations for this indifference. Tumor cells under selection from the immune system can adapt by mutation leading to loss of cognate epitopes, loss of whole antigens or down-regulation of molecules involved in antigen processing and presentation.[8] Many tumors express molecules that can actively dampen the immune system (e.g. transforming growth factor (TGF)-ß and interleukin (IL)-10).[9] Over-expressed tumor antigens may be recognized as self and therefore have been deleted in the thymus or tolerized.[10] The immune system may fail to respond because tumors fail to transmit cues of danger or inflammation.[11,12] And finally, the tumor directed immune response may be inhibited by regulatory T-cells with diverse phenotypes.[13] Immunotherapy hence can be understood as the art of coaxing the immune system to respond to a tumor in a similar vein as a response to an infection – more particularly to simulate the scenario of virally infected cells.

1.2 How can the tumor-specific immune response be induced or enhanced?

Different disciplines of immunotherapy have attempted to trigger immune responses in different ways. One approach is the systemic administration of cytokines, glycolipids or biphosphonate compounds to activate effectors of the innate immune system.[14-16] However, although potent for certain tumors,[17] these strategies can be accompanied by intolerable side effects of high systemic concentrations of cytokines. Modulating the adaptive immune response, as it is antigen specific and results in long-term memory, therefore appears to be a more attractive strategy.

Vaccination strategies aim to actively induce or boost tumor-specific lymphocytes. In initial studies, the tumor antigen in the form of tumor cell lysates, peptides, viral vectors encoding the tumor antigen or plasmid DNA was administrated in combination with adjuvants or cytokines to recruit and activate dendritic cells (DC) for antigen presentation. Although vaccination resulted in an increased number of tumor-reactive T-cells in the majority of cases, clinical responses were induced only sporadically.[2] Epitope enhancement, incorporation of co-stimulatory molecules, ex vivo dendritic cell maturation and antigen loading prior to vaccination have been proposed as strategies for improvement and are currently under investigation in clinical studies.[18] Although vaccination is an attractive approach that can potentially be made available to a large group of patients, it has the inherent difficulty of inducing a potent immune response in the presence of host immune regulatory mechanisms and a hostile tumor environment.

Administering tumor-specific effectors of the immune system that have been generated ex vivo can circumvent this problem. One such approach is the administration of tumor-associated antigen-specific monoclonal antibodies (mAbs). CD20 mAbs have already been successfully implemented in treatment regimens for B-cell malignances.[19-21] The value of mAbs against growth factors and their receptors as treatment for solid tumors including breast cancer is currently under investigation.[22] The technology to humanize murine antibodies facilitates a broader development and clinical use of monoclonal antibodies. Conjugation with immunotoxins, drugs or radioisotopes may further enhance their efficacy but may require strategies to reduce unwanted effects on normal tissue. A major concern regarding treatment with monoclonal antibodies is the development of antigen-loss variants in particular when antigens not essential for tumor cell survival and proliferation are targeted. This has for example been a problem with anti-CD20.[23,24] In addition, antibodies may not be able to penetrate solid tumors well, only intact surface antigens can be targeted and few antigens are specific enough to provide tumor elimination without damage to other essential tissues.

Arguably the most striking successes of immunotherapy so far have been obtained with adoptive T-cell therapy. Donor lymphocyte infusions (DLI) have induced durable remissions in patients with relapsed leukemia after receiving an human leukocyte matched (HLA)-matched allogeneic SCT.[25,26] However, as these lymphocytes are unselected they also contain allo-reactive cells with the inherent risk of graft-versus-host disease (GVHD). T-cell responses to minor histocompatibility (H) antigens, peptides derived from polymorphic intracellular proteins, have been shown to account for both the desirable anti-tumor effect and the unwanted toxicity on normal recipient tissue.[27,28] The identification of minor H antigens with expression limited to the hematopoietic system (HA-1, HA-2, HB-1 and BCL2A1)[29-34] allows for a potential refinement of the DLI approach as treatment for hematological malignancy: in an HLA-matched minor H antigen-mismatched SCT setting, adoptive transfer of selected HA-1-specific donor lymphocytes may provide activity against residual tumor cells (HA-1 positive) without unwanted site effects on donor hematological and recipient non-hematological tissue (HA-1 negative).[35]

However, such a strategy requires procedures for ex vivo reactivation and expansion of antigen-specific T-cells. These have been successfully developed for viral antigens: EBV and cytomegalovirus (CMV)-specific cytotoxic T-cell lines (CTL) were generated by repetitive *in vivo* stimulation of donor lymphocytes with APC expressing viral antigens.[4,36] These *ex vivo* expanded virus-specific CTL proved effective in preventing and treating virus-associated complications in SCT recipients without causing GVHD.[3-5,36] However, application of this strategy to non-viral tumor antigens is more difficult because most tumor antigens are less immunogenic than viral antigens and precursor frequency is much lower. Nevertheless, for a limited number of malignancies tumor-specific T-cells were successfully generated by stimulation of peripheral blood mononuclear cells (PBMC) with DCs pulsed with tumor antigen[37] or by expansion of lymphocytes naturally infiltrating at the tumor site (TIL) in a non-antigen specific fashion.[38] Adoptive transfer of these tumor-specific T-cells was safe, but initial studies showed limited *in vivo* persistence of the infused cells and only transient clinical responses.[39] This observation demonstrates that the commonly used classification of adoptive T-cell therapy as passive immunotherapy is a misnomer; tumor-specific T-cells that have been activated and expanded *in vitro*, even if infused in large numbers, must still home to tumor sites and maintain function and proliferative potential in order to provide a long lasting anti-tumor effect. Therefore, new treatment regimens are being developed that promote *in vivo* expansion and persistence of infused T-cells. Promising results have been obtained by combining adoptive T-cell therapy with prior depletion of endogenous lymphocytes.[40]

Thus, adoptive T-cell therapy appears to have great potential and despite the need for the generation of a therapeutic product for each patient individually, a process that is labor-intensive and expensive, has a number of advantages over other immunotherapeutic approaches. First, ex vivo culture of tumor-specific T-cells circumvents *in vivo* host immune regulatory mechanisms and suppressive tumor influences and allows for creating a well-controlled environment favoring required T-cell characteristics. Second, the resulting therapeutic product can be well characterized prior to infusion. Third, the T-cells can be genetically modified to render them resistant to immunosuppressive cytokines or to engraft them with specificities, which are very difficult to obtain (HA-1) or which would otherwise never exist for T-cells (e.g. for surface antigens such as GD-2). For these reasons, the work in this thesis focuses on the adoptive T-cell therapy approach. It first builds on previously gained experience with T-cell therapy for virus-associated tumors and subsequently addresses the difficulty of generating T-cells specific for less immunogenic tumor antigens.

2. EBV-associated tumors as model to study adoptive T-cell immunotherapy

EBV-associated malignancies provide an excellent model system to develop immunotherapeutic strategies for a number of reasons. First, viral antigens expressed by these tumors and the cellular immune responses against these are well characterized.[41] Second, as over >95% of the adult population is infected with this virus, detectable frequencies of tumor-specific T-cells are circulating in the peripheral blood which simplifies active boosting or ex vivo expansion of these cells. Third, EBV-associated malignancies vary in immunogenicity based on the range of viral antigens expressed. Therefore, proof of principle studies can first be performed in immunogenic tumors and these can then be extended to less immunogenic tumors that are more representative of other non-viral associated tumors.

2.1 The natural course of EBV infection

Epstein Barr virus enters via the oropharynx where it infects resting B-cells and/or epithelial cells.[42,43] This may result in the expression of a full array of nearly 100 replicative (lytic) cycle antigens and release of infectious virus or latency, in which only subsets of the viral genes are expressed. In its most immunogenic form, nine viral proteins, the nuclear antigens (EBNA 1, -2, -3A, -3B, -3C and –LP) and the latent membrane proteins (LMPs)-1 and –2a, -2B are expressed. This type of latency (termed type 3, Figure 1) results in B cell immortalization *in vitro*, is highly immunogenic and stimulates massive expansion of virus-specific and non-specific T-cells *in vivo*, producing mononucleosis and regression of the 'immortalized' B cells.[44] A small proportion of B-cells escape this immune response by expressing a minimal form of latency (type 0) with expression limited to LMP2,[45,46] or possibly no viral antigens at all.[47] Virus can now persist in its latent state for the life of the individual, with frequent release of infectious virus in the oropharynx.[48]

2.2 The association of EBV with malignancies

Primary infection usually occurs during childhood and results in a mild self-limiting illness. When infection is delayed until adolescence infectious mononucleosis, characterized by fever, lymphadenopathy and pharyngytis, occurs in around 50% of individuals.[49] Although harmless in the majority of cases, EBV is associated with a heterogeneous group of tumors including Burkitt's lymphoma, lymphomas associated with immunosuppression, other non-Hodgkin's lymphomas, Hodgkin's disease, NPC, gastric adenocarcinoma, lymphoepithelioma-like carcinomas, and immunodeficiency-related leiomyosarcoma.[50] All of these tumors are associated with the EBV latent cycle, and express a spectrum of latent cycle antigens.
The EBV-associated lymphoproliferative diseases that occur in individuals who are severely immunocompromised after solid organ or stem cell transplantation, or who have congenital immunodeficiency or human immunodeficiency virus infection expresses type 3 latency antigens, like B-cells immortalized by EBV in vitro. A more restricted EBV antigen expression pattern including only EBNA-1, LMP1 and LMP2 called type 2 latency is the hallmark of EBV-positive Hodgkin's disease and NPC (Figure 1). Type 1 latency, including only EBNA-1, is found in EBV-positive Burkitt's lymphoma. The viral antigens expressed by these malignancies provide potential targets for immunotherapy, but the number and type of EBV antigens present have important implications for the strategy to be used and the expected therapeutic effect.

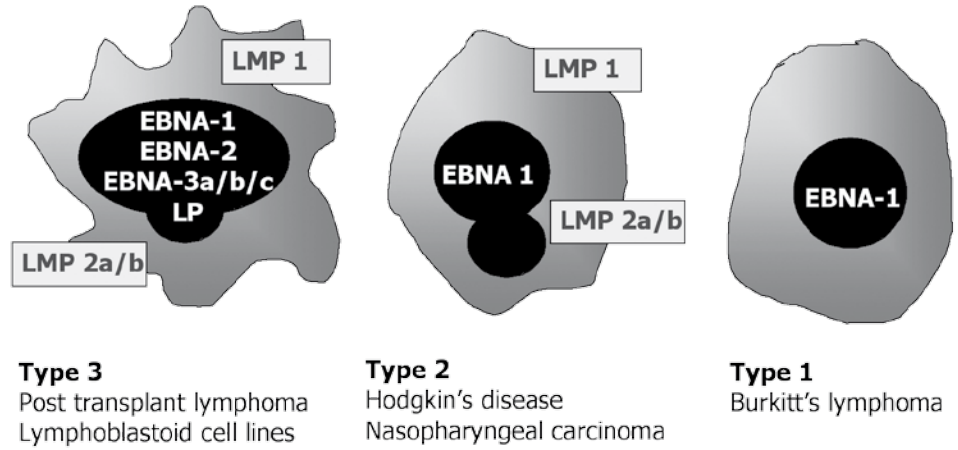

Figure 1. EBV antigen expression in different tumor types
Type 1 latency as seen in Burkitt's lymphoma is defined by the presence of EBNA-1 without expression of other latent EBV antigens. EBNA-1 was considered a poor target antigen for CD8+ T-cells as a glycine alanine repeats inhibits the processing of EBNA-1 through the HLA class I pathway.[124] However, recent studies indicate that incompletely translated EBNA-1 proteins can induce a CD8+ T-cell response.[125-127] A restricted expression pattern including the subdominant EBV antigens LMP1 and LMP2 is de hallmark of Hodgkin's disease and NPC (type 2 latency). Although these tumors express MHC class I/II and costimulatory molecules and can thus be recognized by the immune system,[71,128] the restricted antigen expression pattern and the active immune evasion strategies employed by these tumors are thought to enable these tumors to develop in immunocompetent hosts.[129] In vitro generated EBV-transformed B cell lines (LCL) and lymphomas developing in immunocompromised hosts express the full array of latent EBV antigens (type 3 latency) as well as MHC class I/II and costimulatory molecules which makes these tumors highly immunogenic. In addition to the expression of the indicated EBV antigens, RNAs from the Bam HI A region of the genome with no known protein product and two small nonpolyadenylated RNAs (EBERs) are present in all tumors.

2.3 The success of CTL therapy as prophylaxis and treatment for PTLD

In normal EBV-seropositive individuals, an ongoing balance exists between virus-driven B-cell proliferation and the cellular immune defense mechanisms. However, in individuals with compromised cellular immunity, increased virus reactivation and an increase in the number of latently infected B-cells in the peripheral blood may be seen.[51] Either or both of these factors may account for the elevated levels of EBV-DNA in peripheral blood and plasma by polymerase chain reaction.[52] In some cases, uncontrolled EBV-driven proliferation may then occur, leading to overt lymphoma. The key to prevent and treat post transplant lymphoproliferative disease (PTLD) is to restore the equilibrium between EBV-infected B-cells and EBV-specific T-cells. One strategy to do this is by adoptive transfer of virus-specific T-cells. Initially unmanipulated lymphocytes from the bone marrow donor were given to achieve this.[53] However, although this proved effective in a number of patients, the infused product also contains alloreactive cells and can therefore induce GVHD.[53,54] To circumvent alloreactivity and to obtain sufficient numbers of tumor-specific T-cells, Rooney et al developed a method to reactivate and expand EBV-specific T-cells ex vivo (Figure 2).[55] Donor-derived EBV-specific T-cell lines were used as prophylaxis for EBV-induced lymphoma in over 60 patients post SCT. None of the patients treated with this approach developed PTLD, compared to an incidence of 11.5% in a historical non-treated control group.[5] Gene-marking of donor CTLs facilitated the monitoring of the infused cells *in vivo* and demonstrated persistence of infused CTL for as long as seven years.[3]

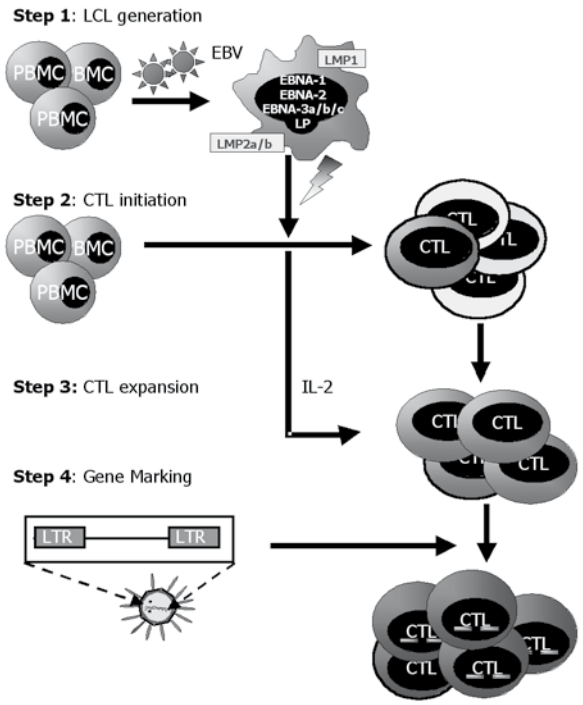

Figure 2. The generation of EBV-specific CTL lines

Step 1: PBMCs are isolated by centrifugation on a ficoll gradient from 30-50 mL of patient blood. A small number of the obtained PBMC (up to 5x10⁶) are infected with a laboratory strain of EBV (B95-8) in the presence of cyclosporin to establish an EBV-transformed B-cell line which generally requires 4-6 weeks.
Step 2: The remaining PBMCs are stimulated with irradiated LCL as APCs. Those T-cells specific for EBV-antigens become activated and start to proliferate.
Step 3: The reactivated T-cells are expanded by weekly stimulation with irradiated LCL and bi-weekly feeding with IL-2 to obtain an EBV-specific CTL line. Within 30-35 days, 5x10⁸ – 1x10⁹ cells may be expanded from 30 mL of blood.[55]
Step 4: Transduction of the CTLs with a retroviral vector, which stably integrates in the genomic DNA, allows for tracking of the infused CTL by PCR. After sterility, absence of replication competent retrovirus, HLA identity and desired phenotype and cytolytic function have been confirmed the CTLs can be administered back to the patients as an i.v. bolus.

Further, five of six patients who received CTL as treatment for overt lymphoma achieved complete remissions. In the patient who failed to respond, the tumor was transformed with a virus that had deleted the two CTL epitopes for which the donor CTL line was specific.[56] The effective treatment of EBV-positive lymphomas with virus-specific T-cells provided a proof of principle for adoptive T-cell therapy. The next step is to investigate if this strategy can also be applied to treat EBV-positive malignancies arising in immunocompetent hosts such as NPC.

2.4 Rationale for adoptive T-cell therapy as treatment for EBV-positive NPC

2.4.1 Epidemiology and clinical aspects of NPC

The incidence of NPC varies widely with geographical location, with an incidence as high as 50 per 100,000 men in Southern China and less than 1 per 100,000 adults in low incidence areas including the USA.[57] NPC is a radiosensitive tumor, and by modern imaging and radiation techniques, local control rates of greater than 80% are obtained.[58] Despite this, distant failures remain the major problem in patients with loco-regional bulky disease, which is the most common form at presentation due to the lack of early symptoms. At present, radiotherapy combined with induction or concurrent chemotherapy results in a 5-year survival of 55-70% in patients with advanced stage disease. However, 40-50% of patients relapse and the treatment-related morbidity and mortality of the current regimens are of major concern.[59,60] It is therefore desirable to develop novel therapies that could improve disease-free survival in relapsed/refractory patients and which might ultimately reduce the incidence of long-term treatment related complications in all patients.

2.4.2 Can T-cells recognize NPC tumor cells?

Multiple factors including EBV exposure, environmental triggers and genetic susceptibility are thought to play a role in the pathogenesis of NPC.[61,62] EBV has been detected in virtually all cases of undifferentiated non-keratinizing NPC and in a proportion of squamous cell NPC.[63] The latter represents a more heterogeneous group of tumors, in which other co-factors such as smoking and HPV contribute to the pathogenic process.[64] Although EBV positive NPC cells lack the expression of the immunodominant EBNA-3 antigens, EBNA-1 is consistently expressed and LMP 1 and 2 are detectable in the majority of tumors.[65-67] Immunohistochemical studies of NPC biopsies have demonstrated expression of MHC class I molecules and TAP-transporters by NPC tumor cells in the majority of cases.[68,69] Processing and presenting endogenously synthesized protein to HLA class I restricted CTL clones by NPC tumor cell lines confirmed the capability of antigen presentation.[70,71] The expression of co-stimulatory molecules and adhesion molecules further contributes to potential interaction with tumor-specific T-cells.[72,73] These observations indicate that NPC cells express viral antigens that can be recognized by the host immune system.

2.4.3 Natural EBV-immunity in NPC patients

If tumor-associated antigens are indeed being presented to immune effector cells, what enables NPC tumors to arise in patients with a functional immune system? T-lymphocytes specific for LMP2 and to a lesser extent for LMP1 are present in the peripheral blood of NPC patients albeit at a lower frequency as compared to healthy donors.[71,74] At the tumor site, large numbers of lymphocytes infiltrate in the malignant tissue. However, T-cells clones expanded from these tumor infiltrating T-cells did not appear to be specific for EBV antigens expressed on the tumor cells.[71] It has been suggested that the T-cell infiltrate, rather than being part of a tumor-specific immune response, supports tumor cell growth by secretion of cytokines.[72,75] The association of the presence of CD8+ T-cells in the tumor with a poor prognosis provides indirect evidence for this.[76] Further, an effective tumor-directed immune response may be suppressed at the tumor site; through expression of fas ligand apoptosis of activated T-cells can be induced upon interaction with the tumor cells [77] and high concentrations of IL-10 may inhibit antigen presentation.[78] Thus, despite the presence of EBV-specific T-cells, NPC patients appear incapable of mounting an effective anti-tumor response.

2.4.5 Immunotherapy for NPC

Amplifying or boosting the pre-existing immune response may overcome these immunosuppressive barriers. In HLA A2/K^b mice, vaccination with an adenoviral vector encoding for multiple LMP1 and LMP2 epitopes was successfully used to reverse the outgrowth of LMP-expressing tumors.[79] In a clinical study, vaccination with DCs loaded with LMP2 peptides induced epitope-specific CD8 T-cell responses in 9 of 16 patients treated. In two patients the increased LMP2-immunity persisted for over 3 months and was accompanied by a partial tumor reduction.[80] These studies demonstrate that tumor-specific T-cell responses can indeed be amplified, but may not be sufficient to elicit a complete clinical response. Adoptive T-cell therapy has been explored in a small pilot study in China. EBV-specific CTL were successfully generated for all 4 patients on study.[81] Administration of the CTL lines was without complications, but did not induce clinical responses possibly due to the large tumor burden in these patients with end-stage disease. Nevertheless, these initial data are encouraging and initiated our further investigation of the feasibility and efficacy of T-cell-based immunotherapy approaches as treatment for this malignancy (Chapters 2, 3 and 4).

3. Broadening the application of T-cell therapy to non-virus associated malignancies

The immunogenicity of EBV-antigens allows for the re-activation and expansion of EBV-specific T-cells using a standardized protocol in almost all EBV-seropositive individuals. However, such immunogenic viral tumor antigens are available for only a small number of other tumors such as HPV16-associated cervical carcinoma. Therefore, the key barrier to using similar methods to target other malignancies is the difficulty of generating large numbers of tumor-specific T-cells when few if any T-cells specific for the target antigen are present in the peripheral blood. An alternative approach for generating tumor-antigen specific T-cell is to redirect their specificity by transduction with tumor-specific TCRs. Thus, T-cells can be engineered to obtain responses that may not be naturally achievable or would require complex strategies for selection and expansion.

3.1 Transfer of tumor-specific TCRs

The α- and β-chain of the TCR can be isolated from a T-cell clone with the desired specificity and subsequently be introduced into other T-cells by retroviral transduction.[82] This strategy has been successfully applied to redirect T-cells to virus- or tumor antigens.[83-87] Adoptive transfer of T-cells redirected by gene transfer can be activated by antigen *in vivo*, home to effector sites and contribute to tumor clearance in a murine model.[88] However, although this strategy seems attractive and the technology has now been available for over 15 years, T-cells transduced with native TCRs have so far not been used in a clinical setting. Obtaining functional expression of the transgenic TCR in a significant number of cells appears the main barrier. In most studies, the TCR α and TCR β chains are expressed from separate vectors. This requires a double transduction and reduces the formation of functional transgenic TCRs. The development of bicistronic vectors that allow expression of both components in equal amounts from the same vector may overcome this problem.[89] Further, transgenic TCR α and β chains appear to cross-pair with native TCR-chains. This not only reduces the expression of transgenic TCRs with the desired specificity but also introduces TCRs with new specificities. As these transduced T-cells are not undergoing the physiological screening and deletion process in the thymus these may include T-cells that are auto-reactive. Methods to prevent cross-pairing including the formation of single chain TCRs or the incorporation of a CD3-component as the intracellular domain are technically difficult.[90,91] Finally, transgenic TCRs, like native TCR, recognize the target antigen in an MHC-restricted fashion. Therefore, TCRs restricted through a range of different HLA alleles need to be isolated to make this approach applicable to a broad patient group. Nevertheless, this approach provides a means to obtain T-cells specific for tumor antigens that otherwise could not readily be obtained and that are expected to have significant anti-tumor activity (e.g. HA-1). Chapter 5 describes our first efforts to overcome the mentioned technical difficulties associated with this approach.

3.2 Transgenic chimeric TCRs render T-cells specific to surface antigens

Conferring antibody-specificity to T-cells allows for application of this same strategy to surface antigens expressed on the tumor (e.g. surface glycoproteins and glycolipids). This requires chimeric TCRs composed of an exodomain that recognizes and binds the target antigen and an endodomain that transmits an activation signal (Figure 3). The exodomain is usually derived from immunoglobulin variable chains and the endodomain most commonly consists of the intracytoplasmic domain of CD3-ς. The lipid bi-layer that naturally divides these extracellular and intracellular components allows linkage of these domains as chimeric proteins without apparently changing their conformation. Multiple chimeric TCRs built from these components or variations thereof have proven functional in vitro studies (reviewed by Pulè et al[92]). Moreover, adoptive transfer of T-lymphocytes transduced with chimeric TCRs have resulted in eradication of tumors in mice.[93] An advantage of this approach compared with the transgenic TCR approach is that antigen recognition in this setting is independent of HLA and can therefore be applied to a large patient group. Its disadvantage is the paucity of molecules that provide sufficient anti-tumor specificity to spare normal tissues.

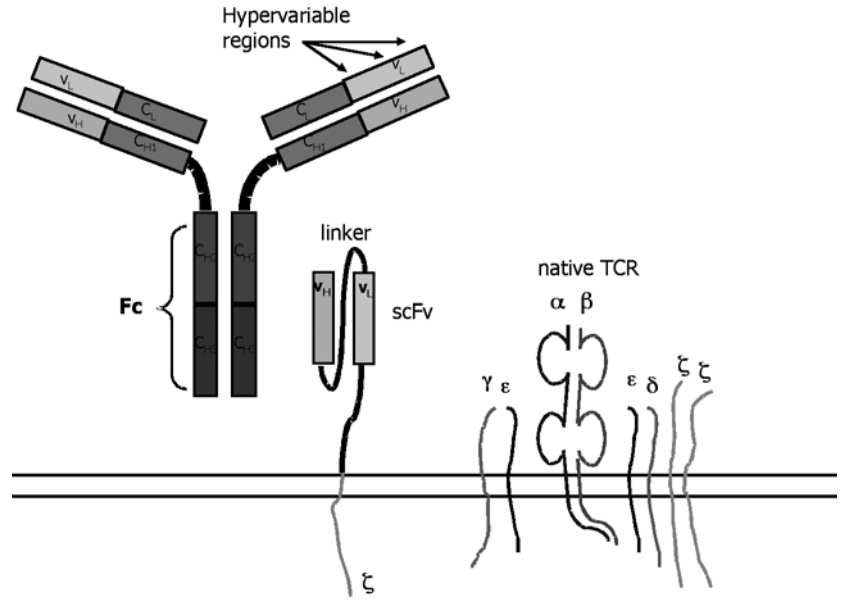

Figure 3. Native and chimeric TCRs for gene transfer
The desired specificity for an intracellular protein derived-epitope or surface antigen can be grafted onto a T-cell by transfer of a native TCR or a chimeric TCR respectively. Transgenic native TCRs, like endogenously expressed TCRs, are TCRα/TCRβ chain heterodimers that form a complex with CD3 γ, δ and ε components in the endoplasmatic reticulum. Subsequently, CD3- ς chains are incorporated and the fully assembled TCR complex is transported to the plasma membrane.[130] A chimeric TCR consist of an exodomain for antigen recognition and an endodomain for signal transduction. In its most common form the exodomain consists of a single chain variable fragment (scFv) derived from the V_H and V_L of an immunoglobulin connected by a flexible linker and the endodomain consists of the transmembrane and intracytoplasmatic part of CD3-ς. The exodomain and endodomain are separated by a spacer that allows different orientations of the scFv to accommodate interaction with the target cell.[92]

3.3 Problems with currently used transgenic TCRs

Administration of T-cells transduced with an HIV envelope-specific TCR transmitting a CD3-ζ signal to HIV patients was the first proof that this approach is feasible in a clinical setting.[94-96] Adoptively transferred T-cells were shown to home to sites of infection and were still detectable in the peripheral blood over one year after treatment. However, the infused T-cells only persisted at low levels and no systemic anti-viral response was induced. Animal studies show that T-cells transduced with a tumor-specific chimeric TCR with CD3-ς as endodomain can lyse tumor cells but fail to expand or secrete physiological amounts of cytokines.[97] This may be explained by the incomplete activation signal to the transduced T-cells upon encounter with the tumor resulting in a trigger for cell lysis but failure to fully activate a proliferative cycle; the transgenic TCR provides a CD3-ς signal but as tumor cells often lack expression of costimulatory molecules additional signals required for optimal T-cell function may be absent. This signaling deficit may be overcome by transducing CTLs specific for endogenous viruses, such as EBV and CMV, rather than unselected lymphocytes to generate bi-specific T-cells. In this case, a background of latent, virus-infected cells may provide antigen receptor stimulation via the native TCRs as well as co-stimulation, promoting a persisting activated state, while co-expression of the transgenic TCR should lead to recognition and killing of tumor cells.[87,98] This concept of bi-specificity to combine proliferation and anti-tumor activity proved effective in a murine study.[99] Alternatively, co-stimulatory domains can be included in the endodomain of the chimeric TCR.[100] Inclusion of CD28 in a prostate carcinoma specific-TCR resulted in proliferation as well as IL-2 production after stimulation with tumor cells. However, the obtained proliferation was only short-term and might indicate that an additional signal is required.[101]

3.4 Can implementation of combined co-stimulation domains improve function?

It has long been acknowledged that for effective T-cell activation both engagement of the TCR (signal 1) and a co-stimulatory receptor (signal 2) is required.[102] However, although signal 2 was initially thought to provide a signal distinct from that initiated by the TCR, it now has become clear that co-stimulation can both enhance TCR activation as well as provide a separate signal to promote cell division, augmen cell survival or induce effector functions.[103,104] Further, co-stimulatory molecules from the immunoglobulin superfamily (such as CD28) and the tumor necrosis receptor family (such as OX40, 4-1BB and CD30) may play a role at different stages of the primary and recall T-cell response.[105] CD28, which is constitutively expressed by naïve T-cells, plays a crucial role in the initial activation phase when the T-cells respond to antigen and clonally expand. The expression of OX40, 4-1BB and CD30 is absent in naïve cells at this initial phase of T-cell activation but peaks at 48 hours after antigen recognition. These molecules then provide signals to prevent activation induced cell death and consequently promote the survival of effector T-cells, resulting in the generation of memory T-cells. According to this model, the absence of TNFR-family member co-signals significantly reduces the number of effector T-cells both during primary and secondary responses.

Tumor cells often lack the expression of these co-stimulatory molecules and create a non-inflammatory environment in which tolerance is induced in tumor-specific T-cells upon encounter with the tumor. These T-cells then fail to expand and enter a state of hypo-responsiveness even if co-stimulatory signals are provided at subsequent antigen encounter.[106] This problem may be overcome by artificially providing co-stimulatory signals.[107] *in vivo* ligation of OX40 in tumor-bearing mice enhanced the anti-tumor effect of immunotherapeutic strategies, leading to tumor-free survival and protection against rechallenge.[108,109] Importantly, this OX40 activation was shown to break pre-existing tolerance and restore normal T-cell functionality.[110] Providing an OX40 signal in addition to CD3-ζ and CD28 within the endodomain of tumor-specific TCR may similarly allow for a more potent and extended anti-tumor response and create the inflammatory environment to overcome hypo-responsiveness of the host T-cells. Experiments to test this hypothesis are described in Chapter 6.

4. Clinical application of genetically modified T-cells requires a safety switch

As a result of newly developed strategies including transgenic expression of tumor-specific TCRs adoptive transfer of antigen-specific cytotoxic T-cells is becoming an attractive treatment strategy for a growing number of malignancies. However, when these cells are genetically modified, e.g. transduced with a tumor-specific artificial TCR or a transgene that enhances their anti-tumor activity, co-expression of a suicide gene may be desired to ensure *in vivo* safety of these cells in clinical trials.

4.1 Currently available suicide genes

Suicide genes that make transduced cells susceptible to a chemotherapeutic agent that is not ordinarily toxic can function as a safety mechanism. A growing list of suicide genes available with the majority being derived from bacteria or viruses (reviewed by Spencer et al[111]). The most commonly used suicide gene is the thymidine kinase gene from herpes simplex virus I (HSV-TK). This enzyme phosphorylates the pro-drug ganciclovir, that is subsequently further activated by cellular kinases and then incorporated into DNA to inhibit DNA synthesis, resulting in death of dividing cells. Donor T-lymphocytes transduced with HSV-TK have been administered to patients either as T-cell add-back post T-cell-depleted SCT or as treatment of relapse of malignancy or EBV-induced lymphoma after SCT.[112,113] In 5 of the 7 patients that developed GVHD in these studies, symptoms resolved after administration of ganciclocir alone. However, the use of ganciclovir (or analogs) as a pro-drug to activate HSV-TK precludes administration of ganciclovir as an anti-viral drug for CMV infections. In addition, HSV-TK-directed immune responses resulted in elimination of HSV-TK transduced cells, even in immunosuppressed HIV and bone marrow transplant patients, compromising the long-lived *in vivo* activity of the infused T-cells.[112,114] Human CD20 has been proposed as a non-immunogenic alternative. Exposure to anti-CD20 antibody, in the presence of complement, rapidly kills of up to 90% of transduced cells.[115] However, this strategy is not selective for transduced T-cells and results in the unwanted loss of normal B-cells for over 6 months.

4.2 Requirements for suicide genes suitable for T-cell therapy

Based on these observed limitations the criteria for the ideal suicide gene that is effective and ensures safety of T-cell-based therapy can be defined. Such a suicide gene must be non-immunogenic, have low basal toxicity to allow for long-term stable expression in transduced T-cells, and its activation should result in the selective and complete elimination of all transduced cells. In the context of a potential therapeutic intervention for GVHD, co-expression of a non-immunogenic selectable marker along with the suicide gene is required to allow for selection of suicide gene-expressing cells. In T-cells transduced with a transgene to enhance their anti-tumor activity or to redirect their specificity, the suicide gene needs to be co-expressed in all genetically modified cells.

4.3 Inducible pro-apoptotic molecules as suicide genes

Suicide genes based on endogenous pro-apoptotic molecules that meet the criteria described above are being developed. Human pro-apoptotic proteins can be linked to modified human FK506-Binding Proteins (FKBP) that contain a binding site for a lipid permeable, chemical inducer of dimerization (AP1903, Ariad Pharmaceuticals).[116] Administration of this dimerizing drug results in the aggregation of two or more chimeric pro-apoptotic molecules, which leads to their activation and thus apoptosis (Figure 4).[117] Activation of

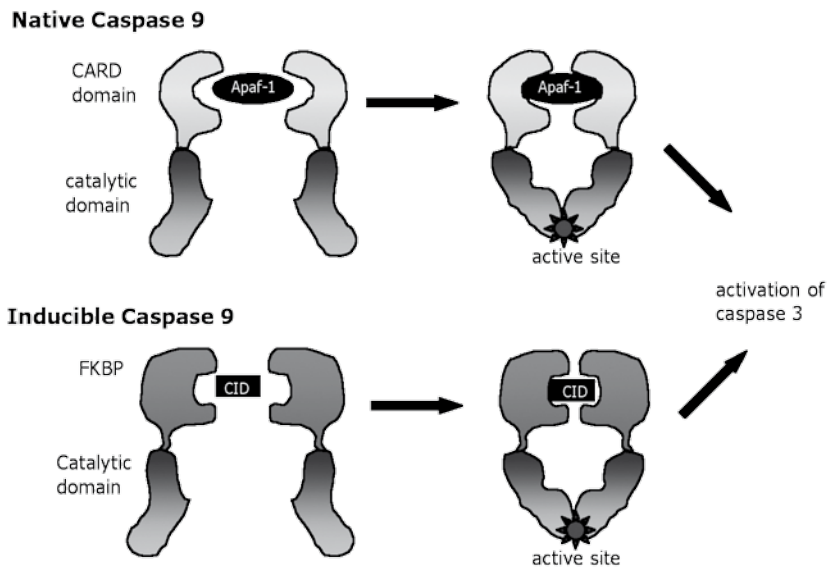

Figure 4. Activation of native and inducible caspase 9
Caspase 9 is the initiator of the intrinsic apoptosis pathway that becomes activated upon exposure of the cell to for example chemotherapeutic agents and ionizing radiation.[131] Following the death trigger mitochondria become selectively permeable and recruit cytochrome c and caspase 9 molecules. Interaction of the apoptotic protease activating factor (Apaf)-1 with the caspase-activation-and-recruitment domain (CARD) of caspase 9 results in dimerization of two caspase 9 molecules.[132] Through this conformational change an active site is formed in the catalytic domain of the caspase molecule. Caspase 9 subsequently activates effector caspase 3, which further induces the signaling cascade leading to apoptosis. In its inducible from, an FK506 Binding Protein (FKBP) that contains a binding site for a chemical inducer of dimerization (CID) replaces the CARD domain. Administration of CID, a small molecule drug, results in dimerization and thereby activation of two inducible caspase 9 molecules.[117] Thus in inducible caspase 9 one mediator of dimerization (CARD) in substituted by another (FKBP).

an inducible fas molecule resulted in elimination of 90% of T-cells transduced with this suicide gene.[118,119] This strategy is attractive for application in T-cell therapy as inducible fas is based on self-proteins and should be minimally or non-immunogenic.[120] Moreover, its inducer, AP1903, selectively eliminates those cells expressing the inducible death molecule without toxicity to other cells.[121] However, as complete elimination of transduced cells has not been obtained using inducible fas (iFas), further optimization of this approach is required. Malignantly transformed T-cells as well as normal T-cells destined to enter the memory compartment overexpress anti-apoptotic molecules that may interfere with the function of inducible death molecules, such as iFas, that act as initiators of the apoptosis pathway.[122,123] Inducible caspase molecules that act downstream of fas may potentially be more potent artificial death switches if they act distal to anti-apoptotic molecules such as c-FLIP and Bcl-2. Chapter 7 describes the development of a suicide gene that meets these desired characteristics.

5. Scope and outline of this thesis

The aim of the studies described in this thesis is to extend the scope of adoptive T-cell therapy to less immunogenic tumors and to harness the T-cells for adoptive transfer with the potential to eradicate tumor cells.
Chapter 2 describes the feasibility of generating autologous EBV-specific CTL lines for patients with advanced NPC. The specificity of LCL-reactivated CTL lines toward LMP2, one of the target tumor antigens, is characterized using a peptide library. New LMP2 epitopes are identified, mainly for HLA-alleles for which no epitopes were previously available. This extended panel of LMP2-epitopes is subsequently used to determine the breadth of LMP2-specificity in the infusion product and to monitor the fate of the tumor-specific T-cells *in vivo* after adoptive transfer. The safety and efficacy of autologous EBV-specific T-cells as treatment for advanced NPC is then studied in a phase I clinical trial.
Chapter 3 describes the clinical, virological and immunological response of the patients treated on this study. Although remissions were induced in a number of patients treated, responses were not induced in all patients and were not always sustained. We hypothesized that increasing the number of tumor-specific T-cells by selectively expanding T-cells specific for LMP2 may improve the efficacy of adoptive T-cell therapy for LMP2-positive tumors.
Chapter 4 describes the development of a clinically feasible protocol to reactivate and expand LMP2-specific CTL from PBMC using DCs and LCLs overexpressing LMP2 as APC.
We next aimed to apply adoptive T-cell therapy to non-virus associated malignancies that express target antigens for which the natural T-cell precursor frequency is low or absent. Redirecting T-cell specificity by TCR transfer provides and alternative method to obtain tumor-specific T-cells in this setting. However, although an elegant principle, for transfer of native TCRs extensive sorting and selection procedures are required to obtain functional expression of the transgenic TCR in a significant percentage of lymphocytes. In **chapter 5** we describe strategies we have explored to address this problem in an attempt to develop a protocol to generate HA-1 specific T-cells that can be translated into a clinical trial.
Chimeric TCRs that engraft antibody specificity onto T-cells allow for application of this same strategy to surface antigens expressed by tumor cells. However, it has now become clear that activation through conventional chimeric TCR that just contain CD3-ζ as endodomain may be insufficient for full T-cell activation in the absence of costimulatory molecules on the tumor cells. We hypothesized that incorporating essential expansion and survival

signals in the endodomain of the TCR can circumvent this problem. **Chapter 6** describes the functional comparison of T-cells transduced with a tumor-specific chimeric TCR with endodomains consisting of CD3-ς, CD3-ς combined with CD28 or OX40, and a combination of all 3 molecules.
Clinical use of T-cells genetically modified to redirect their specificity or to otherwise enhance their function likely requires a safety switch that allows for elimination of the infused *in vivo* in the event toxicity occurs. Currently available suicide genes are not feasible for adoptive T-cell therapy because of their immunogenicity or incomplete function in all T-cell populations. **Chapter 7** describes the optimization of an inducible caspase 9 molecule for use in T-cells and its efficacy in eliminating transduced T-cells in vitro and *in vivo*.
Chapter 8 summarizes the results of the above studies and the conclusions that can be drawn from these and discusses other areas that in my opinion require attention to move the field of adoptive T-cell therapy forward.

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Chapter 2

Characterization of Latent Membrane Protein 2-Specificity in CTL lines from Patients with EBV-Positive Nasopharyngeal Carcinoma and Lymphoma

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Abstract

Viral proteins expressed by EBV-associated tumors provide target antigens for immunotherapy. Adoptive T-cell therapy has proven effective for post transplant EBV-associated lymphoma in which all EBV latent antigens are expressed (type III latency). Application of immunotherapeutic strategies to tumors such as nasopharyngeal carcinoma (NPC) and Hodgkin's lymphoma (HL) that have a restricted pattern of EBV antigen expression (type II latency) is under investigation. Potential EBV antigen targets for T-cell therapy expressed by these tumors include latent membrane proteins (LMP) 1 and 2. A broad panel of epitopes must be identified from these target antigens to optimize vaccination strategies and facilitate monitoring of tumor-specific T-cell populations after immunotherapeutic interventions. So far, LMP2 epitopes have been identified for only a limited number of HLA alleles. Using a peptide library spanning the entire LMP2 sequence, 25 CTL lines from patients with EBV-positive malignancies expressing type II latency were screened for the presence of LMP2-specific T-cell populations. In 21 of 25 lines, T-cell responses against 1-5 LMP2 epitopes were identified. These included responses to previously described epitopes as well as to newly identified HLA-A*0206, A*0204/17, A29, A68, B*1402, B27, B*3501, B53 and HLA-DR restricted epitopes. Seven of the 9 newly identified epitopes were antigenically conserved among virus isolates from NPC tumors. These new LMP2-epitopes broaden the diversity of HLA alleles with available epitopes, and in particular those epitopes conserved between EBV strains provide valuable tools for immunotherapy and immune monitoring.

Introduction

Virtually all undifferentiated NPC, up to 40% of HL and 20-100% of non-Hodgkin lymphomas (NHL) depending on subtype and localization are associated with EBV.[1-4] Regardless of the role of EBV in the pathogenesis of these malignancies the viral antigens expressed by tumor cells provide target antigens for immunotherapeutic strategies. Adoptive transfer of EBV-specific T-cells has proven an effective strategy to prevent and treat EBV-associated lymphomas that arise in immunocompromised patients.[5,6] These lymphomas however are highly immunogenic as they express all latent EBV proteins including the nuclear antigens EBNAs -1, -2, -3A, -3B, -3C and –LP and the membrane proteins LMP1 and 2 (type III latency). In contrast, NPC, HL, and NHL tumors do not express the immunodominant EBNA3 antigens and have an EBV antigen expression pattern restricted to EBNA1, LMP1 and LMP2 (type II latency).

EBNA1, essential for maintaining the latent genome in dividing cells, is present in all EBV-positive malignancies. However, although antigen processing and presentation has been reported for EBNA1 proteins truncated during translation,[7-9] an internal glycine-alanine repeat prevents processing of the full-length protein and thereby inhibits its presentation to CD8+ T-cells.[10-12] LMP1 and LMP2 proteins are present in the majority of EBV-associated NPC and HL tumors and although subdominant antigens, they provide targets for immunotherapeutic approaches.[3,13-15]

LMP2 and to a lesser extent LMP1-specific T-cells are present in the peripheral blood of patients with EBV-positive HL and NPC.[16,17] This implies that these malignancies are able to develop despite the presence of circulating tumor-specific T-cells. Secretion of immunosuppressive chemokines and cytokines and the presence of regulatory T-cells at the tumor site may contribute to this escape from immune surveillance.[18-20] Immunotherapeutic strategies that enhance the LMP-specific immune response may overcome this immunosuppressive environment. LMP-specific T-cells can be actively boosted by vaccination with LMP-peptides.[21,22] Alternatively, LMP-specific T-cells can be removed from the immunosuppressive environment and expanded *ex vivo*. EBV-transformed B-cell lines (LCL) provide an excellent source of APC for this purpose and are readily generated from patients with EBV-positive tumors.[23-25] However, a major concern was whether LMP2-specific T-cells could be reactivated and expanded using LCL_s, in which all other latency proteins including the immunodominant EBNA3 antigens are expressed.[17,26]

To further develop and implement such immunotherapeutic strategies detailed characterization of LMP-specific T-cell immunity is required. So far LMP2 epitopes presented in the context of HLA-A2, A11, A23, A24, A25, B27, B60 and B63 have been described.[27-33] Expansion of this panel of LMP2 epitopes is desirable for multiple reasons. First, this will enable peptide or epitope-based vaccination strategies for all patients regardless of their HLA type. Second, vaccination with multiple rather than a single epitope is desirable to prevent tumor escape as a result of mutation or strain-specific epitope variation.[34] Third, a broad panel of LMP2 epitopes provides useful reagents such as tetramers and peptides that can be used for detailed characterization of *ex-vivo* expanded LMP2-specific T-cells for adoptive transfer as well as for monitoring of LMP2-directed immune responses following therapeutic intervention.

We were successful in generating EBV-specific CTL lines from 25 patients with type II tumors using LCL as APC.[25,35] Here we report the detailed epitope specificities of LMP2-directed immune responses in these patient CTL lines using a peptide library that spans the entire LMP2 protein. Using this strategy we identified 9 new LMP2-derived HLA class I and class II-restricted epitopes and we demonstrate the utility of these LMP2-eptiopes as reagents for immunological monitoring.

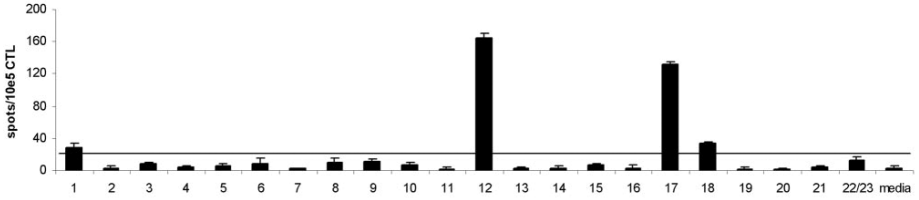
Methods

Patients and EBV status of the tumors
The Baylor College of Medicine Institutional Review Board and the Food and Drug Administration approved the generation of EBV-specific CTL lines for use in clinical studies in patients with EBV-positive NPC, HL and NHL.[25,35] In all patients, tumor samples had been established as EBV-positive, using immunohistochemistry for LMP-1 and/or in situ hybridization for the small non polyadenylated viral RNA EBER1.[23]

Patient CTL lines
EBV-specific CTL lines were reactivated and expanded from PBMC using autologous LCL as APC, as described previously.[36] Briefly, after informed consent, peripheral blood (40-60 ml) was collected from patients with EBV-positive NPC, HL and NHL. First, $5x10^6$ PBMC were incubated with concentrated supernatants from the EBV producer cell line B95-8, and cultured in RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% FBS (Hyclone, Logan, UT) and 200 mM Glutamine (Gibco, Grand Island NY), in the presence of 1 µg/ml cyclosporin A (Sandoz, Vienna, Austria) to establish an LCL. Subsequently, PBMC (2×10^6 per well of a 24 well plate) were stimulated with LCL irradiated (40 Gy) at a responder:stimulator ratio (R:S) of 40:1 in 50%RPMI/50% Clicks media (Irvine, Santa Ana, CA) supplemented with 10% FBS and 200 mM Glutamine. After 9-12 days, viable cells were restimulated with irradiated LCL at a R:S ratio of 4:1 and subsequently further expanded by weekly stimulations with LCL in the presence of recombinant human interleukin-2 (rhIL-2, Proleukin, Chiron Corporation, Emeryville, CA) (40-100 U/ml).

LMP2-peptides
A peptide library consisting of 122 15-mer peptides with 11 amino acids overlap covering the complete sequence of LMP2a (B95-8 strain, swiss prot access P13285) was purchased from Dieter Stoll at Natural and Medical Sciences Institute, University of Tuebingen, Germany. Lyophilized peptides were reconstituted at 20 mg/ml in DMSO. As described previously, these peptides were pooled in a total of 23 pools in such a manner that each 15-mer peptide was represented in 2 pools according to the grid shown in Figure 1b.[37] Single 15-mer peptides were aliquoted at 8 mg/ml. To determine the minimal recognized LMP2 epitope sequence additional peptides, varying in length from 9-14 amino acids, were obtained from Genemed Synthesis Inc. (South San Francisco, CA) and reconstituted at 10 mg/ml in DMSO. Aliquots of peptides were stored at -80°C.

A



B

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13	1	2	3	4	5	6	7	8	9	10	11	12
14	13	14	15	16	17	18	19	20	21	22	23	24
15	25	26	27	28	29	30	31	32	33	34	35	36
16	37	38	39	40	41	42	43	44	45	46	47	48
17	49	50	51	52	53	54	55	56	57	58	59	60
18	61	62	63	64	65	66	67	68	69	70	71	72
19	73	74	75	76	77	78	79	80	81	82	83	84
20	85	86	87	88	89	90	91	92	93	94	95	96
21	97	98	99	100	101	102	103	104	105	106	107	108
22	109	110	111	112	113	114	115	116	117	118	119	120
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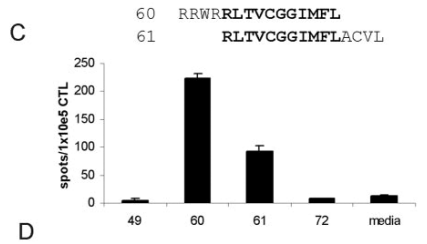
C



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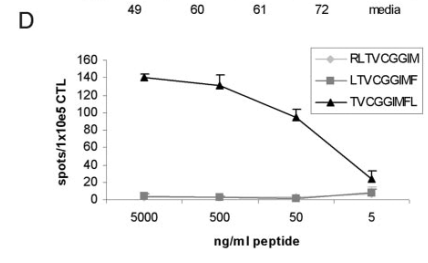

Figure 1. Identification of LMP2 epitopes using an LMP2-peptide library
(a) *LCL-reactivated CTLs (1x10^5/well) from a patient with NPC (HLA-A*0206, A24, B51, B61) were stimulated with an LMP2-peptide library pooled into 23 pools. Responses were measured in an 18-hour IFN-γ ELISPOT assay. Shown is mean and standard deviation of duplicate wells. The black horizontal line shows the threshold level used to determine significance (5x number of SFC/1x10^5 unstimulated CTL).* **(b)** *All peptides were divided into 23 pools in such a way that each peptide is present in 2 pools. This method allows determining those single peptides that likely induced responses to the peptide pools. Thus, responses to pools 1 and 17, 1 and 18, 12 and 17, and 12 and 18 may be induced by single peptides 49, 61, 60 and 72, respectively.* **(c)** *Testing of these individual pentadecamers identifies the sequence of peptides 60 and 61, most likely the overlapping 11 amino acids, as the CTL epitope.* **(d)** *Testing of the 3 potential nonamers within this sequence at indicated concentrations identifies the minimum recognized epitope.*

Enzyme-Linked Immunospot (ELISPOT) assay

96-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated overnight with 10 mg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH). CTL were plated at 1x10^5 cells/well and stimulated with LMP2 peptide pools (1 μg/mL of each peptide) or individual peptides (5-5000 ng/mL as indicated). Irradiated (40 Gy) autologous LCL were used as positive control. After 18-24 hours, the plates were washed and incubated with the secondary biotin conjugated anti-IFN-γ monoclonal antibody (Detector-mAb (7-B6-1-Biotin), Mabtech). After incubation with Avidin:biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit (Standard), #PK6100, Vector Laboratories, Burlingame, CA) plates were developed with AEC substrate (Sigma, St. Louis, MO). Plates were sent for evaluation to Zellnet Consulting, New York, NY. Results are shown as spot forming cells (SFC) per 1x10^5 CTL. For the screening with LMP2 peptide pools, all assays were performed once in duplicate. Prior to using this method as screening for patient CTL lines, the reproducibility of this method was first confirmed using CTL lines from 2 healthy donors (data not shown). Those responses that exceeded 5 x times the background level of non-stimulated CTL and were at least 5 SFC/1x10^5 CTL were regarded as significant. For 2 of 25 CTL lines screened this threshold level was lowered to 1x background level. The relevance of the identified LMP2-specific responses was subsequently confirmed in ELISPOT assays using single LMP2 peptides again using a threshold level of 5 x background and > 5 SFC/1x10^5. Responses to the identified LMP2 epitopes were consistently detected in the CTL lines studied, however, the strength of these responses varied between assays. The average response to each epitope within the same CTL line is reported in Table I.

Determining HLA-restriction of identified epitopes

To determine the HLA-restriction of the novel LMP2 class I peptides, CTL with specificity for the LMP2 peptide, were plated at 1x10^5/well in an IFN-γ ELISPOT assay with partially HLA-matched phytohemagglutinin (PHA)-activated lymphoblasts (40Gy irradiated) used as antigen presenting cells either alone or pulsed with peptide (10 ug/ml for 30 minutes at 37°C). All immunogenic peptides were analyzed for the presence of anchor sites for HLA-alleles expressed by the patient using prediction databases from Dr. Kenneth Parker, National Institute of Allergy and Infectious Diseases, NIH *(http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html_112601)* and Dr. Hans-Georg Rammensee, Heidelberg, Germany *(www.syfpeithi.de)* and the HLA FactsBook.[38] For 7 out of 9 epitopes the HLA-restriction was subsequently confirmed by staining with the HLA tetramer derived from the newly identifed epitope. To confirm HLA-class II restriction of identified epitopes, T-cells were stained with CD4 and CD8 mAbs (BD Biosciences, San Jose, CA) and sorted on a MoFlow Cytometer and subsequently used in an IFN-γ ELISPOT assay. HLA-DR restriction was confirmed by incubating the T-cells for 30 minutes at 37°C with HLA-DR blocking antibody (1 μg/well) before addition of the peptide.

Tetramer staining

Tetramers were prepared by the National Institute of Allergy and Infectious Diseases tetramer core facility (Atlanta, GA), or by the Baylor College of Medicine Tetramer Core Facility (Houston, TX). CTL or PBMC (5-10x10^5) were incubated at room temperature for 30 minutes in PBS/1% FCS containing the PE-labeled tetrameric complex. Samples were co-stained with anti-CD8 FITC and anti-CD3 PerCP. Appropriate isotype controls were included. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. For each sample, a minimum of 100,000 cells was analyzed using a FACS Calibur with the Cell Quest Software (Becton Dickinson).

Table I. LMP2-specific T-cell populations in patient CTL lines

Minimum Epitope	Amino Acids	HLA Restriction	No. Responding/ No. Tested	SFC/105 CTL (range)
CLGGLLTMV	416–434	A*0201/06/07/09	4/12	84 (19 –236)
GLGTLGAAI	293–301	A2	1/12	459
LTAGFLIFL	453-461	A2	0/12	
FLYALALLL	356-364	A*0201	7/12	381 (7-1990)
LIVDAVLQL	**257-265**	**A*0204 or A*0217**	**1/12**	**651**
TVCGGIMFL	**243**	**A*0101/06**	**2/12**	**198 (175–222)**
LLWTLVVL	329-337	A*0101	2/12	19 (14-24)
FTASVSTVV	**144-152**	**A68**	**2/6**	**38 (23-53**
SSCSSCPLSKI	340-350	A11	3/3	43 (8-90)
TYGPVFMSL	419-427	A24	2/5	58 (16-101)
PYLFWLAAI	131-139	A23/24	3/6	462 (12-1132)
ILLARLFLY	**349-358**	**A29**	**1/2**	**6**
RRWRRLTVC	**237-245**	**B*1402**	**1/1**	**41**
RRRWRRLTV	236-244	B*2704/05/09	1/2	16
RRLTVCGGIMF	**240-250**	**B27**	**1/3**	**129**
MGSLEMVPM	**1-9**	**B*3501**	**1/5**	**880**
LPVIVAPYL	**125-133**	**B53**	**1/2**	**5**
IEDPPFNSL	200-208	B60	1/2	918
DYQPLGTQDQSLYLG	**73-87**	**DR4 or DR16**	**1/1**	**57**

Listed are the amino acid sequence of newly identified (in bold) as well as previously described LMP2-epitopes [27,29-33], their location in the LMP2 molecule, HLA-restriction, the number of CTL lines from NPC, HL and NHL patients in which responses to these epitopes were identified and the strength of these responses. When responses to the indicated epitope were found in more than one patient CTL line average response and range are shown.

Results

LMP2-specific T-cells within patient CTL lines

Using LCL as APC, EBV-specific T-cells were reactivated and expanded from the peripheral blood of 25 patients with EBV type II latency tumors: 13 patients with NPC, 10 patients with HL and 2 patients with NHL. The presence of LMP2-specific and thus tumor-specific T-cells within these patient CTL lines was assessed using a peptide library representing the entire LMP2a sequence (B95-8 strain). In 21 of 25 patient CTL lines LMP2-specific T-cells were detectable in an IFN-γ ELISPOT assay after overnight incubation with each of the peptide pools. This result demonstrates that T-cells specific for a subdominant EBV-antigen can regularly be reactivated using LCL even in patients. An example of one CTL line is shown in Figure 1a: T-cells that produced above background levels of IFN-g were detectable after stimulation with pools 1, 12, 17 and 18. In two patient CTL lines the spontaneous IFN-γ secretion of non-stimulated T-cells resulted in a signal to noise ratio that was too high to detect LMP2-specific T-cell responses. In two other patient CTL lines none of the peptide pools induced IFN-γ secretion of the T-cells, although incubation with LCL resulted in a measurable response.

Determining minimal recognized LMP2 sequence

Following the initial screening with each of the LMP2-peptide pools the minimal recognized T-cell epitopes were identified. Based on the LMP2-peptide pools that induced IFN-γ secretion, individual pentadecamers that were present in 2 of the peptide pools that tested positive were selected: for example pentadecamers 49, 60, 61 and 72 for the patient CTL line shown as example (Figure 1b). Stimulation of the T-cells with these single pentadecamers showed that the amino acid sequence of LMP2 that contained the recognized epitope was present in pentadecamers 60 and 61, most likely in the overlapping 11-amino acid sequence of these two adjacent peptides (Figure 1c). In total, 35 LMP2-specific T-cell responses were detected, 24 of which were targeted towards LMP2 sequences representing previously described LMP2 epitopes including FLYALALLL, SSCSSCPLSKI, CLGGLLTMV, IEDPPFNSL, LLWTLVVLL, PYLFWLAAI, TYGPVFMSL, GLGTLGAAI, and RRRWRRLTV [27,29,31-33] (Table I). In 11 CTL lines, the recognized LMP2 sequence did not contain a known LMP2 epitope. The minimal recognized epitope was then identified by testing of the potential nonamers within the overlapping sequence of two adjacent pentadecamers to which responses were detected. For example, within the RLTVCGGIMFL sequence TVCGGIMFL was shown to represent the minimum recognized epitope, whereas RLTVCGGIM and LTVCGGIMF were not recognized by the CTL (Figure 1d). Using this strategy 7 nonamers representing new LMP2 epitopes were identified (Table I, epitopes in bold). Two of the epitopes identified here (LPVIVAPYL and FTASVSTVV) represent the minimum epitope within regions of LMP2 earlier reported as CD8+ T-cells recognition sites in PBMC of healthy donors.[33] For one epitope, RRLTVCGGIMF (aa 240-250) the minimal recognized sequence consisted of 11 amino acids rather than 9 amino acids, analogous to a previously described LMP2-epitope SSCSSCPLSKI (aa 340-350). Interestingly, this epitope is located within a region that contains four overlapping CD8-epitopes VLVMLVLLILAYRRRWRRLTVCGGIMFL ,VLVMLVLLILAYRRRWRRLTVCGGIMFL, VLVMLVLLILAYRRRWRRLTVCGGIMFL and VLVMLVLLILAYRRRWRRLTVCGGIMFL and one CD4-epitope VLVMLVLLILAYRRRRWRRLTVCGGIMFL.[39] Similarly, ILLARLFLY is located in an epitope hotspot: SSCSSCPLSKILLARLFLYALALLL, SSCSSCPLSKILLARLFLYALALLL and SSCSSCPLSKILLARLFLYALALLL.

HLA-restriction of identified CD8-epitopes

To determine the HLA-restriction of the identified LMP2-epitopes we took advantage of described peptide binding motifs (see: Methods). The HLA type of the patient used as an example is as follows: A*0206, A24, B51, B61 (Figure 2a). The identified epitope TVCGGIMFL contains a valine at position 2 and a leucine at position 9, which are anchor residues predicted to bind to HLA-A*0206. We subsequently confirmed this HLA-restriction by using partially HLA-matched phytohemagglutinin-activated lymphoblasts pulsed with this LMP2-peptide as APC in an ELISPOT assay (Figure 2a). T-cells secreted IFN-γ upon stimulation with all peptide-pulsed APC matched for the HLA-A2 allele, but not after stimulation with peptide-pulsed APC matched for HLA-A24. As no HLA-A*0206 matched APC were available, A*0201 typed APC were used in this experiment. Comparing IFN-γ secretion upon stimulation with autologous (A*0206) and A*0201-typed APC pulsed with different concentrations of the TVCGGIMFL peptide demonstrates that although less efficient this epitope can also be presented in the context of HLA-A*0201 (Figure 2b). HLA-A2-restriction was further confirmed by the identification of a T-cell population staining positive with HLA-A*0201-TVCGGIMFL tetramer (Figure 2c). Using this same strategy the other newly identified LMP2-epitopes were found to be HLA-A*0204 or A*0217, HLA-A29, HLA-A68, HLA-B*1402, HLA-B27, HLA-B*3501 and HLA-B53 restricted (Table I).

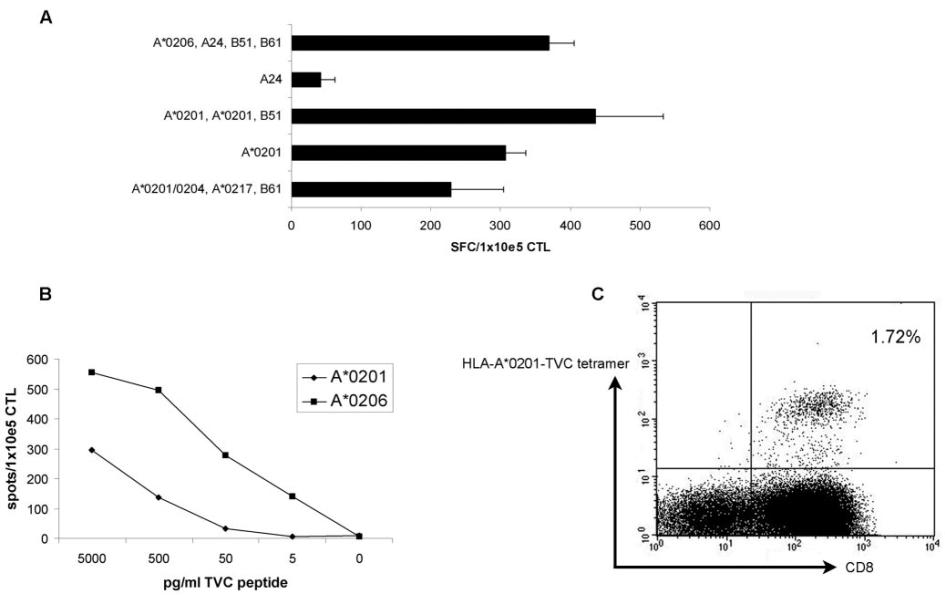

Figure 2. LMP2 epitope TVCGGIMFL is HLA-A*201/06 restricted

(a) *To determine the HLA-restriction of the TVCGGIMFL peptide, T-cells (1x10⁵/well) with specificity for the TVCGGIMFL peptide were stimulated with phytohemagglutinin (PHA)- activated lymphoblasts from donors with the indicated HLA-types with and without peptide (1x10⁵/well). Shown is the mean and standard deviation of the response to peptide-loaded PHA blast after subtraction of the response to unloaded PHA blasts from the same donor.* **(b)** *To determine if this epitope was HLA-A2 subtype-specific, HLA-A*0201 and HLA-A*0206-positive PHA-lymphoblasts were pulsed with indicated amounts of TVCGGIMFL peptide and used as APC in an IFN-γ ELSIPOT with CTL as effectors.* **(c)** *The polyclonal EBV-specific CTL line in which this epitope had been identified was stained with an HLA-A*0201 TVCGGIMFL tetramer. Indicated is the percentage of tetramer positive cells within the CD8+ population.*

Identification of a CD4-epitope

Although the LMP2 peptide library used in this study was designed to identify HLA class I restricted epitopes with a length of 9-11 amino acids, an HLA class II restricted epitope was identified in one of the patient CTL lines. T-cells present in this CTL line recognized LMP2-sequence DYQPLGTQDQSLYLG (aa 73-87) but none of the shorter (9-14 aa) peptides derived form this pentadecamer (data not shown). Therefore, the pentadecamer appeared to represent the minimum recognized epitope. As the binding groove of MHC class II molecules can accommodate peptides with a length of up to 20 amino acids we reasoned that this LMP2-peptide may be recognized in the context of HLA class II. Indeed, separation of CD4+ and CD8+ T-cells within this polyclonal CTL line demonstrated that this peptide induces a CD4-mediated T-cell response, whereas no CD8+ T-cells were activated (Figure 3a). The recognized pentadecamer contains anchor residues that are predicted for binding to HLA-DR4 (DYQPLGTQDQSLYLG) one of the HLA class II alleles of this patient (HLA-DR4/16, DQ5/7, DP not done). Complete abrogation of peptide recognition in the presence of an HLA-DR blocking antibody confirmed this predicted HLA-DR restriction (Figure 3b). Other class II epitopes may have been missed in this study because of the design of the peptide library, which for detection of class II epitopes, should optimally be composed of overlapping 20-mers [39].

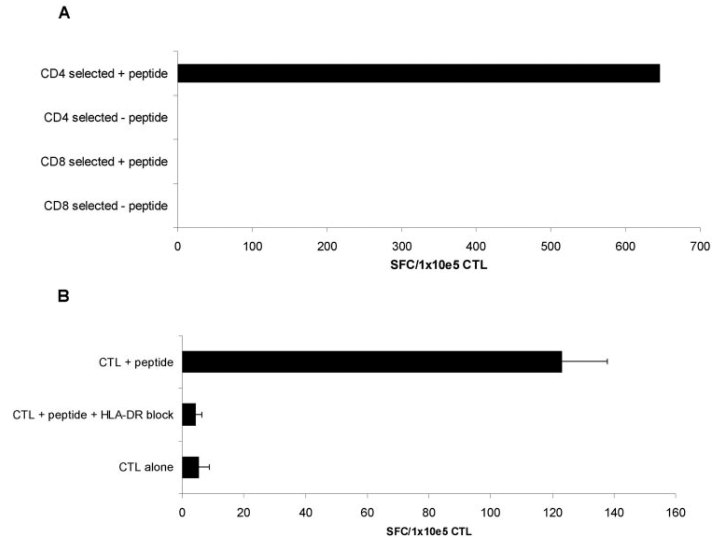

Figure 3. Identification of an HLA-DR restricted LMP2 epitope

(a) *The polyclonal CTL line in which a T-cell population specific for LMP2 peptide DYQPLGTQDQSLYLG was identified was sorted for CD4+ and CD8+ T-cells. Subsequently recognition of this epitope by these separated CD4+ and CD8+ T-cells was determined in an IFN-γ ELISPOT assay.* **(b)** *As this epitope contained anchor sites compatible with HLA-DR4, the HLA-restriction of these epitopes was further characterized by blocking the HLA-DR molecules on the APCs prior to loading with the peptide. EBV-specific CTL from this patient were either non-stimulated or stimulated with APC loaded with peptide with and without prior HLA-DR blocking.*

LMP2-epitopes partially conserved in NPC tumors

The LMP2 peptide library used in this study is based on the prototype EBV type I strain B95-8. However, different EBV strains may be present in the tumor depending on the geographical origin of the patient .[40] For these newly identified LMP2 epitopes to be useful for immunotherapy their sequence must be conserved between the B95-8 strain and the EBV strain present in

the tumor. We compared the amino acid sequence of the newly identified LMP2 epitopes with described LMP2 sequences in EBV isolates from NPC cell lines and biopsy samples [41]. Six of the 9 epitopes were fully conserved, whereas in 3 epitopes one or two mutations were present (Table II). Analysis of the immunogenicity of these variant epitopes with those derived from the B95-8 sequence shows that recognition of the CD8-restricted epitopes LPVIVAPYL and MGSLEMVPM is disrupted when indicated amino acids are altered (Figure 4a-c).
The altered amino acids likely compromise HLA binding (e.g. proline > leucine mutation at an anchor site for B53 binding of the LPVIVAPYL epitope) or TCR recognition of these epitopes. T-cell responses to variants of the CD4-restricted epitope DYQPLGTQDQSLYLG, although reduced in number, appear to be preserved possibly because MHC class II restricted epitopes are often promiscuous in their binding to HLA molecules (Figure 4c). Using those LMP2-epitopes that are conserved between viral isolates for immunotherapeutic strategies is preferred so as to allow for their application in large patient groups worldwide.

Assessing the breadth of the LMP2 response

EBV-specific CTL reactivated and expanded using LCL as APC contain both CD4+ (mean 7.4%, range 0.1-50.0%) and CD8+ (mean 83.8%, range 39.4-98.8%) T-cells that can potentially recognize multiple LMP2-derived epitopes. Screening the patient CTL lines with the LMP2 peptide pools allows for assessment of the breadth of the LMP2-directed specificity. In 12 CTL lines, detectable LMP2 reactivity was directed against a single epitope, whereas in 9 CTL lines T-cell responses against 2-5 LMP2 epitopes were present. (Table III) In two CTL lines that were known to contain a FLYALALLL-specific T-cell population as determined by tetramer staining, no IFN-γ secreting cells were detected upon stimulation with peptide pools that contained a pentadecamer representing the FLYALALLL sequence (data not shown). This observation suggests that screening with peptide pools may underestimate the true breath

Table II. Comparison of LMP2 epitope sequence in B95-8 and virus isolates from NPC tumor (cell lines)

	Strain	Origin	aa 125–133	aa 144–152	aa 237–251	aa 257–265	aa 349–358	aa 1–9	aa 73–87
Cell line/ isolate									
B95-8			LPVIVAPYL	FTASVSTVV	RRWRRLTVCGGIMFL	LIVDAVLQL	ILLARFLY	MGSLEMVPM	DYQPLGTQDQSLYLG
C15	1	Morocco	L**L**VIVAPYL	FTASVSTVV	RRWRRLTVCGGIMFL	LIVDAVLQL	ILLARFLY	MGSLE**VM**PM	DYQPLG**N**QD**P**SLYLG
C18	1	Algeria						MGSLEM**M**PM	DYQPLG**N**QD**P**SLYLG
N10	1	Egypt						MGSLEM**L**PM	DYQPLG**N**QD**P**SLYLG
C19	1	Italy						MGSLEM**M**PM	DYQPLG**N**QD**P**SLYLG
BAT	Ch2	U.S.						MGSLEM**M**PM	DYQPLG**N**QD**P**SLYLG
LIV	Ch2	Alaska						MGSLEMVPM	DYQPLGTQD**P**SLYLG
L2	2	China						MGSLEMVPM	DYQPLG**N**QD**P**SLYLG
D3	Ch1	China						MGSLEMVPM	DYQPLGTQDQSLYLG
D6	Ch1	China						MGSLEMVPM	DYQPLGTQD**P**SLYLG
Si	Ch1	Malaysia						MGSLEM**L**PM	DYQPLG**N**QD**P**SLYLG
Chu	Ch1	Malaysia						MGSLEMVPM	DYQPLGTQDQSLYLG

Amino acid sequences of the identified LMP2 epitopes as derived from the reference B95-8 strain were compared to the previously described sequences of three NPC tumor cell lines (C15, C18 and C19) as well as 8 fresh EBV isolates from NPC tumors.[41] Variations

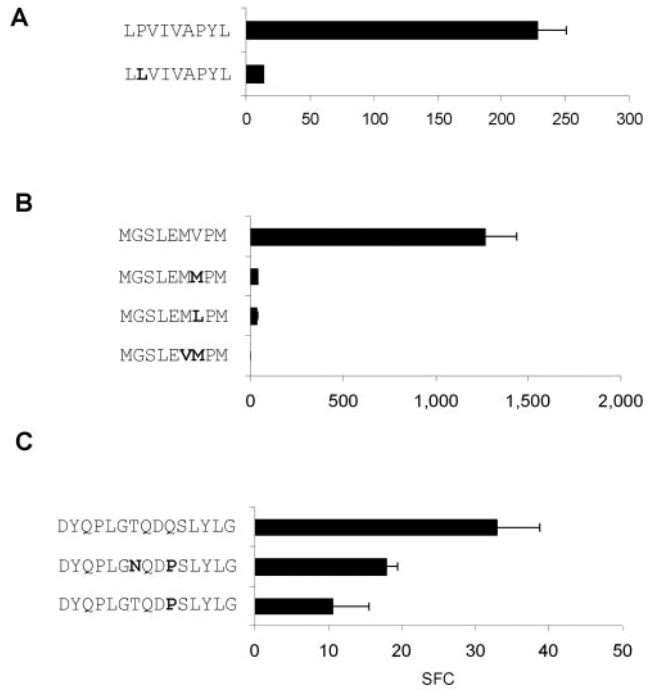

Figure 4. CTL recognition of LMP2 epitopes with altered amino acid sequences

CTL (1x10⁵/well) were stimulated with the B95-8 derived LMP2 epitopes **(a)** *LPVIVAPYL,* **(b)** *MGSLEMVPM and* **(c)** *DYQPLGTQDQSLYLG as well as altered versions as identified in non-B95-8 EBV strains (see: Table II) and responses were measured in an IFN-γ ELISPOT assay. Average and standard deviation of CTL stimulated with 500 ng/ml peptide are shown.*

of the LMP2 response in some cases. An example of a CTL line containing broad LMP2 specificity is shown in Figure 5. Initial screening with the LMP2 peptide pools indicated recognition of multiple LMP2 sequences (Figure 5a). Subsequently these responses were mapped to four LMP2-epitopes: the earlier described HLA-A2-restricted FLYALALLL and LLWTLVVL, and the HLA-B27 restricted RRRWRRLTV epitopes and the newly identified HLA-A29-restricted ILLARLFLY epitope (Figure 5b). The presence of T-cells recognizing multiple epitopes is desirable as this reduces the risk of immune escape by the tumor, and of strain sequence variations.

Table III. Patient Characteristics and Responses to CTL Therapy

Patient ID	Diagnosis	Age (yr)	No. of LMP2 Epitopes Identified in CTL Line	Response to CTL
1	HD	16	1	CRU
2	HD	20	1	Remains in remission
3	HD	27	1	CRU
4	HD	20	None (high background)	Remains in remission
5	HD	8	None (no spots)	CR
6	HD	36	2	SD
7	HD	19 BV-CTL	1	Died before could receive EBV-CTL
8	HD	18	1	CR
9	HD	29	1	NR
10	NHL	66	4	Did not receive EBV-CTL
11	NHL	39	2	SD
12	HD	25	2	Did not receive EBV-CTL
13	NPC	50	2	Remains in remission
14	NPC	59	5	Remains in remission
15	NPC	11	2	Remains in remission
16	NPC	19	None (no spots)	Remains in remission
17	NPC	11	None (high background)	PR
18	NPC	36	1	CR
19	NPC	17	1	CR
20	NPC	46	1	SD
21	NPC	16	1	NR
22	NPC	16	1	PR
23	NPC	18	2	CR
24	NPC	20	2	NR
25	NPC	63	1	Died before could receive EBV-CTL

HD = Hodgkin Disease, NHL = Non Hodgkin Lymphoma, NPC = Nasopharyngeal Lymphoma, NR = No response, SD = Stable Disease, PR = Partial response, CR = Complete response, CRU = Complete remission undetermined. Patients had residual mediastinal masses post autograft at the time they received CTLs which eventually resolved but could not be classified as having definite disease as gallium scans were negative.

Monitoring of LMP2-specific T-cell populations post adoptive T-cell therapy

The ability to monitor the frequency of LMP2-specific T-cell populations in the peripheral blood or infiltrating at the tumor site is crucial to determine the efficacy of immunotherapeutic interventions. In our ongoing Food and Drug Administration- approved clinical studies, patients with relapsed EBV-positive NPC, HD and NHL are being treated with autologous EBV-specific CTL. LMP2-specific T-cell populations are identified in the infusion product by screening with the LMP2 peptide library and subsequent staining with tetramers derived from the identified epitopes. For example, 11.0% of the CD8+ T-cells within the CTL line from a patient with EBV-positive Hodgkin's disease were specific for one of the newly described LMP2 epitope MGSLEMVPM which was found to be HLA-B*3501-restricted. Following infusion of this EBV-specific CTL line, the LMP2-specific T-cell population was monitored in peripheral blood using tetramer analysis (Figure 6). Pre infusion, 1.98% of CD8+ T-cells in the peripheral blood were specific for this LMP2-epitope. The frequency of MGSLEMVPM-specific T-cells increased to 5.37% six weeks post CTL infusion. These results indicate that the infused CTL ($4x10^7/m^2$) proliferate and persist for at least 6 weeks post infusion and demonstrate the value of monitoring tools derived from LMP2-epitopes.

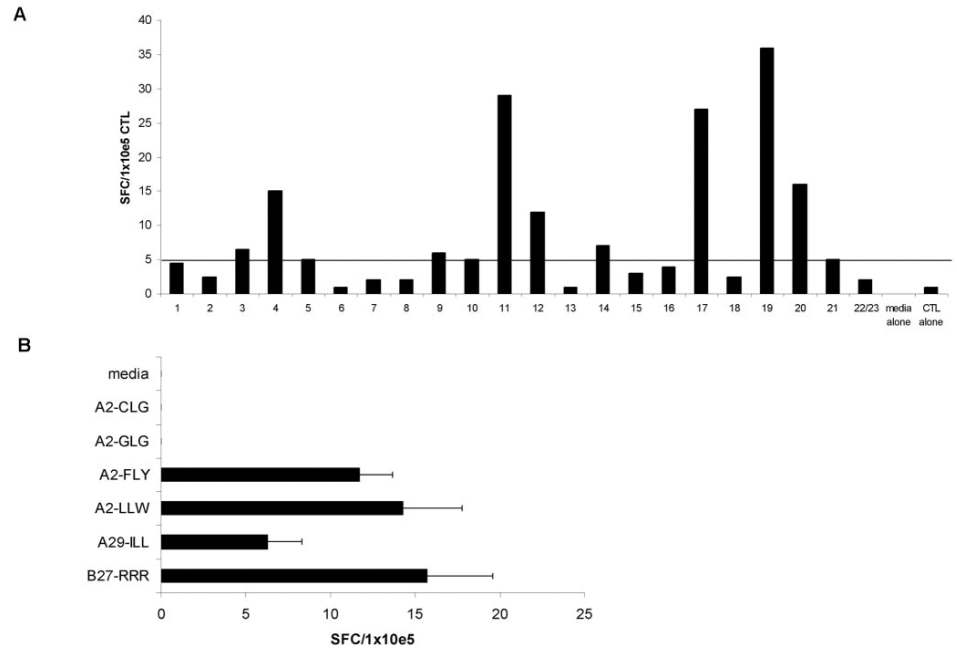

Figure 5. Breadth of the LMP2-specific T-cell responses in patient CTL line.
(a) *EBV-specific CTL from a patient with NHL (HLA type A2, A29, B13, B27) ($1x10^5$/well) were stimulated with the indicated LMP2 peptide pools. The black horizontal line shows the threshold level used to determine significance (5x number of SFC/$1x10^5$ unstimulated CTL).* **(b)** *Responses to these peptide pools could subsequently be mapped to 4 different HLA-A2 (FLYALALLL and LLWTLVVL), A29 (ILLARLFLY) and B27 (RRRWRRLTV)-restricted LMP2 epitopes demonstrating a broad LMP2-specificity in this CTL line.*

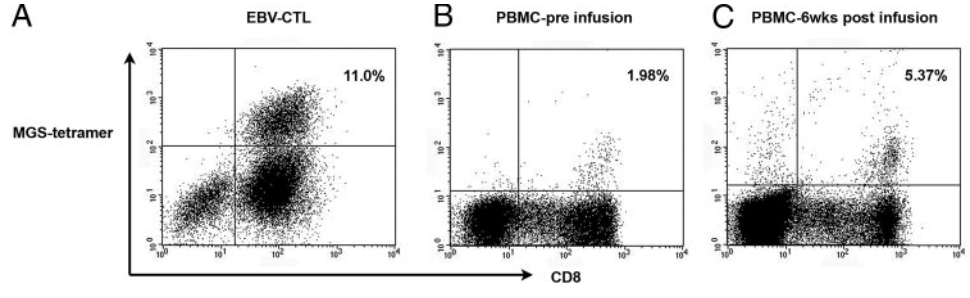

Figure 6. Monitoring of an LMP2-specific T-cell population in PBMC post CTL infusion.
(a) *Using an HLA-B35 LMP2aa 1-9: MGSLEMVPM tetramer, an LMP2-specific T-cell population was detected in the CTL line from a patient with HL.* **(b)** *Using the same HLA-B35 tetramer the number of T-cells specific for this epitope were determined in PBMC before and* **(c)** *6 weeks after the infusion of this CTL line to monitor the persistence and expansion of the infused CTL .*

Discussion

Detailed characterization of LMP2-directed T-cell specificity will greatly enhance the potential application of immunotherapeutic strategies and our ability to evaluate their effect as treatment of EBV latency type II malignancies. Stimulation of patient EBV-specific Cytotoxic T-cell lines with an LMP2-peptide library using IFN-γ secretion as read-out proves to be a fast and sensitive method to evaluate the strength and breadth of the LMP2-specific immune response. As this technique is effective regardless of the patient's HLA type, it can be applied to all patients. LMP2-specific T-cell responses were detectable in 84% of LCL-reactivated CTL lines. This result may be viewed as surprising as these patients have developed EBV-positive malignancies in the presence of a competent immune system. However, EBV+ve tumors expressing type II latency, use multiple strategies to evade the immune response. For example, Hodgkin Lymphoma cells secrete the immunosuppressive cytokine, TGFβ, and recruit regulatory T-cells, which together have devastating effects on CTL proliferation and function.[42] The apparent lack of efficacy of endogenous tumor-specific CTL circulating in the patient peripheral blood provides a rationale for ex vivo expansion of the tumor-specific CTL in isolation from tumor-derived immune suppressive factors.

The LMP2-specific T-cell responses were mapped to previously described epitopes and to 9 newly identified HLA class I and class II restricted epitopes. The latter were shown to be HLA-A*0204/17, A*0206, A29, A68, B*1402, B27, B*3501, B53 and DR (likely DR4)-restricted, mostly alleles for which no LMP2-epitopes have previously been identified. Interestingly, the new class II epitope identified, which partially overlaps with a previously reported CD8+ recognition site [33], and the class I epitope MGSLEMVPM identified in this study, are the only LMP2 epitopes located in the cytoplasmic region of LMP2, whereas all other LMP2 epitopes are located in the transmembrane region. (Figure 7)

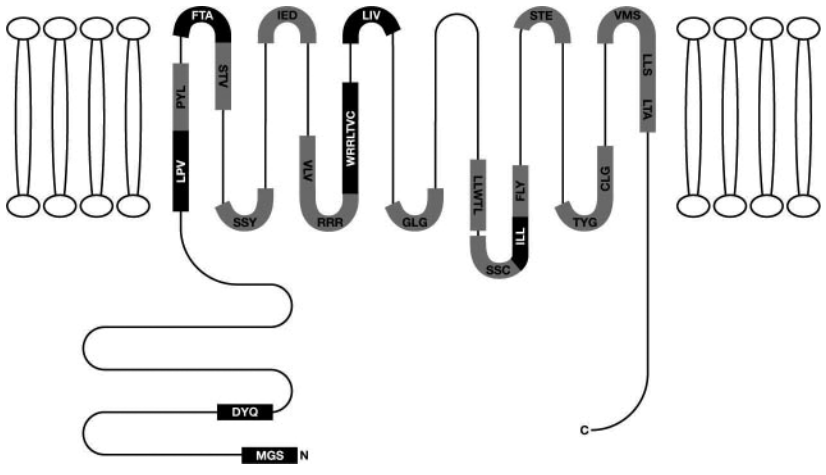

Figure 7. Location of epitopes with the LMP2 molecule
The location of newly identified (in black) and previously described (in gray) CD4- and CD8-epitopes within the LMP2 molecule is shown. Note that the amino acid sequences of multiple epitopes including the newly identified LPVIVAPYL, FTASVSTVV, RRLTVCGGIM, TVCGGIMFL and ILLARLFLY epitopes partially overlap with the sequences of other LMP2 epitopes.

Although NPC, HL and NHL occur worldwide, NPC is endemic in Southern China,[43] whereas EBV-positive HL is more common among Hispanics.[44] As HLA-A*0206 is a common HLA-allele in the Asian population and HLA-A29, A68 and B*3501 are common HLA-alleles among the Hispanic populations [45] these are valuable additions to the panel of LMP2-epitopes particularly when conserved among geographically separated virus isolates.

Initial methods to identify LMP2 epitopes relied on the generation of CTL clones expanded from LCL-reactivated T-cell lines from healthy donors; a relatively time-consuming process. [27,29,30] More recently, an epitope screening strategy based on a peptide library developed by Kern et al [37] was used to analyze EBNA1, LMP1 and LMP2-specific immune responses in the peripheral blood of healthy donors.[33, 39] This method with IFN-γ release as measured in an ELISPOT assay as read-out significantly simplified the epitope identification and was therefore our method of choice. LCL-reactivated T-cell lines, increased the frequency of the LMP2-specific T-cells compared to that in PBMC. Nevertheless, LMP2-specificity could rarely be detected in cytotoxicity assays because of the low frequency of the LMP2-specific component. However, LMP2 tetramer reactive cells that were isolated and expanded demonstrated LMP2-specific cytotoxic effector function (not shown). Here we have shown that, using LCL as APC, LMP2-specific T-cells can be reactivated and expanded for the vast majority of patients with type II latency malignancies irrespective of the patient's age, sex, type of cancer, and disease stage. LMP2-specific T-cell populations that represent <0.1% of CD3+ T-cells could be detected using the ELISPOT assay suggesting under detection of LMP2 specificity in previous studies.

One potential problem with the screening method used is that not all epitopes may be detected using the 15-mer peptide pools. In two CTL lines that contained a FLYALALLL -specific T-cell population detectable by tetramer staining, no IFN-γ secreting cells were detected upon stimulation with peptide pools that contained a pentadecamer containing the FLYALALLL sequence. However, upon stimulation with the minimum 9-mer peptide IFN-γ secretion was induced. The pentadecamers used in this study are C- and/or N-terminus extended versions of potential CD8-epitopes. Whereas peptide trimming by aminopeptidases is sufficient for MHC class I presentation of N-terminus extended epitopes, proteosomes are required for presentation of C-terminus extended epitopes.[46-48] The FLYALALLL-epitope differs from the other epitopes described, in that it relies on the immunoproteasome for its processing from whole antigen.[29] Although T-cells appear to be capable of peptide trimming to a certain extent, it is not clear whether they can express the immunoproteasome after IFN-γ induction,[49,50] and thus may be unable to complete C-terminal trimming of the FLYALALLL epitope. Therefore, the absence of professional APC in our screening assay may explain why FLYALALLL-specific T-cell responses were not detected in all cases. In addition, when APC are exposed to peptide cocktails competition for binding to HLA molecules may lead to underdetection of LMP2-specificity.

If T-cells specific for LMP2 epitopes are to have anti-tumor effects, epitopes originating from the Caucasian derived B95-8 variant of LMP2,[51] must be conserved in the tumor strain of EBV. Comparison of the LMP2 epitope sequences in B95-8 with Asian and Mediterranean EBV isolates from NPC tumors showed that one or two amino acids were altered in 3 of the newly identified epitopes. Similarly, previous analysis showed amino acid alterations in 6 of 11 described/predicted LMP2 epitopes (including LPVIVAPYL characterized in this paper)

in isolates from NPC and HL tumors.[30,52] We and others [30] have shown that for 5 of these epitopes the alterations did not disrupt CTL recognition. Mutations in 2 epitopes (LPVIVAPYL and MGSLEMVPM) were shown to compromise their interaction with CTL, and for one epitope (RRRWRRLTV) CTL recognition is predicted to be decreased.[52] Overall, the majority of but not all LMP2 epitopes appear to be antigenically conserved among different isolates. These results imply that one should ideally use the LMP2 protein as expressed in the tumor as source of antigen for immunotherapeutic strategies. However, as this is not feasible in the manufacturing of a clinical grade therapeutic product, multiple LMP2 epitopes including isolate-specific variant epitopes should be used to activate tumor-specific T-cells.

The ability of tumor cells to delete certain antigens or epitopes to escape from the immune response as described both for the melanoma antigen MART1 and an immunodominant HLA-A11 restricted EBV EBNA3 epitope, further stresses the importance of targeting multiple tumor epitopes preferentially from multiple tumor antigens.[34,53] Although broad LMP2-specificity was found in a number of the LCL-reactivated CTL lines studied, in a significant number of CTL lines the LMP2 response was targeted towards a single epitope and 4 CTL lines lacked detectable numbers of LMP2-specific T-cells. LMP1-specific responses were only detected in 1 out of 25 CTL lines (data not shown). This is most likely a result of the preferential activation of immunodominant EBNA3 and lytic EBV-antigen specific T-cells using LCL as APC to establish these CTL lines. To improve tumor-antigen reactivity, reactivation and expansion methods using APC overexpressing LMP1 and LMP2 have been developed. Using this approach, the frequency of LMP-specific T-cells and the number of epitopes these are targeted towards can be increased.[54,55] Similarly, for vaccination approaches, vectors encoding whole protein or, to avoid possible oncogenicity of the antigen, multiple LMP1 and LMP2 epitopes (polytope approach) are being developed instead of single peptides, to boost LMP-specific T-cells with a broad specificity.[21] Incorporation of the here-identified LMP2-epitopes into current polytopes, will enhance the number of tumor antigen-derived epitopes targeted and allow for application of this strategy to an even broader patient group.

Valuable tools for immune monitoring following immunotherapeutic interventions can be derived from LMP2-epitopes. These include tetramers and peptides for stimulation of T-cells in quantitative and functional assays to detect cytokine secretion, and here we have demonstrated how the expansion and persistence of LMP2-specific T-cells can be monitored in the peripheral blood using LMP2-tetramers. Such immune studies that provide insight into functional changes in tumor immunity are crucial to evaluate efficacy and further optimize immunotherapeutic strategies. These newly identified LMP2 epitopes will contribute to a detailed characterization of the LMP2-directed T-cell immunity required to achieve this goal.

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Chapter 3

Treatment of Nasopharyngeal Carcinoma with Epstein-Barr Virus-Specific T-Lymphocytes

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Abstract

Conventional treatment for nasopharyngeal carcinoma (NPC) frequently fails, and is accompanied by severe long-term side effects. Since virtually all undifferentiated NPCs are associated with Epstein Barr virus (EBV), this tumor is an attractive candidate for cellular immunotherapy targeted against tumor-associated viral antigens. We now demonstrate that EBV-specific cytotoxic T-cell lines (CTL) can readily be generated from individuals with NPC, notwithstanding the patients' prior exposure to chemotherapy/radiation. Ten patients diagnosed with advanced NPC were treated with autologous CTL. All patients tolerated the CTL, although one developed increased swelling at the site of pre-existing disease. Four patients treated in remission from locally advanced disease remain disease free 19 to 27 months after infusion. Of 6 patients with refractory disease prior to treatment, 2 had complete responses, and remain in remission > 11-23 months after treatment, 1 had a partial remission that persisted for 12 months, 1 has had stable disease for > 14 months and 2 had no response. These results demonstrate that administration of EBV-specific CTL to patients with advanced NPC is feasible, appears to be safe and can be associated with significant anti-tumor activity.

Introduction

Nasopharyngeal carcinoma (NPC) occurs worldwide and is the third most common malignancy in Southern China, where the incidence is as high as 50 per 100,000.[1] NPC is a radiosensitive tumor and local control rates of greater than 80% can be obtained. However, a significant number of patients relapse, particularly when disease is advanced at diagnosis - the commonest presentation due to a lack of early symptoms.[2] Moreover, radiation and chemotherapy are accompanied by severe short and long-term side effects including secondary malignancies.[3] Hence there is a need for therapies that will improve disease-free survival and that may be associated with reduced toxicity.

Epstein Barr Virus (EBV) is present in virtually all poorly and undifferentiated non-keratininzing NPCs regardless of geographical origin[4] and the viral antigens expressed by the tumor provide potential target antigens for immunotherapy. Adoptive transfer of cytotoxic T-cells (CTL) specific for EBV antigens has proven safe and effective as prophylaxis and treatment for EBV associated lymphoproliferative disease in bone marrow and solid organ transplant recipients.[5-11] These highly immunogenic lymphomas express all latent EBV antigens, including the immunodominant EBNA3 antigens, and are therefore ideal targets for immunotherapy. By contrast, NPC expresses a restricted set of less immunogenic viral antigens, namely EBNA1, LMP1 and LMP2. EBNA1 is expressed in all NPCs and although its processing through the HLA class I pathway is inhibited by a glycine-alanine repeat, peptides derived from incompletely translated proteins may be presented to CD8+ T-cells.[12-15] Expression of LMP1 and/or LMP2 is detectable in at least 50% of NPC tumors.[16,17] Since NPCs also express MHC class I molecules as well as the peptide transporters TAP1 and TAP2, they are capable of processing and presenting these antigens in the context of HLA class I molecules for recognition by CTL.[18] LMP1 and LMP2 specific T-cells are indeed present in the peripheral blood of NPC patients, albeit at lower frequency than in normal donors,[19,20] and could potentially be activated and expanded for immunotherapeutic strategies.
We hypothesized that ex vivo expansion of EBV-specific CTL in the absence of tumor inhibitory factors[21,22] and the subsequent adoptive transfer of these cells may be of benefit to patients with EBV-positive NPC. Here we confirm the feasibility of this approach, and in 10 patients show evidence for safety and activity.

Patients, material and methods

Study entry criteria and patient details

This protocol was approved by the institutional review board at Baylor College of Medicine and the Food and Drug Administration. Patients were eligible for study if they had stage III or IV nasopharyngeal carcinoma at diagnosis (according to American Joint Committee for Cancer Staging and End-Results Reporting staging system 1997[23]) and were either in remission or had refractory or relapsed disease, and if their tumor was EBV-positive as determined by in situ hybridization or PCR-amplification for Epstein Barr Virus-Encoded RNA (EBER). Patients were treated on 3 escalating dose levels and received either 2 doses of $2x10^7$ CTL/m^2 (dose level 1), or one dose of $2x10^7$ CTL/m^2 and 1 dose of $1x10^8$ CTL/m^2 (dose level 2) or 1 dose of $1x10^8$CTL/m^2 and 1 dose of $2x10^8$/m^2 (dose level 3). CTL were given intravenously with a 2-week interval between each dose. Peripheral blood was obtained pre and at multiple time points post CTL infusion for evaluation of toxicity and EBV-immunity.

Generation of EBV-transformed B cell lines and EBV-specific CTL

After informed consent, peripheral blood (40-60 ml) from patients with EBV-positive NPC was used to generate both EBV-transformed lymphoblastoid B-cell lines (LCL) and EBV-specific CTL lines.[24] Briefly, for LCL generation, $5x10^6$ peripheral blood mononuclear cells (PBMC) were incubated with concentrated supernatant of B95-8 cultures, in the presence of 1 µg/ml cyclosporin A (Sandoz, Vienna, Austria) to establish an LCL. Subsequently, PBMC (2×10^6 per well of a 24 well plate) were stimulated with LCL irradiated at 4000 rads at an effector: stimulator ratio of 40:1. After 9-12 days, viable cells were restimulated with irradiated LCL (at 4:1 E:S ratio). Subsequently, CTL were expanded by weekly stimulations with LCL (at 4:1 E:S ratio) in the presence of recombinant human interleukin-2 (rhIL-2, Proleukin, Chiron Corporation, Emeryville, CA) (40-100 U/ml). After expansion, CTLs were tested for sterility, HLA identity, immunophenotype, and EBV specificity and cryopreserved. Specificity was tested in a 4-hour Cr^{51} release assay. In 8 lines, the CTL showed a significantly higher killing of the autologous LCLs (mean 56.6%: range 38-92%) as compared to HLA antigen mismatched LCLs (mean 6.1%, range 0-27%, $p<0.0001$) or to HSB-2 (mean 21.5%, range 6-55% $p<0.005$) at an E/T ratio of 20:1. In two CTL lines, lysis of the HLA-mismatched LCL was observed, which was significantly reduced by depletion of TCRγδ-positive cells. Auto-reactivity was excluded by the absence of lysis of autologous Phytohemagglutinin (PHA)-stimulated lymphoblasts in all 10 CTL lines.

Peptides

The following peptides were used for analysis of EBV-specific T-cell populations according to the patients HLA specificity: **LMP1**: HLA-A2: YLQQNWWTL, YLLEMLWRL, LMP2: HLA-A2: LLWTLVVLL, CLGGLLTMV, FLYALALLI, GLGTLGAAI, TVCGGIMFL, LTAGFLIFL, LIVDAVLQL, HLA-A11: SSCSSCPLSKI, HLA-A24: TYGPVFMCL, HLA-A23/24: PYLFWLAAI, HLA-A68: FTASVSTVV, ASCFTASVSTVVTAT (15-mer), HLA-B27: RRRWRRLTV, RRWRRLTVCGGIMFL (15-mer), RRLTVCGGIMFL, HLA-B60: IEDPPFNSL, **EBNA1**: HLA-B35: HPVGEADYFEY, **EBNA2**: HLA-A2: DTPLIPLTIF, **EBNA3**: HLA-A2: LLDFVRFMGV, HLA-A3: RLRAEAQVK, HLA-A11: AVFDRKSDAK, IVTDFSVIK, LPGPQVTAVLLHHEES, DEPASTEPVHDQLL, NPTQAPVIQLVHAVY, HLA-A24: RYSIFFDY, TYSAGIVQI, HLA-B7: RPPIFIRLL, QPRAPIRPI, HLA-B27: RRIYDLIEL, HLA-B35: YPLHEQHGM, AVLLHEESM, HLAB44: VEITPYKPTW, EGGVGWRHW, EENLLDFVRF, KEHVIQNAF, **BZLF1**: HLA-B35: EPLPQGQLTAY, **BRLF1**: HLA-A2: YVLDHLIVV, HLA-A11: ATIGTAMYK, HLA-A24: DYCNVLNKEF, **BMLF1**: HLA-A2: GLCTLVAML, **BMRF1**: HLA-A2: TLDYKPLSV (listed in Khanna *et al*[25] and Houssaint *et al*[26], and Straathof *et al*, manuscript in preparation). HLA-A2-restricted Cytomegalovirus pp65-derived peptide NLVPMVATV was used as a control. Peptides were either synthesized by Martin Campbell, Synthetic Antigen Laboratory, The University of Texas MD Anderson Cancer Center, Houston, TX, or Genemed Synthesis Inc. (South San Francisco, CA). In this paper the peptides are referred to by the first 3 amino acids as underlined.

Tetramer staining

To identify LMP1 and LMP2-specific T-cells a selection from the following tetramers was used, as determined by the HLA-type of the patient: **LMP1**: HLA-A*0201-YLQQNWWTL, and **LMP2:** HLA-A*0201-CLGGLLTMV, HLA-A*0201-FLYALALLI, HLA-A*0201-LLWTLVVLL, HLA-A*0201- TVCGGIMFL, HLA-A*1101-SSCSSCPLSKI, HLA-A*2301-PYLFWLAAI, HLA-A24-TYGPVFMCL, HLA-A68-FTASVSTVV, HLA-B*2705-RRRWRRLTV, and HLA-B*2705-RRLTVCGGIMF. Tetramers were prepared by the National Institute of Allergy and Infectious Diseases (NIAID) tetramer core facility (Atlanta, GA), or by the Baylor College of Medicine Tetramer Core Facility (Houston, TX). CTLs or PBMCs ($5\text{-}10x10^5$) were incubated at RT for 30 minutes in PBS/1% FCS containing the PE-labeled tetrameric complex. Samples were costained with anti-CD8 FITC and anti-CD3 PerCP. Appropriate isotype controls were included. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. For each sample, a minimum of 100,000 cells was analyzed using a FACS Calibur with the Cell Quest Software (Becton Dickinson).

Enzyme-Linked Immunospot (ELISPOT) assay

The frequency of EBV- and LMP2-specific T-cells in the infusion product as well as in the peripheral blood pre and at multiple time points post CTL infusion was measured using an IFN-γ ELISPOT assay. 96-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated overnight with 10 µg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH). PBMC were thawed 24 hours before the assay in complete media supplemented with 50 U/ml Benzonase (Novagen, Madison, WI), rested overnight in complete media, and plated at $1\text{-}2x10^5$ cells/well and 2-3 serial dilutions for LCL targets and $3\text{-}4x10^5$/well for peptide targets. CTL were rested overnight in complete media and plated at $1x10^5$ cells/well and 2 serial dilutions. Cells were stimulated with either irradiated (40 Gy) autologous LCL ($1x10^5$/well) or 5 µg/mL peptide. In HLA-A2-positive patients the Cytomegalovirus (CMV)-pp65 encoded HLA-A2 restricted peptide NLVPMVATV was used as control. After 18-24 hours, the plates were washed and incubated with the secondary biotin conjugated anti-IFN-γ monoclonal antibody (Detector-mAB (7-B6-1-Biotin), Mabtech). After incubation with Avidin:biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit (Standard), #PK6100, Vector Laboratories, Burlingame, CA) plates were developed with AEC substrate (Sigma, St. Louis, MO). Plates were sent for evaluation to Zellnet Consulting, New York, NY. Spot-forming units (SFC) per $1x10^5$ CTL or per $1x10^6$ PBMC were calculated by linear regression analysis when serial dilutions were performed and subsequent subtraction of background of non-stimulated T-cells. If an epitope-specific T-cell population had been identified in the infusion product, EBVand LMP2-specific immunity was monitored in patient peripheral blood using this IFN-γ ELISPOT assay and, when enough PBMC were available and HLA type was informative, by tetramer staining.

PCR for EBV-load in PBMC

PBMC were isolated from peripheral blood on a Ficoll (Lymphoprep, Axis-Shield, Oslo, Norway) gradient and washed with PBS. DNA was isolated from 3-5x10^6 PBMC using an anion exchange column (Qiagen, Valencia, CA). Five hundred nanograms of DNA was then used for real time polymerase chain reaction (PCR) to quantitate EBV genome copy number and was reported as copies (cp)/µg DNA.[27]

Results

Patient characteristics

Ten patients were enrolled on the study and all had poorly differentiated or undifferentiated nasopharyngeal carcinoma (WHO II/III) at diagnosis. Four patients at high risk for relapse were in remission at the time of CTL infusion and six patients had failed multiple rounds of radiotherapy and chemotherapy and had relapsed/refractory disease. Patient characteristics and previous treatment are summarized in Table 1.

Table 1. Characteristics of patients on study

Patient no.	Dose	Sex/age	HLA	Ethnicity	Stage	Previous treatment
Treated in remission						
729	2 x 10^7/m^2 x 2	M/50	A2/11 B56/61	Asian	IV	RT, cisplantin, 5-FU
606	2 x 10^7/m^2 x 2	F/29	A2/2 B60/61	White	IV	RT, cisplantin, 5-FU
697	2 x 10^7/m^2 x 2	F/11	A1/2 B37/44	African American	III	RT, cisplantin, MTX, 5-FU
815	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	M/19	A33/36 B53/72	African American	IV	RT, cisplantin, MTX, 5-FU
Treated with relapsed or refactory disease						
845	2 x 10^7/m^2 x 1	M/11	A3/68 B7/35	White	IV	RT, cisplantin, MTX, 5-FU, paclitaxel, carboplantin, VP16, vinorelbine, gemcitabine
894	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x 1	M/36	A1/32 B27/35	White	III	RT, cisplantin, 5-FU, carboplantin, ifospamide, paclitaxel, radioactive seed implants, gemcitabine
389	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x **1***	F/17	A2/3 B44	White	IV	RT, cisplantin, MTX, 5-FU, carboplantin, paclitaxel
918	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x 1	M/16	A11/68 B49/52	Hispanic	IV	RT, cisplantin, MTX, 5-FU
1042	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	F/46	A2/24 B51/61	Asian	IV	RT, cisplantin, 5-FU, docetaxel, CPT-11
1046	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	M/16	A30/68 B18/42	African American	IV	RT, cisplantin, MTX, 5-FU, docetaxel, oxaliplantin, epirubicin, gemcitabine, etoposide

*M = male, F = Female, RT = radiotherapy, MTX = Methotrexate, 5-FU = 5-Fluoruracil. Stage according to American Joint Committee for Cancer Staging and End-Results Reporting staging system 1997.[23] * = this patients received additional doses of 1x10^8 CTL/m^2 at 6 months, 9 months and 12 months after the initial CTL infusions*

CTL lines contain LMP2-specific T-cell populations

Autologous LCL and EBV-specific CTLs were successfully generated from 10 of 10 NPC patients. The phenotype of these CTL lines is shown in Table 2. The presence of LMP1 and LMP2-specific T-cells within these CTL lines was evaluated by IFN-γ ELISPOT after stimulation with LMP1/2-peptides. In 8 of 9 CTL lines for which informative peptides were available based on HLA type, T-cells specific for at least 1 LMP2 epitope were detected (Table 3). In addition, in 1 out of 5 CTL lines evaluable for LMP1-specificity an LMP1-YLL-specific T-cell population was identified. As measured by tetramer staining, up to 5.5% of the total CD8+ population was specific for a single LMP2 epitope (data not shown). In 4 lines, T-cells specific for multiple (up to 5) different LMP2 epitopes were present, in 2 cases these were restricted through different HLA alleles. Such T-cell responses targeted towards multiple tumor antigen-derived epitopes are important to reduce the risk of tumor escape through antigen deletion. Overall the T-cell responses against these subdominant LMP-antigens were weaker than those against epitopes derived from the immunodominant lytic and EBNA3 latent antigens (Table 3), but in the same range as detected in LCL-reactivated CTL lines from healthy donors.[28] Moreover, the identified T-cell populations specific for individual peptides reflect the minimum LMP2-specificity present and likely underestimate the total number of LMP2-specific T-cells.

Table 2. Phenotype of patient CTL lines for infusion

Patient no.	$CD3^{+/-}$ $TCR\alpha\beta^{+}$,%	$CD3^{+/-}$ $TCR\gamma\delta^{+}$,%	$CD3^{+/-}$ $CD4^{+}$,%	$CD3^{+/-}$ $CD4^{+}$,%	$CD3^{+/-}$ $CD56^{+}$,%	$CD3^{-/-}$ $CD56^{+}$,%	$CD3^{-/-}$ $CD16^{+}$,%
729	92.3	5.6	5.4	87.8	46.9	3.1	3.4
606	96.4	0.9	1.0	95.1	5.7	2.6	2.7
697	85.9	8.6	17.1	73.2	31.7	5.1	5.5
815	86.1	12.0	3.7	78.7	10.1	1.9	1.6
845	71.7	30.2	4.6	67.2	25.4	0.2	0.0
894	83,8	2.4	11.1	72.9	12.8	13.8	10.4
389	98,0	0.1	0.2	97.0	24.9	0.1	0.1
918	95.7	1.4	4.8	91.7	9.7	0.7	0.6
1042	84.2	15.6	1.4	83.5	22.4	2.8	2.7
1046	92.9	1.4	0.3	94.9	31.7	0,9	0.0

Safety of EBV-specific CTL

Upon administration of EBV-specific CTL no immediate or long-term toxicity was observed in the 4 patients without detectable disease and in 5 out of 6 patients with refractory/relapsed disease (Table 4). However, in one patient (P845) with bulky disease, pre-existing facial swelling increased markedly two days after infusion of the first dose of CTL ($2x10^7/m^2$) requiring a tracheostomy. A needle biopsy of this mass showed tumor cells and no inflammatory cells suggesting tumor progression as the causative factor, but a contributory effect from CTL cannot be excluded.

Table 3. T-cell populations specific for EBV antigens (SFC/1x10^5 CTLs) in infusion product

Patient no.	LMP1	LMP2	EBNA1	EBNA2/3	Lytic cycle
729	YLQ: 0	CLG: 0	ND	DTP: 0	TDL: 3.5
	YLL: 0	GLG: 0		LLD: 0	YVL: 31
		FLY: 1988		AVF: 0	GLC: 1236
		LLW: 0		IVT: 0	ATI: 0
		LTA: 0		NPT: 0	
		TVC: 0		LGP: 0	
		LIV: 0		DEP: 0	
		IED: 830			
606	YLQ: 0	CLG: 45	ND	DTP: 24	TDL: 50
	YLL: 30	GLG: 0		LLD: 0	YVL: 45
		FLY: 6			GLC: 1824
		LLW: 0			
		LTA: 0			
		TVC: 82			
		LIV: 750			
		IED: 830			
697	YLQ: 0	CLG: 33	ND	DTP: 0	TDL: 12
	YLL: 0	GLG: 0		LLD: 256	YVL: 60
		FLY: 156		VEI: 0	GLC: 480
		LLW: 4		EGG: 96	
		LTA: 0		KEH: 0	
		TVC: 0		EEN: 3	
		LIV: 0			
845	ND	FTA: 0	HPV: 0	RPP: 125	EPL: 0
				QPR: 0	
				RLR: 0	
				YPL: 0	
				AVL: 0	
894	ND	RRR: 3	HVP: 0	RRI: 160	EPL: 1124
		RRL: 52		YPL: 26	
				AVL: 72	
389	YLQ: 0	CLG: 26	ND	DTP: 0	TDL: 4
	YLL: 0	GLG: 0		LLD: 0	YVL: 7
		FLY: 0		VEI: 0	GLC: 934
		LLW: 0		EGG: 398	
		LTA: 0		KEH: 0	
		TVC: 0		EEN: 506	
		LIV: 0		RLR: 0	
918	ND	SSC: 8	ND	AVF: 1214	ATI: 0
		FTA: 0		IVT: 1420	
				NPT: 0	
				LPG: 0	
				DEP: 0	
1042	YLQ: 0	CLG: 0	ND	DTP: 0	DYC: 0
	YLL: 0	GLG: 0		LLD: 0	TDL: 0
		FLY: 0		RYS: 0	YVL: 0
		LLW: 0		TYS: 0	GLC: 0
		LTA: 0			
		TVC: 317			
		LIV: 0			
		PYL: 0			
		TYG: 0			
1046	ND	FTA: 29	ND	ND	ND

CTL lines were screened for the presence of T-cell populations specific for the indicated antigens by IFN-γ ELISPOT. The panel of peptides used for stimulation was based on the HLA type of the patient (see: Table 1). The sequence of the peptides referred to by the first 3 amino acids is listed in the Methods section. ND = not done as no informative peptides available.

Changes in EBV immunity after CTL administration

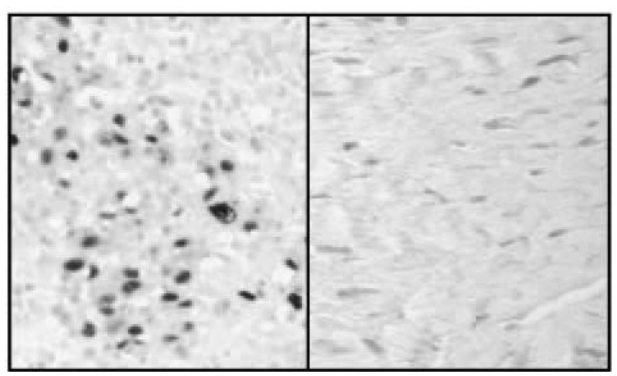
Viral load and the frequency of EBV-specific T-cells were monitored in the peripheral blood at multiple time points post CTL infusion to evaluate persistence and activity of the infused CTL. Of 9 patients with a detectable amount of EBV-DNA in PBMC prior to CTL infusion, EBV load fell within 6 weeks post infusion in 6 patients (Table 5). A decrease in EBV viral load in the peripheral blood likely reflects the lysis of EBV-infected B-cells, and therefore demonstrates activity of the infused EBV-specific CTLs.

In 9 of 10 patients the low normal frequency of EBV-specific T-cells in the peripheral blood (mean: 274, range: 197-384 SFC/1x10^5 PBMC), as measured by IFN-γ secretion of PBMC upon stimulation with autologous LCL, remained unchanged post CTL infusion (data not shown). In one patient (P845) with a low number of circulating EBV-specific CTL prior to CTL infusion (24 SFC/1x10^5 PBMC) a transient 3-fold increase in the number of EBV-specific CTL was measured. In addition, the LMP2-specific T-cell populations identified in the infusion product were monitored in the peripheral blood post CTL infusion. In 5 HLA-A2+ patients, using IFN-γ ELISPOT analysis, the number of T-cells specific for a Cytomegalovirus pp65-derived epitope was determined at the same time points to control for natural variations in viral immunity. In 4 of 8 evaluated patients the number of T-cells specific for LMP2 epitopes increased > 2-fold whereas the pp65-specific immunity remained stable over this time period (Table 5). However, this increase in LMP2-immunity was transient as the number of LMP2-specific T-cells was similar to baseline 6 weeks after CTL infusion in 3 of these 4 patients. Additional tetramer analysis of the frequency of LMP2-specific T-cells in the peripheral blood after CTL infusion in 3 patients failed to detect a persistent increase in LMP2-immunity (data not shown).

Clinical responses post CTL therapy indicate anti-tumor activity

Clinical responses were evaluated from CT and MRI scans pre and post CTL therapy, using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee.[29] All 4 patients who were in remission at the time of enrollment on the study remain in complete remission 19-27 months post CTL therapy (Table 4). Of the 6 patients with refractory/relapsed disease, two patients had no response, 1 patient has stable disease for > 14 months without additional therapy, 1 patient had a partial response sustained for 12 months and 2 patients attained complete remission (CR). One of the patients who attained CR (P389) with refractory relapsed disease had a 24% reduction in tumor size after the initial 2 CTL infusions on dose level 2. Because of this partial response, this patient received 3 additional doses of 1x10^8 CTL/m^2 at 6 months, 9 months and 12 months after the initial CTL infusions with IRB and FDA approval. During this period the patient did not receive other

Figure 1. Absence of NPC tumor cells in nasopharynx post treatment
Biopsies taken pre (left) and post (right, representative of 7 biopsies) the administration of EBV-specific CTL as adjuvant treatment in a patient with refractory NPC (P894) were analyzed for the presence of EBV-positive tumor cells by in situ hybridization for EBER 1 (EBV-encoded small nuclear RNA). EBER-positive cells stain red-brown. The absence of EBER-positive cells post treatment demonstrates a complete response.
Full colour image available at: http://www.bloodjournal.org/cgi/content/full/105/5/1898



Before **After**

Table 4. Toxicity and clinical responses after CTL therapy

Patient no.	Toxicity	Clinical response	Outcome
Treated in remission			
729	None	N/A	Remains in remission > 27 mo
606	None	N/A	Remains in remission > 26 mo
697	None	N/A	Remains in remission > 25 mo
815	None	N/A	Remains in remission > 19 mo
Treated with relapsed or refactory disease			
845	Swelling at tumor site	No response then PR after chemotherapy	PR for 4 months then progressed and died at 12 mo
894	None	CR	Remains in remission > 23 mo afther CTLs
389	None	CR	Remains in remission > 11 mo afther CTLs
918	None	PR	PR for 12 mo after CTLs then relapsed
1042	None	Stable disease	Stable disease for > 14 mo
1046	None	No response	Died of disease at 3 mo

N/A = not applicable, CR = complete remission, PR = partial response according to the international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee.[29]

Table 5. Virological and immunological response to CTL infusion

	EBV load (cp/µg DNA) in PBMCs			LMP2-specific T cells (SFCs/1x10^6 PBMCs)				pp65-specific (SFCs/1x10^6 PBMCs)			
Patient no.	**Before**	**2 wk after**	**6 wk after**	**Epitope tested**	**Before**	**2 wk after**	**6 wk after**	**Epitope tested**	**Before**	**2 wk after**	**6 wk after**
729	10	46	0	FLY	8	26	15[a]	NLV	2623	2521	1896[a]
606	295	114	324	IED	9	5	50	NLV	995	958	1181
				LIV	4	ND	26				
697	31	193	519	FLY	14	0	9	NLV	144	143	96
815	367	174	147	ND				ND			
845	797	286	103	ND				ND			
894	0	0	0	RRL[c]	11	44	3[b]	ND			
389	347	120	156	CLG	16	10	18	NLV	114	100	95
918	87	27	0	SSC	15	63	20	ND			
1042	664	120	367	TVC	116	171	84	NLV	2510	2960	2596
1046	67	56	54	FTA[c]	0	0	0	ND			

wk = weeks, cp = copies, [a] = 3 months post CTL, [b] = 8 weeks post CTL, as not sufficient number of PBMC available at 6 weeks post CTL time point, [c] = pentadecamers containing minimum epitope were used for stimulation. ND = not done as not enough PBMC or no informative peptide available.

treatment and showed continuing response. PET Imaging at 15 months after the first CTL infusion showed normal isotope uptake consistent with a complete response and residual fibrosis. In the second patient who had a CR (P894) a biopsy of the nasopharynx prior to CTL infusion showed poorly differentiated EBER-positive NPC. Multiple biopsies taken 6 months post CTL therapy were all negative for tumor indicating a complete remission (Figure 1). Of the two patients who had no direct response to CTL infusion, one (P845), came off study at 2 weeks because of progressive disease, but subsequently developed a partial response to palliative chemotherapy (Gemcitabine and Carboplatin) to which the disease had been previously unresponsive. The condition of this patient remained stable for 4 months until the tumor again progressed.

Discussion

Although patients with advanced, relapsed NPC have been exposed to intensive radiation and chemotherapy, EBV-specific CTL can readily be reactivated from their PBMC. Adoptive transfer of these CTL lines appears safe in this patient group, although caution may be required in patients with bulky disease. The infused lines contained cytotoxic T-cells specific for LMP2 (an EBV antigen usually expressed by NPC tumor cells), and were biologically active , reducing levels of EBV DNA in peripheral blood mononuclear cells. Although there was no persistent rise in the frequency of circulating T-cells specific for LMP2 after infusion, the CTL appeared to have significant anti-tumor activity. Two of six patients with disease that was resistant to, or had relapsed after, intensive chemotherapy and radiation, have had complete and sustained remissions. A third patient had a partial response and a fourth has stable disease. All 4 patients who were in remission at the time of CTL infusion remained disease free after 19-27 months.

The EBV-specific CTL used in this study were reactivated using LCL that express all EBV latent antigens. LCL are excellent antigen presenting cells that are readily available for all patients as only a limited amount of blood is required to establish an LCL line. As expected using this method only a minority of the expanded T-cells were specific for the subdominant antigen LMP2. However, upon encounter with NPC cells *in vivo* these LMP2 specific T-cells may expand in number. Although such an increase in the frequency of LMP2-specific T-cells was not detectable in the peripheral blood in the majority of patients using ELISPOT assays or tetramers, only a small number of T-cells were infused ($4\text{-}30x10^7$ CTL/m^2) and less than 10% were LMP2 specific. An expansion of several logs would be required to detect a significant increase in the peripheral blood, and it may be that the infused T-cells instead accumulate and expand at local sites of tumor antigen presentation rather than circulate in the periphery. In addition to LMP2-directed immune responses, immunity to other EBV antigens may have contributed to these tumor responses. Recent insights in the processing and presentation of EBNA1 suggest that although a glycine-alanine repeat prevents the processing of the full-length protein, peptides derived from incompletely translated proteins may be available for T-cell recognition.[12-15] Of note, the CTL line from P894, who attained a complete response, contains a relatively large T-cell population specific for an EBNA1-derived, HLA class I-restricted epitope (Table 3). In addition, clinically relevant doses of chemotherapy can induce the expression of EBV lytic cycle antigens in NPC tumors .[30] Similarly, gamma-irradiation at clinically relevant doses can induce lytic EBV infection in EBV-positive B-cell tumors .[31] Patient 845, who progressed 2 days after CTL therapy, received chemotherapy shortly after CTLs. These chemotherapeutic agents had no anti-tumor effect at an earlier stage, whereas when combined with CTL a partial tumor response was induced. This might be the result of chemotherapy-induced expression of lytic EBV antigens and thus sensitization of the tumor for lytic antigen specific T-cells present in the CTL lines and would provide a rationale for combination of CTL therapy with chemotherapy and/or radiation to enhance CTL efficacy.

Previous efforts have been made to recruit the immune system to destroy EBV-positive NPC cells *in vivo*. Adoptive transfer of similar quantities of autologous EBV-specific CTL as used in this study induced anti-viral responses but no clinical responses in 4 NPC patients treated on a pilot study in China.[32] This lack of tumor response may be explained by the fact

that these patients all had end-stage disease with a large tumor burden. Adoptive transfer of an allogeneic EBV-specific CTL line, in one patient with relapsed NPC resulted in a temporary stabilization of disease.[33] Vaccination with dendritic cells loaded with LMP2 peptides induced or boosted LMP2-specific CD8+ T-cell responses in 75% of the patients with advanced stage NPC.[34] In 2 of these patients in whom the LMP2-directed immune response was sustained for 3 months a partial tumor response was induced. How may the success rate of immunotherapy for NPC be increased? The CTL we transfer may undergo only limited *in vivo* expansion, so that strategies aimed at increasing the number of LMP1 and LMP2 specific T-cell in the infusion product may be of value. We are currently using dendritic cells and/or LCL that over express these subdominant antigens to produce order of magnitude increments in the proportion of cells in CTL lines specific for the EBV latency antigens that are expressed by the tumor.[35,36] In addition, anti-tumor activity after CTL infusion may be augmented by vaccinating patients with an LMP1 polyepitope adenovirus vaccine,[37] LMP2 peptide-loaded dendritic cells[34] or EBNA1-LMP2 transduced dendritic cells.[38] Finally, depletion of the patients endogenous T-cells may promote the expansion of the subsequently infused CTL, a strategy that has been successfully explored by Dudley *et al.*,[39] and which may underlie the greatly increased expansion of infused T-cells after hemopoietic stem cell transplantation.[7,40] Given the feasibility and apparent safety of preparing and administering EBV-specific CTL to patients with advanced NPC, it will be of interest to discover if these and other manipulations further increase the tumor response rate.

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Chapter 4

The Generation and Characterization of LMP2-specific CTLs for Use As Adoptive Transfer From Patients with Relapsed EBV-Positive Hodgkin Disease

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Introduction

Adoptive immunotherapy with cytotoxic T-lymphocytes (CTL) has been used successfully to treat Epstein-Barr virus associated malignant disease. The results have been most striking for the EBV positive B-immunoblastic lymphomas occurring in immunocompromised hosts following stem cell transplant (SCT). In this disorder, the malignant cells express the full spectrum of nine EBV latent cycle antigens (latency Type III) and may also express lytic cycle EBV antigens. Hence, EBV specific CTL lines (EBV-CTL) can be generated simply by stimulating peripheral blood mononuclear cells (PBMC) with irradiated EBV-transformed B-cells (EBV-lymphoblastoid cell lines or LCL), which express an identical pattern of viral genes, and are excellent antigen presenting cells.

In the immunocompetent host, EBV is associated with malignancies that express a more limited array of viral genes. For example, in EBV-positive Hodgkin's Lymphoma, only subdominant EBV antigens such as latent membrane antigens (LMP) 1 and 2, and Epstein-Barr nuclear Ag (EBNA) 1 are expressed – termed Type II latency. Our clinical studies of CTL for the treatment of relapsed EBV positive Hodgkin Disease (HD) showed that *ex vivo* expanded EBV-CTL survived in the patients' circulation for up to 9 months, homed to sites of disease and reduced EB viral load, but produced limited tumor responses.[1] This limitation may be because the bulk of each EBV-CTL line was directed against the immunodominant EBV antigens (EBNA 3A, 3B, 3C) that are strongly expressed by the stimulator LCL or against early lytic cycle antigens, which are lacking from the tumor cells themselves.[2;3]

Of the potential CTL target antigens expressed in HD, EBNA-1- is not processed for presentation on class I antigens by tumor cells[4] while LMP1 displays heterogeneity between virus strains,[5] so that CTL raised against B-cell (B95-8 prototype) derived LMP1 may not recognize the LMP1 tumor variants.[6] LMP2A, however, is consistently expressed on Reed-Sternberg cells and its epitopes are conserved between viral strains and amongst Hodgkin's lymphoma biopsy samples.[7-9] The existence of LMP2-specific CTLs in patients with HD has previously been reported.[10] We have also reported the production of LMP2-specific CTL lines from healthy donors, using Ad5-LMP2-transduced DC as APC. However, this method was ineffective when applied to mononuclear cells from patients with relapsed HD. We were unable to produce sufficient DC for the expansion of adequate numbers of LMP2-specific CTL and CTL restimulated with transduced DC failed to expand.

We now describe how LMP2-specific CTLs, from patients with relapsed HD, can be reactivated from patient peripheral blood using LMP2-expressing DC and expanded using autologous LCLs overexpressing LMP2 from an adenovirus vector. We used a chimeric adenoviral vector Ad5F35 that has been optimized for the transduction of human hematopoietic cells, and effectively expresses transgene in both DC and LCL.[11,12] This modification increases the frequency of LMP2-specific T-cells produced and ensures that every patient line contains an LMP2-specific component. The CTL lines contained both CD4+ T-helper cells and CD8+ CTLs that and recognized multiple LMP-2 epitopes, favoring prolonged *in vivo* survival and reducing the risk of tumor-antigen loss variants respectively.[13]

Material and methods

Cell lines and blood donors
The LMP2-specific CTL lines were derived from 2 patients with relapsed EBV-positive Hodgkin disease. Both patients had received standard HD chemotherapy and radiotherapy and patient 1 had also received an autologous stem cell transplant. At relapse, peripheral blood was collected to generate the cell lines before instituting rescue chemotherapy. The HLA class I types of the patients were as follows: Patient 1 HLA-A2, -A3, -B7, -B51 and patient 2: HLA-A11, -29, -B7, -40(61). EBV-transformed Lymphoblastoid Cell Lines (LCL) were established, using a standard protocol.[14] As positive controls, LMP2-specific CTL lines were generated from normal EBV seropositive donors with HLA types A2, B27, B51 and A11, A34, B35, B40(61). Primary skin fibroblasts were isolated and cultured as previously described.[15]

Construction of the recombinant adenovirus AdLMP2A
A recombinant adenovirus Ad5f35LMP2A for the transduction of DC and LCL was generated by Dr Alan Davis in our Gene Vector Laboratory (Baylor College of Medicine, Houston, TX). The structure and synthesis of the Ad5f35 vector has been described previously.[11] The 1.8 kb fragment containing the LMP2A gene was removed via Hind III and Xba I sites from pBluescriptLMP2A[15] and cloned into the *Hind III* and *Xba* I sites of the pShuttle-X (Clontech) shuttle vector. Clontech's adeno-X expression system protocol was used and the I-Ceu/PI-SceI fragment of pShuttle-X was cloned into Adeno-X viral DNA backbone containing an Ad5 gene with a modified fiber containing the knob and shaft of the Ad35 and the tail of Ad5.[11;16] The titer of the vector was 1 x 10^{12} vp/ml. The recombinant vector was stored at -80°C until use. The recombinant adenoviruses Ad5GFP for the transduction of fibroblast targets in cytotoxicity assay were also supplied by Dr. Alan Davis (Baylor College of Medicine, Houston, TX).[15]

Generation of Dendritic Cells (DC)
DCs were generated as previously described by Gahn et al with some modifications.[15] 40-60 mls of peripheral blood was collected from the patients. The PBMCs were then purified by Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient separation and frozen at 5x10^6/ml in Origen® DMSO freeze medium (Irvine Scientific, Santa Ana, CA). Once the LCL line was established 10-20x10^6 PBMC were thawed and washed twice in CellGenix media (CellGenix USA, Antioch, IL) and plated at 5x10^6 cells per well in DC medium (CellGenix media plus 2 mM L-glutamine) (GlutaMAX, Invitrogen, Carlsbad, CA) in a 6-well plate (Costar, Corning, NY) for 2 hr at 37°C in a humidified CO2 incubator. Nonadherent cells were removed by rinsing with 1X PBS (GibcoBRL, Gaithersburg, Maryland), and loosely adherenT-cells were cultured in DC media with 800 U/ml GM-CSF (Sargramostim Leukine; Immunex, Seattle, WA) and 500 U/ml IL-4 (R&D Systems, Minneapolis, MN, USA) for 7 days. IL-4 and GM-CSF were again added on day 3. On day 5, cells were harvested by vigorous washing.

Adenoviral transduction of Dendritic Cells
Immature DCs were harvested on day 5, counted and resuspended at 2x10^6/ml in DC media. 500 µl of cells were then added to 24-well plates (Costar, Corning, NY) and transduced with Ad5f35LMP2A at a multiplicity of infection (MOI) of 30,000 vp/cell. Plates were incubated 37°C for 90 minutes. Afterward, DCs were cultured in DC media for 2 days supple-

mented with 1000 U/ml IL-4, 800 U/ml GM-CSF, 10 ng/ml TNF-α (R&D Systems) and 1 μg/ml PGE2 (Sigma, St Louis, MO, USA) and then irradiated and used to stimulate LMP2-specific CTLs.

Adenoviral transduction of LCLs
Two days before CTL stimulation, LCL were harvested, pelleted and incubated with Ad-5f35LMP2A at a multiplicity of infection (MOI) of 100,000 vp/cell for 90 minutes at 37°C. The cells were then resuspended at $5x10^5$ cells/ml of complete media and transferred to a 24 well plate at 2 mls per well.

Initiation and expansion of LMP2-specific CTL (LMP2-CTL)
Frozen PBMC were thawed and washed and resuspended in 45% RPMI (Hyclone) and 45% CLICKS (Irvine Scientific) with 10% FCS plus GlutaMAX™-I (CTL medium). Cells were resuspended at $2x10^6$/ml and co-cultured with autologous, transduced DCs at a ratio of 40 PBMC to 1 DC. Cultures were restimulated on day 10 with Ad5f35:LMP2-transduced LCL at a responder-to-stimulator ratio of 4:1 and after that weekly with irradiated autologous Ad5f35LMP2-transduced LCL at a responder-to-stimulator ratio of 4:1. IL-2 (50-100 U/ml, Proleukin; Chiron, Emeryville, CA) was added 3 days after the second stimulation and added twice weekly.

Generation of EBV-specific CTL cultures (EBV-CTL)
EBV-CTLs were prepared by stimulating PBMC with the autologous EBV-transformed LCL as previously published.[17;18] Briefly, PBMC ($2x10^6$) were co cultured with $5x10^4$ gamma-irradiated (40 Gy) autologous LCL per well in a 24-well plate. Starting on day 10, the responder cells were restimulated weekly with irradiated (40Gy) LCL at a responder: stimulator ratio of 4:1. Two weekly doses of rhIL-2 (50-100 IU/ml) were added from day 14.

Enzyme-Linked Immunospot (ELISPOT) assay
ELISPOT analysis was used to estimate the frequency of T-cells secreting IFNγ in response to LMP2 peptides. To analyze the HLA-restriction of the LMP2 CTL clone, we measured IFNγ release after culture with peptide pulsed allogeneic LCL lines, matched at only one class I antigen. Ninety six-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated with 10 μg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH) overnight at 40C and then washed and blocked with RPMI 1640 containing 5% Human Serum and L-glutamine (ELISPOT medium) as previously described.[19] To determine the LMP2 peptide specificity of the CTL lines, the responder LMP2-CTL were thawed and cultured in CTL media with 25 U/ml IL-2 for 24 hour. The CTL were harvested and resuspended at $1x10^6$/ml ELISPOT medium without IL-2. Peptides were initially diluted in 1ml 1XPBS and 1ml ELISPOT medium to 10 μg/ml. To identify the HLA A29 LMP2 peptide epitope, serial logarithmic dilutions of 8-mer, 9-mer and 10-mer peptides were made. 100 μl of CTL were then added to 100 μl of diluted peptide. LCL generated as previously described were used either alone, or pulsed with peptide. The LCL were washed, irradiated (40 Gy), washed twice and then transferred to sterile polypropylene tubes at (500 μl of $1x10^6$/ml) and 10 ug peptide (diluted to 20 μg/ml in PBS) was added. 1hr later, the cells were washed and resuspended in 2mls ELISPOT medium. Responder and stimulator cells were first prepared in a replica 96-well U-bottom plate and then transferred to coated 96-well plate. After 20hr incubation, the plates were washed, incubated with the secondary biotin conjugated anti-IFN-γ monoclonal antibody (DetectormAB (7-B6-1-Biotin), Mabtech) followed by incubation with Avidin: biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit (Standard), #PK6100, Vector Laboratories, Burlingame, CA) and then developed with AEC substrate (Sigma, St. Louis,MO) as previously described.[19] Plates were sent for evaluation to Zellnet Consulting, New York, NY. The frequency of peptide-specific T-cells was expressed as specific Spotforming units (SFC).

LMP2-peptides and LMP-2 peptide Pools
Panels of 15-mer peptides (overlapping by 11 amino acids) covering the entire amino acid sequence of LMP2 from the Caucasian prototype EBV strain B95-8 were synthesized as described by Meij et al.[20] Twenty-three peptide pools comprising 2 to twelve 15-mer peptides were prepared, so that each 15-mer peptide was represented in two pools, as previously described.[21] To identify the minimal epitope from a reactive 15-mer HLA-A29 LMP2 epitope, 8-mer, 9-mer and 10-mer peptides were created using published predictions of peptide binding to HLA A29.[22] All peptides were synthesized by Genemed Synthesis Inc. (South San Francisco CA) and dissolved in DMSO under nitrogen.

T-cell Cloning and Identifying the HLA restriction of LMP2 epitope
$CD8^+$ T-cell clones were generated from the LMP2-CTL line of patient 2 by limiting dilution cloning of the CTL on allogeneic feeder cells in interleukin 2-conditioned medium with irradiated LCL transduced with the Ad5f35 LMP2A adenoviral vector and pulsed with the 10-mer peptide KILLARLFLY identified from the reactive 15-mer. The specificity and HLA restriction of the clones was then determined by ELISPOT assay.

Flow cytometry
Expression of the LMP2A protein was analyzed by FACS 2 days after transduction. The anti-LMP2A MAb, derived from clone 8c3, was kindly provided by E. Kremmer (Munich, Germany). Briefly, transduced and control DCs or LCL were fixed for 20 minutes at RT with 4% paraformaldehyde, permeabilized for 30 minutes with 1% saponin (Sigma) and stained with primary rat anti-LMP2A Mab as described previously.[15] Expression of the surface molecules on DC were measured on non-fixed, non-permeabilized DCs using PEconjugated MAbs: anti-CD3, -CD16, -CD19, -CD56, (Becton Dickinson, Mountain View, CA) -CD83, (Caltag, San Francisco, CA) and -DR PerCP (Becton Dickinson) and FITC-, PE- and PerCP-conjugated, isotype-matched mouse IgGs were used as controls (Becton Dickinson). CTL lines were analyzed with anti-CD8 FITC, -CD56 PE, -CD3 PerCP, and CD4 PE antibodies (Becton Dickinson). The TCR Vβ analysis was performed on the CTL lines using the IOTest® Beta Mark TCR Vβ Repertoire Kit according to the manufacturers instructions (Immunotech, a Beckman Coulter Company, Marseille, France). Samples were acquired on a FACScan flow cytometer (Becton Dickinson) and the data analyzed using *CellQuest* software (Becton Dickinson).

Tetramer staining
Soluble HLA-A02*01-CLGGLLTMV, A02*01-FLYALALLL, A29*01-ILLARLFLY, A11*01-IVTDFSVIK, A11*01AVFDRKSDAK and B07*01-RPPIFIRRL PE-conjugated tetramers were prepared by the National Institute of Allergy and Infectious Diseases (NIAID) tetramer core facility (Atlanta, GA) and the Baylor Tetramer Core Facility (Houston TX). The CLGGLLTMV peptide was also prepared by Synthesis Inc. (South San Francisco CA). CTLs (1×10^6) were in-

cubated at RT for 30 min in PBS with 1% FCS containing the PE-labeled tetrameric complex. Samples were additionally incubated with anti-CD8 FITC and anti-CD3 PerCP. IgG-PE was used for isotype control. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. Samples were analyzed by FACS as described.

Cytotoxicity assay

CTLs were tested for specific cytotoxicity against fibroblasts, either uninfected or infected with Ad5LMP2A or Ad5GFP. Autologous LCLs, and HLA class I-mismatched LCLs were also tested.[51] Cr-labeled target cells were mixed with effector cells at doubling dilutions to produce the effector: target (E: T) ratios specified. TargeT-cells incubated in complete medium or 5% Triton X-100 (Sigma) were used to determine spontaneous and maximal ^{51} Cr release, respectively. After 4 hours (LCLs) or 6 hours (fibroblasts), supernatants were collected and radioactivity was measured on a gamma counter. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release - spontaneous release)/ (maximal release - spontaneous release).

Statistical analysis

The Student *t* test was used to test for significance in each set of values, assuming equal variance. Mean values ± SE are given unless otherwise stated.

Results

Expansion of LMP2-CTL in patients with relapsed Hodgkin Disease

We previously generated LMP2-specific CTLs (LMP2-CTL) in normal donors by repeated stimulation of peripheral blood mononuclear cells (PBMCs) with DCs expressing LMP2a from a recombinant adenovirus (Ad5).[15] This method induced CTLs that lysed LMP2a-expressing target cells more effectively than CTLs stimulated with LCLs. However, this approach required large numbers of dendritic cells as antigen presenting cells to activate and expand LMP2-specific CTL and Ad5LMP2-transduced DC were unable to expand LMP2-specific T-cells from patients with relapsed HD (data not shown).

To reduce the requirement for large numbers of DC, we used LCL over expressing LMP2 to expand CTL. Thus HD patient PBMCs were stimulated with Ad5f35LMP2A-transduced autologous DCs on day 0 and then with Ad5f35LMP2A-transduced autologous LCL on days 10 and 17, 25 and 32. The transduction efficiencies of the DC and LCL ranged from 48.8% to 63% (average 55.9%) and 0.8% to 8.7% (average 4.2%) respectively (data not shown). The resultant CTL lines, termed LMP2-CTL, were compared phenotypically to EBV-CTL lines generated using unmodified LCL as stimulators from day 0. Figure 1 shows that on day 24, all lines contained both CD4 and C8 cells, and that apart from a small increase in CD4 positive cells in the LMP2-CTL lines, there were no striking differences between LMP2 and EBV CTL.
To assess the repertoire diversity of our CTL lines, we used antibodies against the T-cell receptor beta chain v region. Flow cytometric analysis (Figure 2) confirmed that both EBV and LMP2-specific lines were polyclonal, and that the majority of Vβ families were represented. However, all the lines were dominated by relatively few families, the most extreme example being the LMP2-CTL line of patient 2, which was dominated by Vβ 5.1 and Vβ 13.6. Vβ usage differed between the LMP2 and EBV-specific CTL lines consistent with a skewed antigen specificity in the LMP-2 stimulated lines. Interestingly in both patients 1 and 2, only

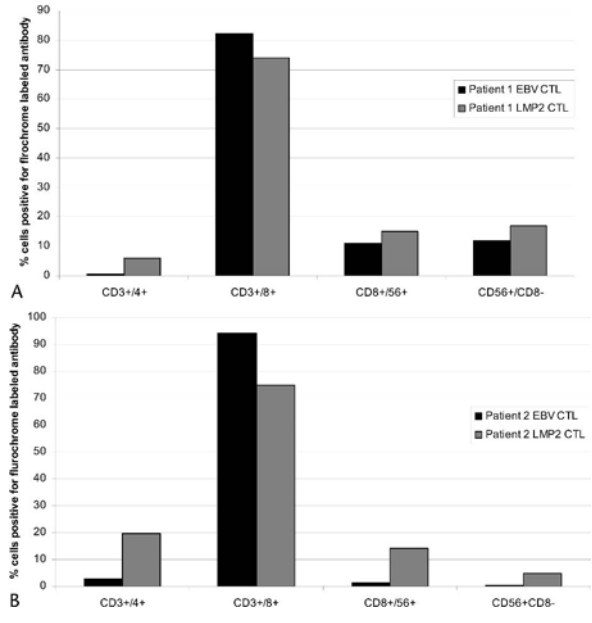


Figure 1. LMP2-CTL lines have more CD4 positive cells compared to EBV-CTL lines generated from the same patient. *LMP2 specific CTL (gray) and EBV specific CTL (black) lines generated from patients 1 (Figure 1a) and patient 2 (Figure 1b) were stained with antibodies against T-cell surface antigens CD3, CD4, CD8, and CD56. Surface immunofluorescence was analyzed by flow cytometry.*

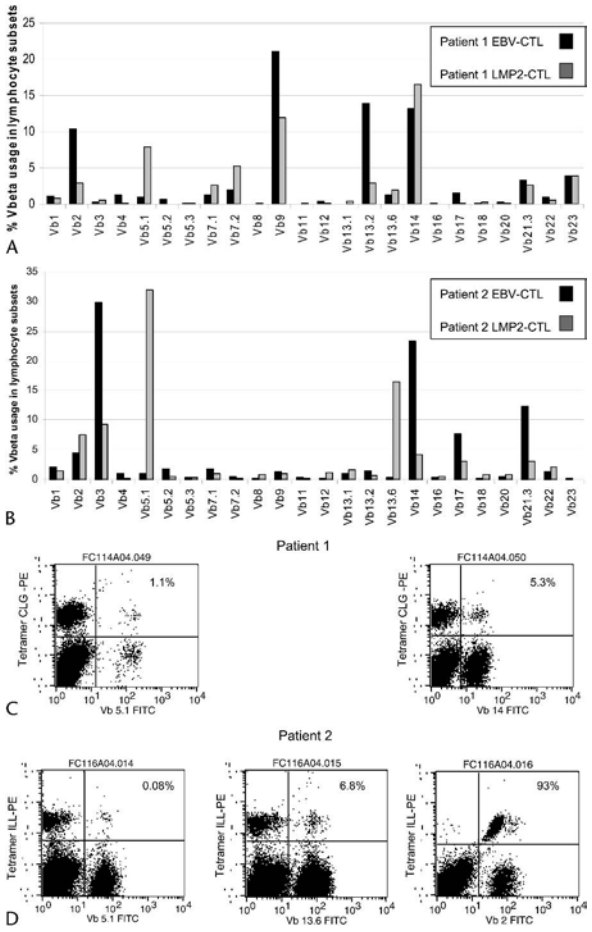


Figure 2. All CTL lines generated were polyclonal with unique Vβ repertoires. *Toanalyze the Vβ repertoires of the LMP2 specific (gray) and EBV specific (black) CTL lines, the CTL generated from patient 1 (Figure 2a) and patient 2 (Figure 2b) were stained with anti-CD3 and 24 Vβ antibodies grouped into 8 vials and surface immunofluorescence was analyzed by flow cytometry. To analyze the Vβ specificity of LMP2 tetramer positive cells the polyclonal LMP2 specific CTL lines from patient 1 (Figure 2c) and 2 (Figure 2d) were stained with anti-CD8 PerCP and either CLGtetramer- PE (patient 1) or ILL-tetramer-PE (patient 2) and single FITC-labeled Vβ antibodies.*

a relatively small proportion of the tetramer positive cells were positive for the dominating Vβ's tested on the polyclonal lines and the epitope specific T-cells demonstrated varying Vβ specificities (Figures 2c and 2d). In patient 1, Vβ 5.1, 9 and 14 predominated on the polyclonal LMP2-CTL line (Figure 2a). A FITC-labeled Vβ 9 antibody was not available. However, using the HLA A2 restricted LMP2 tetramers CLG and FLY we showed that no FLY positive cells were positive for these Vβ subtypes. (data not shown) In contrast, 1.1% of CLG tetramer positive cells were positive for Vβ5.1 and 5.3% were positive for Vβ14. (Figure 2c) In patient 2, the HLA A11 restricted LMP2-tetramer (SSC) positive population in the polyclonal line was negative for both Vβ5.1 and 13.6 (data not shown). Only 0.08% of the HLA A29 restricted LMP2-tetramer (ILL) positive population in the polyclonal line were positive for Vβ5.1, 7% were positive for Vβ13.6 and 93% positive for Vβ 2. (Figure 2d) However, as shown in Figure 2d there are substantial populations that are positive for Vβ 5.1, and 13.6 that are negative for this tetramer.

Figure 3a and 3b shows that the LMP2-CTL and EBV-CTL generated from patients 1 and 2 grew at similar rates. Hence, while the growth characteristics of the patient CTL lines were essentially the same, the Vβ usage differences were suggestive of an altered specificity.

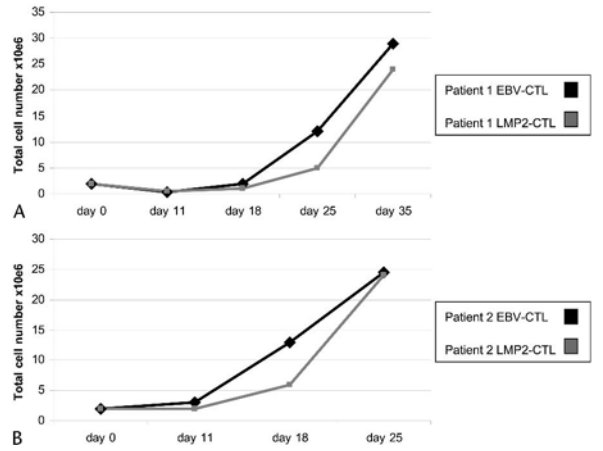
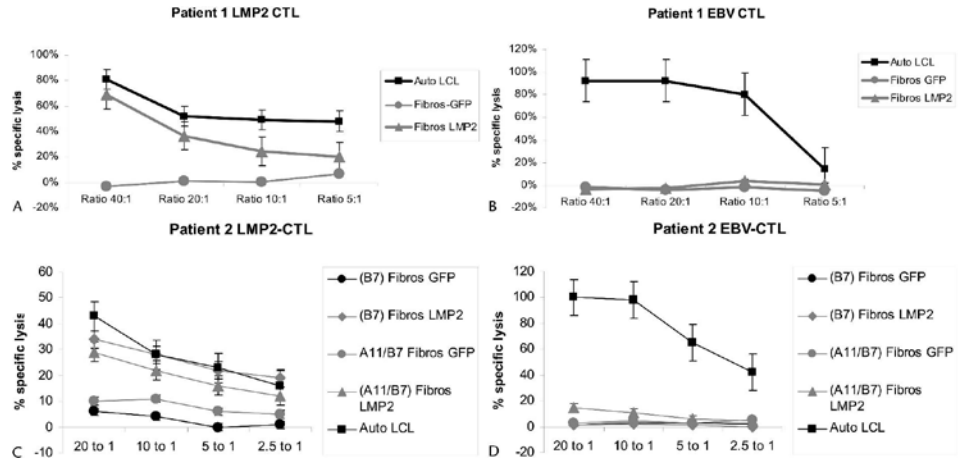


Figure 3a and 3b. The expansion rates of autologous EBV-specific versus LMP-2–specific CTL were similar. *For the generation of EBV-specific CTL (EBV-CTL), a total of 2x10⁶ peripheral blood mononuclear cells (PBMC) were activated with autologous lymphoblastoid cell lines (LCL) and were stimulated weekly with LCL and fed twice weekly with IL-2. LMP2-specific CTL (LMP2-CTL) were activated with Ad5f35LMP2- transduced dendritic cells (DC) followed by weekly stimulations with Ad5f35LMP2- transduced LCL (LMP2-CTL) and twice weekly IL-2 feeds. Figure 3a (patient 1) and Figure 3b (patient 2) represent CTL cell numbers (x10⁶) recorded from weekly cell counts comparing the expansion rates of EBV-CTL (black) versus LMP2-CTL (gray).*

Patient-derived LMP2A-specific CTLs kill Ad5LMP2 Fibroblast targets.
The cytolytic activity of the responder cells was tested against a panel of ^{51} Cr-labeled autologous and allogeneic target cells. LMP2-specific CTLs generated from patient 1 killed autologous fibroblasts only if transduced with Ad5LMP2A (68% at an E: T ratio of 40:1 (Figure 4a)) whereas the EBV-specific CTL line demonstrated no LMP2 specific activity (Figure 4b). Both EBV and LMP2 CTL lines lysed autologous LCLs. Similarly, LMP2-CTLs generated from patient 2 lysed both autologous LCLs and Ad5LMP2Ainfected fibroblasts matched at HLA A11*01 and B07*01 (Figure 4c). By comparison, EBV-CTL generated from patient 2 showed strong cytolytic activity against autologous LCL (100% at E: T ratio of 20:1) but little lysis of HLA A11 fibroblasts expressing LMP2 (15% specific at an E: T ratio of 20:1) and no killing of

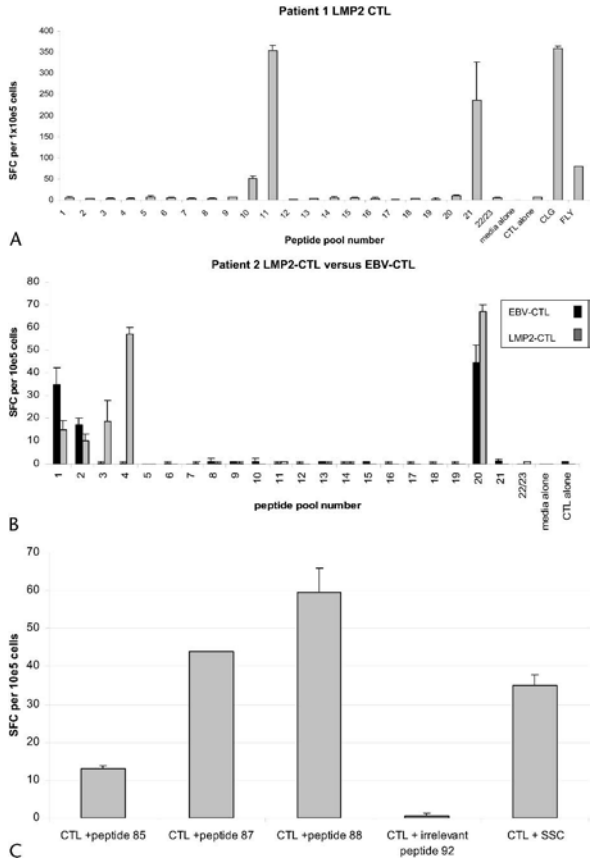


Figures 4a-4d LMP2-specific CTL demonstrate specific lysis of Ad5LMP2- transduced fibroblasts but not Ad5GFP-transduced fibroblasts.
Percent specific ^{51}Cr release was determined 6-hours after coincubation with fibroblasts transduced with Ad5LMP2 (gray triangle), or Ad5GFP (gray circle) and autologous LCLs (black square). The percent specific lysis at the indicated effector to target ratios for the LMP2-CTL line (Figure 4a) and EBV CTL line (Figure 4b) from patient 1 and the LMP2-CTL line (Figure 4c) and EBV CTL line (Figure 4d) from patient 2 are shown. The fibroblast targets used for patient 1 were autologous. In absence of autologous fibroblasts for patient 2, allogeneic fibroblasts class I HLA matched at A11 and B7 (gray triangle) or B7 only (gray diamond) were transduced with Ad5LMP2 and used as targets. As controls, HLA A11;B7 matched (gray circle) and HLA B7 matched (black circle) fibroblasts transduced with Ad5GFP were also tested in this assay.

HLA B07*01-matched fibroblasts expressing LMP2 (Figure 4d). Killing was not due to adenovirus-directed CTLs, since fibroblasts infected with recombinant adenovirus encoding GFP were not recognized. There was no cytotoxic activity against untransduced fibroblasts or HLA-mismatched LCLs (data not shown).

LMP2 epitope specificity was detected in patient CTL lines using LMP2 peptide pools

To characterize the epitope specificity of the CTL lines, they were incubated with LMP2 peptide pools and IFNγ release was measured by ELISPOT assays. Patient 1 had a detectable T-cell response to 15-mer peptides from pools 10, 11 and 21 (Figure 5a). The 15-mers common to these three pools were 106 (GPVFMCLGGLLTMVA) and/or 107 (MCLGGLLTMVAGAVW) spanning the region 421-439. Both peptides contain the known HLA A02*01 LMP2 9-mer epitope CLGGLLTMV (CLG). Subsequent ELISPOT analysis revealed that the LMP2-CTL line shows specificity for the CLG peptide (Figure 5a). There was additional specificity for another known HLA A02*01 LMP2 9mer epitope FLYALALLL (FLY) (Figure 5a), even though reactivity with peptide pools 4, 5, and 20, which also contain the FLY peptide, was not seen. By contrast, analysis of lines prepared with nontransduced LCL stimulators, showed no IFNγ release with any LMP2 peptide pool as targets (data not shown).



Figures 5a-5c. Identification of CD8+ T-cell epitopes within LMP2 using IFNγ- ELISPOT and a 15-mer (11-mer overlapping) peptide library.

*CTL were plated at 1x10⁵ cells per well and results are expressed as spot forming cells (SFC) per 10⁵ cells. Figure 5a shows the reactivity of the LMP2-CTL line from patient 1 with the peptide pools and two known HLA A02*01 restricted 9mer peptides CLGGLLTMV (CLG) and FLYALALLL (FLY). For patient 2 (Figure 5b) the EBV-CTL line (black) and the LMP2-CTL line (gray) are screened against the 15-mer LMP2 peptide library. LMP2-specific CTL from patient 2 were then tested against separate 15-mer peptides within pools 1, 3, 4 and 20 and the known HLA A11*01 restricted 11-mer peptide SSCSSCPLSKI (SSC)(Figure 5c). As a negative control the CTL are also incubated with an irrelevant 15-mer peptide not contained within pools 1, 3, 4, and 20.*

The LMP2 CTL line from patient 2 had a detectable T-cell response to pools 1,2,3,4 and 20 (Figure 5b). The most prominent responses were seen with peptide pools 3, 4 and 20, all of which contain peptides 87 (CPLSKILLARLFLYA) and/or 88 (KILLARLFLYALALL) (region 345-364 of LMP2). Peptide pools 1, 2 and 20 also contained the 15-mer peptides LICSSCSSCPLSKIL and SCSSCPLSKILLARL (#87 and 88) which incorporate the known HLA A11*01 restricted LMP2 epitope SSCSSCPLSKI (SSC) (region 337-352). There is a marked response to two 15-mer peptides (#87 and 88) tested separately and to another peptide (#85) that contained the SSC epitope. (Figure 5c) Testing of this patient's EBVspecific CTL line generated using non-transduced LCL as stimulators, showed a more limited ability to recognize LMP-2 epitopes, with responses only to LMP2 peptide pools 1,2 and 20 consistent with specificity for the SSC epitope only (Figure 5b).

Detection of a new HLA A29*01 LMP2-specific epitope using ELISPOT assay

To identify the minimal epitope contained in peptides 87 and 88 (CPLSKILLARLFLYA and KILLARLFLYALALL), we used shortened sequences within the 345-364 region of LMP2 as shown in Table 1 predicted based on published HLA A29 restricted T-cell epitopes.[22] Serial dilutions of the peptides were incubated with the LMP2 CTL from patient 2. CTL incubated with peptide 2 KILLARLFLY (KIL), secreted IFN-γ at all peptide concentrations in contrast to other peptides tested (Figure 6a). In limiting dilution, the 9 mer ILLARLFLY (ILL) (peptide 7) and 10-mer KIL induced similar levels of IFNγ release, (Figure 6a) therefore ILL was determined to be the minimal epitope.

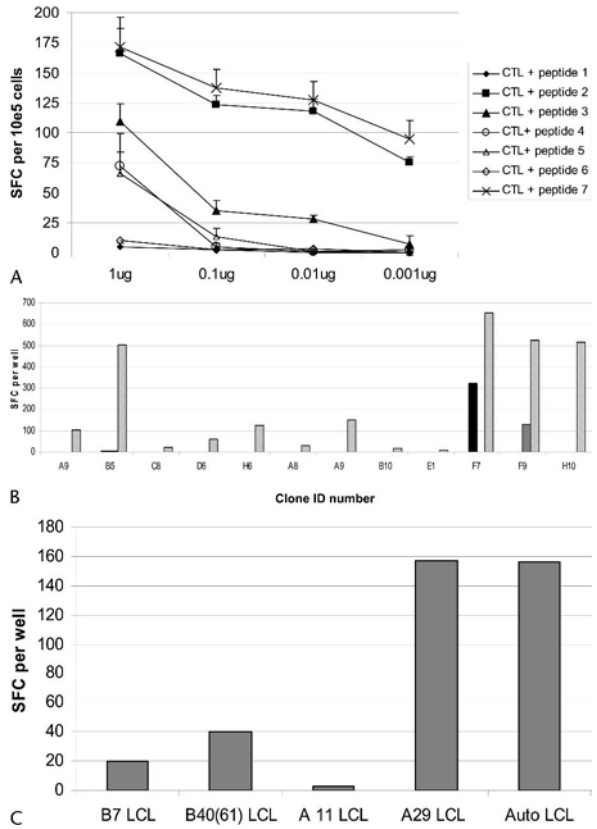


Figure 6a-6c. Identification of new HLA29*01 restricted LMP2 epitope.

Figure 6a shows the polyclonal LMP2-specific CTL line generated from patient 2 screened against shortened peptides listed in Table 1 comprising the region 345-360 of the LMP2 antigen using an IFNγ ELISPOT assay. Log dilutions of each peptide were performed before adding to the CTL. The CTL were plated at 1x10⁵ cells/well and results are expressed as spot forming cells (SFC) per 10⁵ cells. In Figure 6b LMP2-CTL clones generated from patient 2 were screened against autologous LCL (gray bars), KILLARLFLY (KIL) peptide (black bar) and PMA-ionomycin (gray stripes). As a negative control, clones were incubated in media only (black stripes). The CTL clones were plated at 10 μL/well and results are expressed as spot forming cells (SFC). Figure 6c shows the HLA restriction of clone F7. Allogeneic LCL matched at one class I locus with patient 2 whose HLA type was A11, A29, B7, B40(61) were irradiated, incubated with the KIL peptide for 1 hour and then washed and plated at 1x10⁵ cells per well. As a positive control, irradiated autologous LCL pulsed with KIL peptide were also plated as above. 10 μl of clone F7 was added to each LCL target and results are expressed as spot forming cells (SFC).

Table 1. Minimization of the LMP2-Specific CD8+ T-Cell Epitope KILLARLFLYA (Region 345–364) Detected Using the IFN-γ ELISPOT and the 15 Mer LMP2 Peptide Library

Peptide identification number	Sequence	Region
1	KILLARLF	349–356
2	KILLARLFLY	349–358
3	LLARLFLY	351–358
4	LLARLFLYA	351–359
5	LLARLFLYAL	351–360
6	KILLARLFL	349–357
7	ILLARLFLY	350–358

To determine the HLA restriction of this new LMP2 epitope, single cell clones were expanded from the LMP2-CTL line from patient 2 and tested against the KIL peptide or LCL (Figure 6b). One clone (F7) recognized KIL but not the autologous LCL. This clone was then incubated with 4 allogeneic LCL lines, matched at one class I locus each. These LCL were also pulsed with KIL. Clone F7 reacted most strongly to the HLA A29*01 LCLpulsed with KIL peptide cells, and to a minor extent with LCL targets expressing A11*01, B7*01 or B40 (61). These data indicate that this LMP2 epitope is A29*01 restricted (Figure 6c). Despite the ELISPOT results showing that ILL and KIL and induced equal responses at all concentrations, the ILL peptide was able to fold easily into the HLA A29 tetramer compared to the KIL peptide thus confirming that ILL was the minimal epitope of the KIL peptide.

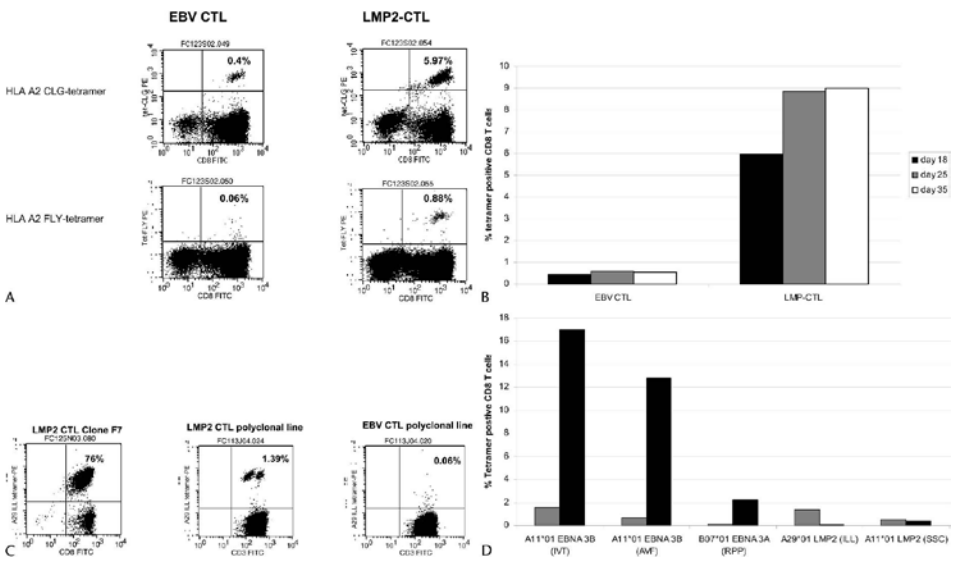


Figure 7a-7d LMP2-expressing dendritic cells effectively increase the LMP2 tetramer-specific CTL populations compared to unmodified LCL.

The specificity of EBV CTL or LMP2 CTL from patient 1 were compared by tetramer analysis using HLAA02 01-CLGGLLTMV (CLG) and HLA-A02*01-FLYALALLL (FLY) tetramers on days 18, 25 and 35 (Figures 7a and 7b). In Figure 7a, the percentage of FLY and CLG tetramer+CD8+ cells is indicated on histograms. Figure 7b shows the percentage CLG tetramer+CD8+ cells over time in the EBV-CTL line versus the LMP2-CTL line. In Figure 7c the EBV-CTL line, the polyclonal LMP-CTL line and the LMP2-CTL clone F7 generated from patient 2 were analyzed using the A29*01-ILLARLYFLY (ILL) tetramer. The percentage of ILL tetramer+CD8+ cells is indicated on histograms. Figure 7d compares the percentage of tetramer positive CD8+ cells specific for the HLA 11*01 restricted LMP2 peptide SSC and the HLA A29*01 restricted LMP2 peptide ILL with HLA A11*01 restricted EBNA 3B peptides IVTDFSVIK (IVT) and AVFDRKSDAK (AVF) and the B07*01 restricted EBNA 3A peptide RPPIFIRRL (RPP) in the EBV-CTL versus LMP2-CTL lines generated on patient 2.*

The frequency of LMP2 epitope-specific CTL is increased by stimulation with Ad5f35LMP2 transduced stimulator cells

To confirm the results of the ELISPOT assays we used tetramer analysis with the CLGA02* 01 (A2-CLG) and FLY-A02*01 (A2-FLY) tetramers to estimate the frequency of LMP2A-specific CTLs before and after stimulation with LMP-2a transduced DCs and LCL. In the unstimulated peripheral blood lymphocytes of patient 1, 0.05% and 0% of CD8+ T-cells were positive for the A2-CLG tetramer and A2-FLY tetramers respectively (data not shown). After stimulation with Ad5f35LMP2A-transduced DC and LCL, 5.97% of $CD8^+$ cells reacted with the A2-CLG tetramer and 0.88% with the A2-FLY tetramer (Figure 7a,b). In comparison, unmodified autologous LCLs as stimulators induced only 0.4% of EBV-CTLs specific for the A2-CLG tetramer and 0.06% for the A2-FLY tetramer. For Patient 2 we used the 9-mer ILL-A29*01 tetramer (A29-ILL). A29-ILL tetramer reactive T-cells could not be detected in unstimulated PBMC nor in the EBV-specific CTL line generated using non-transduced LCL. By comparison, 1.39% of CD8+ cells were detected in the LMP2-CTL line (Figure 7c). Only 0.5% of the LMP2-CTL line from patient 2 reacted with the known HLA-A11*01 restricted SSC epitope using tetramer analysis. This was similar to the frequency seen in the EBV-CTL line (Figure 7d). A significant population of the EBV-CTL from Patient 2 were specific for the known immunodominant HLA A11*01 EBNA 3B epitopes IVTDFSVIK (IVT) and AVFDRKSDAK (AVF) as well as for the B07*01 EBNA 3A peptide RPPIFIRRL (RPP) (Figure 7d). In contrast, LMP2-CTLs generated from this same patient show a decreased frequency for these immunodominant EBNA epitopes (Figure 7d). These data indicate that stimulation of patient T-cells using DC and LCL expressing LMP-2a selectively expands clones capable of recognizing LMP2 specific tetramers/peptides, whilst reducing the proportion of CTL specific for conventionally immunodominant EBV antigens such as EBNA 3A, B or C. Finally, we also observed that the frequency of peptide positive cells is higher using tetramer assay compared to ELISPOT analysis. In the LMP2-CTL line from patient 1 at total of 5.97% of CD8+ve cells recognized the CLG peptide using tetramer analysis (Figure 7a) compared to 352 spots/ 100,000 cells (0.35%) observed using ELISPOT (Figure 5a). A difference was also seen in the LMP2-CTL line from patient 2 where 1.39% of CD8+ve cells were positive for the ILL peptide (Figure 7c) versus 0.08% cells producing IFNγ in response to this peptide (data not shown). Thus ELISPOT can be used to identify specificity, but does not reveal the frequency of CTL specific for the identified epitopes.

Discussion

One means of improving immunotherapeutic strategies against EBV+ tumors in immunocompetent patients is to specifically target subdominant antigens such as LMP2 that are expressed as part of the Type II latency pattern of virus gene expression. Efforts to expand LMP2-specific CTL by stimulating PBMC with Ad5LMP2 transduced DC and expanding with transduced DC or non-transduced LCL were successful for normal donors, who have measurable level of CTL reactive with LMP2 and whose DC and CTL are uncompromised and expand readily [15]. However this approach consistently failed when patient samples were used. We now describe an approach to generate LMP2 specific CTL that is effective in patient with relapsed HD and that avoids the requirement for multiple stimulation with DCs, a technical challenge in patients heavily treated with chemoradiotherapy. [23] Our method takes advantage of an Ad5F35 vector that readily transduces B-LCL, allowing them to express high levels of transgenic LMP2a. [12] A sequence of one stimulation with LMP2a expressing DCs, to specifically reactivate LMP2- specific T-cells, followed by expansion with multiple stimulations with LMP2a-LCL substantially enriches CTL specific for LMP2a protein and peptides, whilst also decreasing the proportion of cells specific for other, conventionally immunodominant, EBV antigens. Hence by transducing DCs and LCL with LMP2a and using them as stimulators, we can bias recognition towards the subdominant LMP2A antigen rather than the highly immunodominant EBNA epitopes. Our approach significantly reduced the volume of patient blood necessary to generate the LMP2-specific CTL (LMP2-CTL) when compared to generating the LMP2-CTL using DC as the primary APC. Further, the LMP2-CTLs were generated in large numbers with growth kinetics similar to the conventional EBV-CTL lines generated from the same patients. The use of whole protein antigen expressed as a transgene from recombinant adenovirus is advantageous over single peptide antigens, since host alleles are able to select multiple HLA-restricted epitopes for presentation. By contrast, the use of single peptides restricts patient eligibility based on their HLA type and increases the chance that the antigen chosen may not be expressed by endogenous virus.

Analysis of single HLA-restricted tetramers within a polyclonal CTL line may significantly under-estimate the frequency of LMP2-specific CTLs. This is highlighted by patient 2 for whom the only previously identified LMP2-restricted epitope was the HLA A11*01- restricted epitope SSC. Only 0.5% of LMP2-CTLs from patient 2 were specific for this SSC epitope using tetramer analysis (data not shown). To identify other LMP2 epitopes we screened the LMP2-CTL lines using LMP2 peptide pools spanning the entire LMP2A antigen. We identified a new LMP2 epitope which mapped to HLA A29*01, and revealed a frequency of CTL specific for the new LMP2 epitope (ILL) that was 2 fold greater than for the previously defined A11*01-LMP2 SSC epitope. Thus the cocktail library will aid in characterizing LMP2 epitopes in CTL lines for which LMP2 epitopes have not yet been identified, and will assist the tracking of infused cells in peripheral blood.

While LMP2 peptide pools may identify multiple epitopes recognized by CTL, even this method underestimates the true breadth of the response to the viral antigen. Class I presented peptides are usually 8-10 amino acids long, and degradation of the 15-mer peptides relies on the proteasome and surface proteases.[24] It is however not clear how effective this machinery is in T-cells versus professional antigen presenting cells. Hence, 15-mer peptides added directly to T-cells may fail to elicit responses because of a lack of proteolysis of the 15-mer to an presentable 8 to 10-mer.[24 25] This problem was observed in patient 2, where the detection of HLA-B7 restricted LMP2 killing implies that epitopes not identified by the cocktail pools were also targeted. Similarly, in patient 1 only one epitope (the HLA A02*01 restricted CLG peptide) was identified using the peptide pool screening, but additional testing revealed reactivity with the known HLA A02*01 restricted FLYALALLL (FLY) 9-mer LMP2 peptide which is unusual in its dependence upon the immunoproteasome.[26;27] These problems may be specific to T-cells as APC. Of note, comparison of peptide-specific T-cell precursor frequencies by tetramer and ELISPOT analysis (Figures 5a, 5c, and 6a versus Figures 7a, 7c and 7d) shows that in concordance with previously published results performed on PBMC, the ELISPOT assay underestimates epitope-specific T-cell frequency in CTL lines by about tenfold, compared to the tetramer assay. [28;29] This may be because fewer activated T-cells secrete γ-IFN in response to specific antigen or because of activation-induced cell death in the responder population. Thus, while the ELISPO assay is useful in characterizing the epitope specificity of the CTL line, the frequencies obtained by ELISPOT do not reflect the true frequency of epitope-specific T-cells within each CTL line.

The HLA restriction of the ILL epitope was determined using T-cell clones derived from the polyclonal LMP2-CTL line. It is of note that the ILL-specific clone secreted IFNγ when incubated with the ILL peptide but not with the autologous LCL and that the EBV-CTL line generated using autologous LCL also lacked detectable ILL–specificity. This may be because a low affinity clone was selected by the cloning procedure, or that the unmodified LCL does not present the ILL peptide. Thus to generate ILL-specific CTL, over expression of LMP2a following gene transfer was required. Low-level expression of LMP2 epitopes by unmodified LCLs may be related to requirements such as immunoproteasome dependence for the generation of this peptide within LCL and/or tumor cells. [26] This is supported by the fact that the ILL peptide was calculated to be highly hydrophobic (8.73) which is predictive for proteasome dependence.[26;30] However the existence of precursor T-cells specific for this LMP2 epitope suggests that it may be relevant *in vivo*. Further work is therefore required to study the requirements for the generation of this and other "tumorassociated" epitopes within cells and determine whether these requirements are met in the Hodgkin Reed Sternberg cells themselves to allow killing of the tumor cells by the epitope-specific CTL.

In conclusion, the use of DCs and LCLs transduced with an LMP2A-encoding adenovirus vector efficiently stimulates and expands LMP2-specific CTLs derived from patients with relapsed Hodgkin Disease. Expression of the whole protein allows the activation of both CD4 and CD8 T-cells important for persistence and presentation of as yet undefined antigen epitopes, such as the new HLA A29*01 restricted epitope we describe. This approach is now being used in clinical adoptive immunotherapy protocols targeting EBV+ Hodgkin disease.

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Chapter 5

Epstein Barr Virus-Specific T-cells grafted with Minor Histocompatibility Antigen HA-1 Specificity by Retroviral T-cell Receptor Transfer

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Abstract

Minor histocompatibility (H) antigens are peptides derived from immunogenic polymorphic cellular proteins that elicit allo T-cell responses. In the HLA-matched stem cell transplantation (SCT) setting, hematopoietic tissue-restricted minor H antigens, such as HA-1, provide excellent tumor-specific target antigens. Indeed, HA-1 specific T-cells have been shown to contribute to the graft-versus-leukemia (GVL) effect induced by allogeneic SCT. However, graft-versus-host disease (GVHD) is a major complication of allogeneic SCT. Administration of donor-derived T-cells *ex vivo* selected for HA-1 specificity, instead of unselected stem cells, may induce a GVL effect while minimizing the risk of GVHD. An efficient and practical methodology that consistently accomplishes this has not yet been described. We developed a method to obtain sufficient numbers of HA-1 specific T-cells by a single transduction of Epstein Barr virus (EBV)-specific cytotoxic T-cell lines (CTL) with an HA-1 specific T-cell receptor (TCR). These CTLs have dual specificity with cytolytic activity against natively expressed EBV and HA-1^{H} antigens. We further propose a number of strategies to prevent promiscuous cross-pairing of transgenic TCR α and β chains with native TCR β and α chains to further enhance functional expression of the HA-1 TCR while eliminating the risk of introducing novel TCR specificities. This TCR transfer method facilitates the implementation of adoptive cell therapy strategies using T-cells grafted with specificity for HA-1^{H} or other target antigens into clinical studies.

Introduction

Allogeneic BMT is an established curative therapy for various hematologic malignancies.[1] However, the curative GVL effect is often accompanied by GVHD, a severe complication of allogeneic BMT. Minor H antigens, defined as peptides derived from polymorphic proteins, are responsible for both the desired GVL effect as well as GVHD.[2] The tissue distribution of minor H antigens may provide a means to separate these. In a human skin graft model it was demonstrated that T-cells specific for the ubiquitously expressed minor H antigen H-Y caused GVHD. In contrast, GVHD was minimal or absent using T-cells specific for the hematopoietic tissue-restricted minor H antigen HA-1.[3] Therefore, administration of T-cells specific for HA-1 or for other minor H antigens with expression restricted to hematopoietic tissue, instead of non-selected T-cells, is expected to minimize the risk of GvHD while preserving the GvL effect. Indeed, in a murine model, adoptive transfer of T-cells specific for a single minor H antigen was shown to eradicate leukemia cells without causing GVHD.[4]

Although methods have been developed for the generation of HA-1 specific T-cells,[5,6] these have a variable success rate likely depending on the precursor frequency of T-cells specific for HA-1.[7] Redirecting T-cell specificity by TCR transfer provides an alternative means to obtain T-cells with the desired specificity. However, although functional minor H antigen-specific T-cells have been generated using this method, extensive manipulation of the T-cells was required, making clinical application complex.[8,9] The difficulty of obtaining bright expression of a transgenic TCR in polyclonal human T-cell populations may be attributed to a number of factors. First, efficient retroviral transduction with bright transgene expression in primary human T-cells has been difficult to accomplish. Secondly, when the TCRα and β chains are expressed from separate vectors, only a limited number of T-cells will have a sufficient expression of both TCR chains to form a functional TCR. Vectors with dual promoters or internal ribosomal entry sequences (IRES) may not provide balanced expression. Thirdly, transgenic α and β chains may cross pair with native β and α chains, which reduces expression of the correct TCR. The observation that transgenic TCR expressing T-cells were detectable by tetramer staining in transduced T-cell lines lacking native TCRs[10] but not in non selected human T-cells supports this hypothesis.

The aim of this study is to develop a protocol that facilitates the generation of a sufficient number of T-cells expressing a functional HA-1 TCR after a single transduction step. We grafted EBV-CTLs with HA-1 TCRs, as we have extensive expertise in generating these CTL lines that have proven effective in prevention and therapy of EBV-associated disease in SCT recipients.[11,12] Further, transduced CTL with dual specificity will provide both protection against EBV-associated disease and provoke a GVL effect. Here, we describe a method that takes advantage of a foot-and-mouth disease virus-derived auto-cleavage sequence, and a retroviral expression system that allows for high, efficient transgene expression to obtain CTLs with dual specificity for natively expressed HA-1 and EBV antigens.

Methods

HA-1 TCR construct

HA-1 is a nonapeptide derived from a diallelic gene with a single amino acid polymorphism: HA-1^H is the immunogenic allele expressed in the context of HLA-A2 at the cell surface, whereas HA1R is not expressed.[13] An HA-1 specific HLA-A2 restricted T-cell clone (3HA15) was previously isolated from a patient with GVHD post HLA-identical SCT.[14] Plasmids containing the genes encoding for the α and the β chain derived from this T-cell clone were provided by Els Goulmy, Leiden University Medical Center, Leiden, the Netherlands. This TCR consists of an AV3S1 chain and BV7S9, the latter commonly used by HA-1 specific TCRs.[15] The β chain and α chain were fused by a foot-and-mouth disease virus-derived 2A sequence in frame by splicing using overlap PCR.[16] The construct was subsequently cloned into the retroviral vector SFG[17] (Figure 1). CD3 elements were all cloned separately by PCR from IMAGE EST clones (5745355, 5214563, 5214563, 5752844 for δ, γ, ε, ζ respectively, Invitrogen, Carlsbad, CA) into SFG vectors as NcoI / MluI fragments. All cloning steps were checked by sequence analysis.

Cells

NIH-293T cells were obtained from ATCC and cultured in IMDM (Biowhittaker, Cambrex, Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT) and Glutamax (Gibco, Grand Island, NY). EBV-specific CTL lines were reactivated and expanded from peripheral blood mononuclear cells (PBMC) in 50% RPMI (Hyclone)/50% Clicks media (Irvine, Santa Ana, CA)(supplemented with 10% FBS and Glutamax) using autologous LCL as antigen presenting cells, as described previously.[12] T lymphoblasts were generated by stimulating PBMC ($1x10^6$/well) with Phytohemagglutinin (PHA) (1 µg/ml). From day 2 onwards, IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, 100 U/ml) was added every 3-4 days. PHA blasts cultured for > 7 days were used as targets cells in ELISPOT and cytotoxicity assays. Typing of the HA-1 allele on HLA-A2 positive target cells was performed by allele-specific PCR as previously described.[18]

Retroviral supernatant

Transient retroviral supernatant was produced by co-transfection of NIH 293T-cells with the MoMLV gag-pol expression plasmid PeqPam3(-env), the RD114 envelope expression plasmid RDF, and SFG vectors at a ratio of 2:3:3 respectively, with a total of 10 µg DNA. The transfection was facilitated with gene-juice reagent (Calbiochem, San Diego, CA). The supernatant was harvested 2 and 3 days after transfection, snap-frozen and stored at –80°C in 5 mL aliquots.

Transduction

Non-tissue coated 24-well plates were coated with 1 mg/mL retronectin (Takara Biochemicals, Shiga, Japan) in PBS overnight at 4°C. Retronectin-coated wells were then pre-incubated twice with 0.5 mL of retroviral supernatant at room temperature for 30 minutes. Subsequently EBV-specific CTL, day 3-4 post antigenic stimulation, were plated on the pre-coated wells ($3x10^5$/well in 0.5 mL of CTL media) and 1 mL of retrovirus/well was added. Plates were centrifuged at 1000 x g for 30 minutes at room temperature and then incubated for 2-3 days at 37°C in the presence of 100 U/mL IL-2. After transduction T-cells were transferred to a tissue-culture treated plate, facilitated by cell-dissociation medium (Sigma, St Louis, MO) and expanded by weekly antigenic stimulations with LCL and bi-weekly IL-2.

TCRαβ and tetramer staining/sorting

293T-CD3 were transfected with 2 µg of indicated plasmid DNA using GeneJuice transfection reagent. Forty-eight hours post transfection cells were stained with TCRαβ-antibody (BD Biosciences, Pharmingen, San Diego, CA) and appropriate isotype controls. Transduced EBV-specific CTL were stained with APC-labeled HLA-A2 HA-1^H tetramer (generated by Michel Kester and obtained from Els Goulmy, LUMC, the Netherlands, 1:50 dilution) for 30 minutes at room temperature after pre-staining with CD8 mAbs (BD Biosciences, San Diego, CA). Stained cells were either analyzed on a FACSscan flow cytometer (BD Biosciences, San Diego, CA) or sorted for the desired phenotype on a MoFlo® cytometer (Dako Cytomation, Ft Collins, CO).

Cytotoxicity assay

The cytotoxic activity of transduced and non-transduced CTL was evaluated in a standard 4-hour ^{51}Cr release assay. Target cells incubated in complete medium or in 1% Triton X-100 (Sigma, St Loius, MO) were used to determine spontaneous and maximum ^{51}Cr release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release - spontaneous release)/(maximal release - spontaneous release).

Enzyme-Linked Immunospot (ELISPOT) assay

96-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated overnight with 10 µg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH). Non-transduced and HA-1 TCR transduced CTL were plated at indicated concentrations. Target cells were non-pulsed or pulsed with HA-1^H peptide (30 minutes, at 370C at indicated concentrations), irradiated (40 Gy) and plated at $5x10^4$ cells/well. After 18-24 hours, the plates were washed and incubated with the secondary biotin conjugated anti-IFN-γ monoclonal antibody (Detector-mAb (7-B6-1-Biotin), Mabtech). After incubation with Avidin:biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit (Standard), #PK6100, Vector Laboratories, Burlingame, CA) plates were developed with AEC substrate (Sigma, St. Louis, MO). Plates were sent for evaluation to Zellnet Consulting, New York, NY. Results are shown as spot forming cells (SFC) per $1x10^3$ T-cells.

Results

Expression of transgenic HA-1 TCR in CD3-293T-cells

First, a retroviral vector was constructed that allows for expression of both HA-1 TCR chains. The coding sequence for the β and the α chain of an HA-1 specific TCR was linked in frame with a foot-and-mouth disease virus-derived 2A sequence (Figure 1).[16] Self-cleavage at the C-terminus of the 2A-sequence during translation allows for equal expression of both TCR chains within transduced cells. This TCR construct is expressed from SFG, a splicing oncoretroviral vector, that drives high transgene expression upon integration in human T-cells.[17] To allow for rapid testing of expression of transgenic TCRs, NIH-293T-cells were multiply transduced with the γ, δ, ε and ζ components of CD3 and subsequently selected for CD3 expression (Figure 2a-b). This 293T-CD3 cell line accommodates TCR complex formation and expression: upon co-transfection with the HA-1 TCR α and β chain expression of the TCR was detectable by staining with a TCRαβ antibody, while TCRαβ expression was absent after transfection with the HA-1 TCR α chain or β chain alone (Figure 2 c-f). As anticipated, when the HA-1 TCR α and β chain were expressed from a single vector the number of 293T-CD3 cells staining positive for TCRαβ approximately doubled (Figure 2 f).

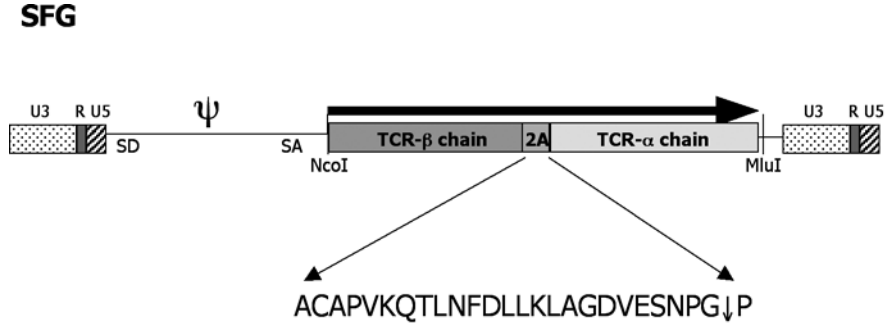

Figure 1. Vector design to obtain expression of TCRα and TCRβ from a single retroviral vector using a 2A-sequence

The HA-1 TCR β-chain is cloned in frame with the HA-1 TCR α-chain using a foot-and-mouth disease virus-derived 2A-sequence (amino acid sequence is indicated). Upon translation this protein undergoes auto-cleavage between the Glycine and the Proline at the C-terminus of the 2A-sequence (indicated by the ↓ symbol). The N-terminal part of the 2A-sequence remains attached to the C-terminus of the TCRβ chain, whereas a Proline is attached to the N-terminal sequence of the TCRα chain and is subsequently cleaved with the signal peptide. The HA-1 TCRβ-2A-TCRα cassette was cloned into the SFG vector as a NcoI-MluI fragment. SD = splicing donor, SA = splicing acceptor.

Expression of transgenic HA-1 TCR on EBV-specific CTL

To facilitate transduction of EBV-specific CTL, RD114-pseudotyped retrovirus was produced using this HA-1 TCRβ.2A.α vector.[19] CTL from an HLA-A2^{neg} donor (HLA-A1, A24, B8, B18) were used to prevent lysis of transduced and non-transduced CTL by CTL grafted with HA-1 specificity and to demonstrate proof of principle. However, this strategy should also be applicable to the more clinically relevant HLA-identical setting with an HLA-A2^{pos}HA-1^{R} donor and an HLA-A2^{pos}HA-1^{H} recipient. On day 3 after retroviral transduction, non-transduced and HA-1 TCR transduced CTL were stained with HLA-A2 HA-1^{H} tetramer to evaluate transgenic TCR expression: 3.58% of HA-1 TCR transduced CTL stained positive with the HLA-A2 HA-1^{H} tetramer as compared to 0.25% of non-transduced CTL (Figure 3).

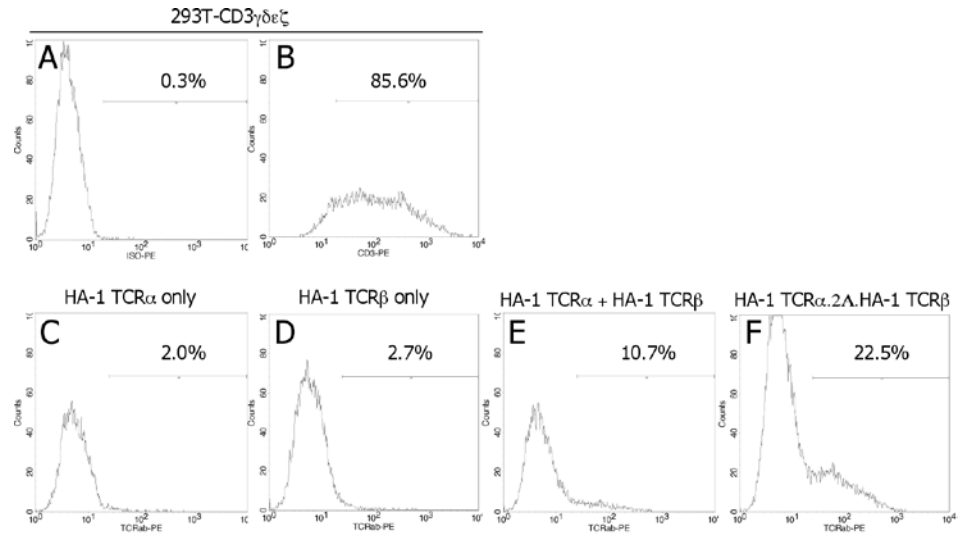

Figure 2. Expression of HA-1 TCR on CD3-293T-cells

NIH-293T were multiply transduced with RD114-pseudotyped retrovirus from the SFG vectors expressing CD3 γ, δ, ε, and ζ. Subsequently 293T-cells staining positive for CD3-PE were selected using the MACS system. Isotype IgG1 (A) and CD3 Mab (B) staining of this cell line are shown. 293T-CD3 cells were transfected with SFG/HA-1 TCRα alone (C), SFG/HA-1 TCRβ alone (D), SFG/HA-1 TCRα and SFG/HA-1 TCRβ (E) or SFG/HA-1TCRb.2A.α (F) and stained with TCRαβ Mab. Isotype controls were performed (data not shown).

Dual specificity of HA-1 TCR transduced CTL for EBV+ and HA-1H targets

To assess the function and specificity of the transgenic TCR, HA-1^{H} tetramer-positive T-cells were selected. IFN-γ secretion upon incubation with HLA-mismatched (HLA-A2, A3, B7, B51) HA-1^{R} PHA blasts and HLA-mismatched (HLA-A2, B27, B51) HA-1^{H} PHA blasts was determined in an ELISPOT assay. HA-1 TCR transduced CTL but not non-transduced CTL recognized HLA-A2^{pos} HA-1^{R} PHA blast pulsed with 1 μg/mL HA-1^{H} peptide (Figure 4). Importantly, IFN-γ secretion was also induced upon stimulation with PHA blast natively expressing HA-1^{H}. In addition, EBV-transformed B cells (LCL) were recognized by both non-transduced and HA-1 TCR transduced T-cells in an HLA restricted manner demonstrating dual specificity of HA-1 TCR transduced CTL for HA-1^{H} and EBV-antigens.

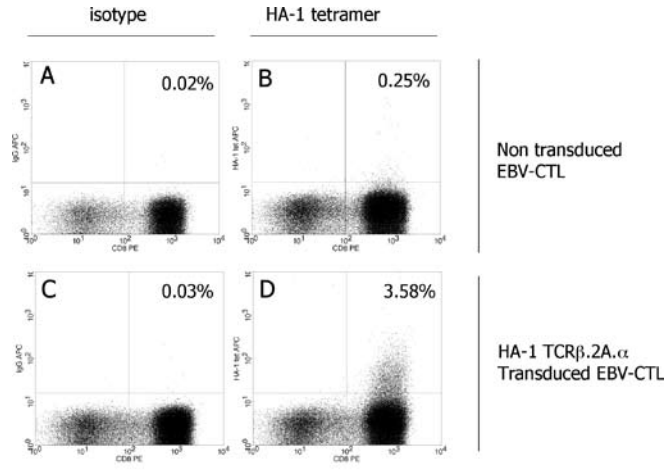

Figure 3. Transgenic HA-1 TCR expression in human EBV-specific CTL

Non-transduced (A+C) and HA-1 TCRβ.2A.α transduced (B+D) EBV-specific CTL (day 3 post transduction) were stained with APC-labeled HLA-A2 HA-1^{H} (VLHDDLLEA) tetramer (B+D) or with isotype control (A+C) after pre-staining with CD8-PE. Indicated are the percentages of total T-cells staining positive with tetramer.

HA-1 TCR transduced CTL lyse hematopoietic cells natively expressing HA-1H

Cytolytic capacity upon recognition of targets cells through both native and transgenic TCRs was evaluated in a 4-hour ^{51}Cr release assay using non-selected HA-1 TCR transduced CTL. Both non-transduced and HA-1 TCR transduced CTL lysed autologous LCL (Figure 5). HA-1 TCR transduced CTL lysed not only HA-1^{H} peptide pulsed HLA-$A2^{pos}$ HA-1^{R} target cells but also PHA blasts with endogenous HA-1^{H} expression, whereas HA-1^{H} directed lysis was absent in non-transduced CTL. These results demonstrate that despite a relatively low percentage of CTL staining positive with HA-1^{H} tetramer, specific lysis of targets cells expressing HA-1^{H} at a physiological level can be obtained.

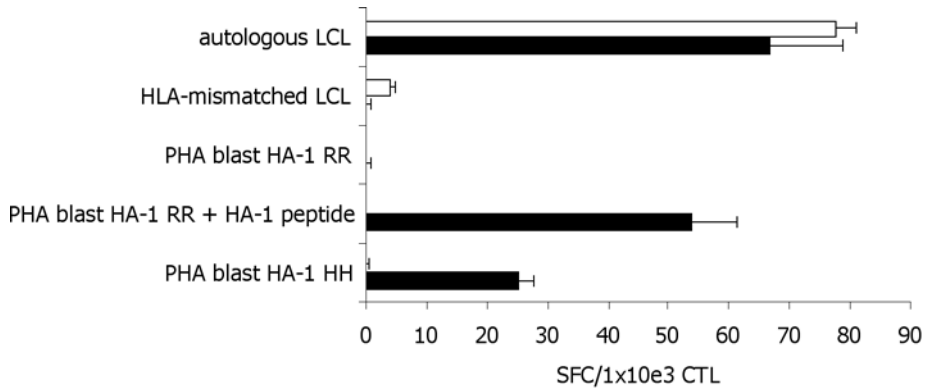

Figure 4. IFN-γ secretion of HA-1 TCR transduced EBV-CTL upon stimulation with EBV^{+} and HA-1^{H} target cells

IFN-γ secretion by non-transduced CTL (open bars) and HA-1 TCR transduced CTL sorted for HLA-A2 HA-1^{H} tetramer expression (closed bars) upon incubation with autologous LCL, HLA-mismatched LCL, non pulsed and HA-1^{H} peptide pulsed (1 μg/mL) HLA-mismatched $A2^{pos}$-HA-1^{R} PHA blasts and HLA-mismatched $A2^{pos}$HA-1^{H} PHA blasts was measured in an ELISPOT assay. Indicated is the mean and standard deviation of triplicate wells after subtraction of background from stimulator cells alone as Spot Forming Cells (SFC) per 1x10^{3} CTL.

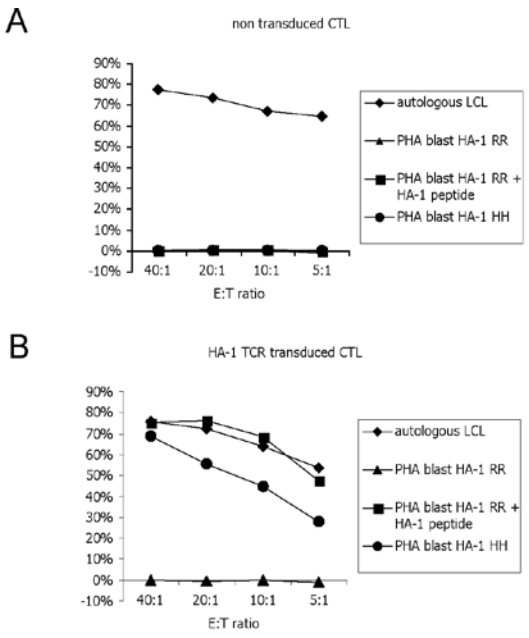

Figure 5. HA-1 TCR transduced EBV-CTL lyse both EBV^{+} and HA-1^{H} targeT-cells

Cytolytic activity of non-transduced (A) and HA-1 TCR transduced EBV-specific CTL (B) was tested in a 4-hour ^{51}Cr assay against autologous LCL, non pulsed and HA-1^{H} peptide pulsed (10 μg/mL) HLA-mismatched $A2^{pos}$HA-1^{R} PHA blasts and HLA-mismatched A2posHA-1H PHA blasts targeT-cells at indicated effector:target (E:T) ratios.

Discussion

A method that allows for generation of HA-1 specific T-cells from all HA-1 negative (HLA-$A2^{neg}$, HLA-$A2^{pos}$HA-1^{R}) SCT donors would facilitate adoptive T-cell therapy in combination with allogeneic SCT as treatment for HA-1^{H}-positive malignancies. TCR transfer provides an attractive strategy to generate HA-1 specific T-cells, but its feasibility has been hampered by difficulty obtaining functional transgenic TCR expression in a high number of T-cells. We addressed this problem by using RD114-pseudotyped retrovirus that facilitates efficient transduction of T-cells, a splicing SFG vector that accommodates high transgene expression and a 2A sequence to obtain equal expression of both TCR chains from a single vector. A single transduction of EBV-specific CTL was sufficient to achieve HA-1 TCR expression detectable by tetramer staining. HA-1 TCR transduced CTL lyse target cells natively expressing HA-1^{H} and EBV-antigens demonstrating expression of the transgenic TCR at functional levels. Importantly, similar EBV-specific responses in non-transduced and HA-1 TCR transduced CTL demonstrated that T-cell activity mediated through endogenous TCRs was not affected by the expression of transgenic HA-1-specific TCRs.

Although functional expression of the HA-1 TCR was clearly obtained, the number of T-cells staining positive with HA-1 tetramer indicated lower transduction efficiency as regularly obtained with a marker gene using this same procedure. This observation suggests that not all transduced T-cells express the TCR at a sufficient level to allow its detection by tetramer staining. This reduced expression may be the result of pairing of the transgenic TCR chains with native TCR chains. Cross-pairing of native and transgenic TCR chains further possesses the risk of forming TCRs with new specificities including auto-antigens. Strategies to preferentially pair transgenic TCRα and TCRβ chains may overcome these problems. These include the generation of chimeric TCRs consisting of human variable domains and murine constant regions. However, the immunogenicity of such chimeric TCRs hinders their clinical application. The incorporation of CD3-ζ in the intracellular part of the TCRα and β chain or the formation of a single chain TCR, have been proposed by others.[20,21] This was not successful in our hands. Alternatively, leucine zipper motifs can be included in the constant region of the transgenic α and β chain to force their paring,[22] a strategy we are currently exploring.

Once the strategy of TCR transfer has been further refined, this approach should be transferable to any TCR. This requires an efficient high through put screening of TCRs with the desired characteristics. We are currently generating variants of the 293T-CD3 cell lines that co-expresses CD4 and CD8 to allow for evaluation of TCR expression by tetramer staining. T-cells with the desired specificity can be obtained from donors with a detectable precursor frequency by tetramer sorting. By PCR, a plasmid library coding for the TCRα and β chains obtained from the cDNA of the tetramer-positive cells can be generated. This plasmid library could be screened by transfection into 293T-CD3-CD8 cells, and subsequent staining with TCRαβ MAb and tetramer. Tetramer-dissociation assays in the transfected 293T-CD3-CD8 may allow for selection of high affinity TCRs. Finally, a group of good candidates are cloned into the SFG vector using the 2A sequence and tested for cytolytic function in primary T-cells against target tumor cells.

The large *in vivo* expansion of HA-1 and HA-2 specific T-cells in responding patients treated with donor lymphocyte infusion for relapsed leukemia after SCT indicates the contribution of minor H antigen-specific T-cells to the GVL effect.[23] The observation that HA-1^{H} is aberrantly expressed on malignant epithelial cells but absent on normal tissue[24] opens the possibility of allogeneic SCT combined adoptive transfer of HA-1 specific T-cells as treatment for solid tumors.2 Indeed, minor H antigen-specific T-cells could be isolated in patients that underwent allogeneic SCT for renal cell carcinoma.[25]
Depending on risk factors such as T-cell depletion and EBV status prior to transplantation, SCT and solid organ transplant recipients are at risk for developing EBV-associated lymphoproliferative disease.[26] In those patients, using T-cells with dual HA-1^{H} and EBV-specificity would provide an ideal approach. In settings were EBV-reactivity is not a major concern, HA-1 TCR transduced polyclonal lymphocytes may be used as infusion product. However, although infusion of unselected donor lymphocytes has proven anti-tumor activity[23], this approach has the inherent risk of inducing GVHD. Further, depending on the activation protocol used to enable transduction, T-cell receptor repertoire and T-cell subset profiles can be altered in transduced T-lymphocytes, which may impair their function or persistence.[27-29] In contrast, EBV-specific T-cell populations do not induce GVHD and antigenic stimulation with LCL to accommodate transduction does not appear to affect CTL function. Finally, the presence of latently infected B-cells in EBV-seropositive recipients may prolong the persistence of infused EBV-specific CTL[30], an effect that could be enhanced by vaccination with irradiated LCL. We therefore propose to use EBV-specific CTL as the source cell product to develop immunotherapeutic strategies based on transgenic TCRs.

In conclusion, TCR transfer provides an attractive and feasible means to obtain tumor antigen specific T-cells for large patient groups. Further optimization steps to enforce correct TCR pairing and to enhance expression are required to allow evaluation of the clinical anti-tumor potential of HA-1 specific T-cells in both SCT and solid organ transplant settings.

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Chapter 6

A Chimeric T-cell Antigen Receptor that Augments Cytokine Release and Supports Clonal Expansion of Primary Human T-cells

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Abstract

The transduction of primary T-cells to express chimeric T–cell receptors (cTCR) for redirected targeting of tumor cells is an attractive strategy for generating tumor-specific T-cells for adoptive therapy. However, tumor cells rarely provide co-stimulatory signals and hence cTCRs that transmit just a CD3ζ signal can only initiate target cell killing and Interferon-γ release, and fail to induce full activation. Although incorporation of a CD28 component results in IL-2 release and limited proliferation, T-cell activation remains incomplete. OX40 transmits a potent and prolonged T-cell activation signal and is crucial for maintaining an immunological response. We hypothesize that the CD28-OX40-CD3ζ tripartite cytoplasmic domain will provide a full complement of activation, proliferation and survival signals for enhanced anti-tumor activity.

Introduction

Adoptive immunotherapy with T-cells appears effective for treatment of certain malignant and infectious disorders.[1-4] There are however, considerable barriers to expanding the scope of this approach, not least in generating high affinity T-cells with appropriate anti-tumor effector function. This problem may be overcome by the use of chimeric T-cell receptors (cTCRs). cTCRs are fusions between an antigen-recognizing ectodomain and a signaling endodomain (Figure 1a).[5] In their simplest form, these chimeric molecules connect the antigen-recognition properties of antibodies with the signaling endodomain of CD3ζ. T-cells transduced with cTCR are redirected to almost any target, providing an attractive strategy to generate large numbers of tumor-specific T-cells. CD3ζ-based cTCRs have been tested in clinical studies to target HIV infected cells[6-8]. Although findings from these studies may not be directly applicable to cancer immunotherapy strategies, it is notable that cTCR-transduced T-cells did not appear to proliferate significantly , and that they only had a marginal therapeutic effect.

Tumor cells rarely express co-stimulatory molecules. Moreover, the lack of an innate inflammatory response to most tumors means that chimeric T-cells do not receive co-stimulatory cues from accessory immune cells. Instead cTCR-expressing T-cells targeting a tumor must function and survive solely on signals received through the chimeric receptor. Although signals generated by CD3ζ cTCRs can recruit the cellular killing machinery[9], these constructs failed to fully activate resting T-cells.[10,11] As a consequence T-cells fail to divide, lose activity and perform poorly. Improved function requires signaling through the antigen receptor to be accompanied by co-stimulatory stimuli.[12] CD28 signaling is a critical initial co-stimulatory signal, and incorporation of the endodomain of CD28 leads to proliferation and IL-2 release.[13,14] Nonetheless, activation remains suboptimal, so that T-cells fail to maintain their expansion and function following engagement of their chimeric receptor.

Physiologically, optimal activation requires CD28 engagement to be followed by co-stimulation through other T-cell signaling molecules. Amongst the most important of these is X40 (CD134)[15], which is expressed on T-cells 24 hours after antigen and CD28 stimulation.[15-17] The ligand for OX40 (OX40L) is expressed on antigen-presenting cells several hours to days after their own activation.[18-20] *In vitro* and studies have detailed how OX40 signaling can further augment CD28-activated T-cell responses, enhancing proliferation, cytokine secretion and survival[21-23]. Hence, CD28 signaling provides the initial co-stimulus for proliferation, while subsequent OX40 signaling allows effector T-cells to survive and continue proliferating.

We hypothesized that antigen engagement of a chimeric antigen receptor linked to an endomain supplying CD3ζ, CD28 and OX40 signals *in cis* would produce sustained activation, proliferation and effector function in resting T-cells. This would allow chimeric receptor mediated recognition of a tumor cell lacking costimulatory molecules, but expressing the target antigen, to result in transmission of a full proliferative signal. We used a model of human T-cells transduced with a GD-2 targeting ectodomain. When coupled to a conventional CD3ζ endodomain, these T-cells kill GD2+ neuroblastoma lines but fail to proliferate or sustain their function. We now show that inclusion of endodomains capable of acting sequentially in the T-cell activation cascade lead to greatly increased proliferation, cytokine release and effector function.

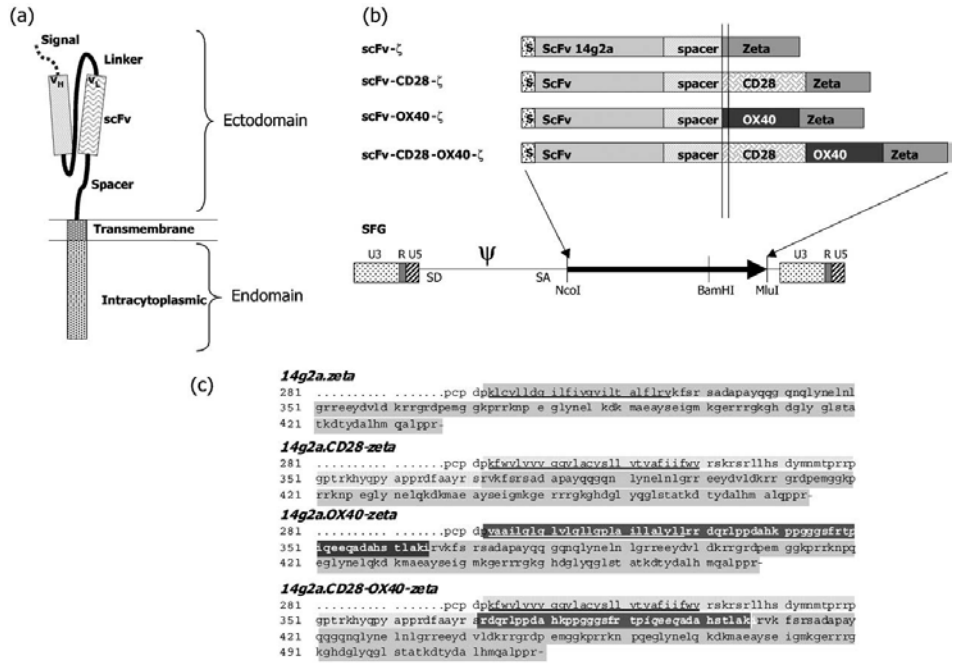

Figure 1. Receptor and vector design.

***(a)** Schematic diagram of cTCR. The ectodomain is a single-chain variable fragment (ScFv) derived from a monoclonal antibody 14g2a linked to a flexible spacer derived from IgG1 hinge. The ectodomain recognizes and binds target antigen diasialoganglioside (GD2). The endodomain transmits intracellular signals and is connected to the ectodomain via a hydrophobic transmembrane region. The lipid bilayer isolates the two domains. **(b)** Maps of cTCRs and retroviral vector. SFG is a splicing MoMLV based retroviral vector. Gag/pol start codon has been mutated out. **(c)** amino-acid sequence of transmembrane and endodomains of different receptors. Underlined residues form the transmembrane domain. Residues piqee form the TRAF-2 consensus binding domain.*

Methods

Constructs

cTCR were generated using splicing by overlap PCR. The scFv derived from 14g2a has been described previously.[24] The coding sequence for CD28 was derived from Jurkat cDNA, while the coding sequence for the OX40 endodomain was derived from IMAGE clone 4334567 (Invitrogen, CA). Annotated amino-acid sequences for all constructs are provided in Figure 1c. All constructs were cloned into SFG NcoI site[25] and were all identical down to the BamHI site, which marks the junction between the ectodomains and the transmembrane domain (Figure 1b). The NFκB-luciferase reporter retrovirus was constructed by PCR cloning NFκB-RE-luciferase (Clontech, CA) as a BamHI/HindIII fragment into the SIN retroviral vector pSuper.retro (Oligo-engine, WA). FLAG-tagged TRAF2 was generated by cloning a TRAF2 coding sequence PCR product as HindIII/BamHI fragment into pFLAG3xCMV (Sigma, MO). cTCR V5-tagged constructs were generated by TOPO cloning PCR fragments of the entire receptor except the stop-codon into pCDNA3.1-V5-His expression plasmid (Invitrogen, CA). All PCR products were generated using Phusion polymerase (MJ Research, MA). All PCR generated cloned fragments were sequenced by primer extension sequencing (ABI Prism 3300). RDF – an expression plasmid for RD114 retrovirus envelope was a generous gift of Mary Collins. PeqPam3(-env) – an expression plasmid for Moloney Leukemia virus gagpol was a generous gift of Elio Vanin.

Cell lines

NIH 293T, LAN-1 and LAN-5 cells were obtained from ATCC and cultivated in IMDM (BioWhittaker) supplemented with 10% heat-inactivated FCS (Hyclone, UT) and 5 mM L-glutamine. Peripheral blood (PB), obtained from normal donors with informed consent was processed by centrifugation over Ficoll gradients. PB mononuclear cells (MC) and T-cells were cultured in 45% RPMI (Hyclone, CA), 45% Click's(Irvine, CA) medium and 10% heat-inactivated FCS and 5 mM L-glutamine.

Immunoprecipitation

$1.5x10^6$ NIH-293T-cells were transfected with 1 μg of pFLAG3xCMV-Traf2 and 8 μg of V5-tagged receptors. Transfection was facilitated by GeneJuice transfection reagent (Calbiochem, CA). 24 hours after transfection, cells were co-cultured 1:1 with GD-2 positive LAN-1 cells to cross-link the receptor. Twelve hours later, cells were lysed and proteins precipitated with anti-V5 antibody (Invitrogen, CA) using a co-immunoprecipation kit (Sigma, MO). The immunoprecipitate was run on a SDS-PAGE gel and blotted with anti-FLAG peroxidase conjugated antibody and M5 antibody. Input lysate was also analysed on the same gel and blotted for V5 and FLAG.

Retroviral supernatant

Transient retroviral supernatant was produced by co-transfection of NIH 293T-cells with the MoMLV gagpol expression plasmid PeqPam3(-env), the RD114 env expression plasmid RDF and SFG vectors at a ratio of 2:3:3 respectively, with a total of 10 μg DNA. The transfection was facilitated with gene-juice reagent (Calbiochem, CA). The supernatant was harvested 2 and 3 days after transfection, snap-frozen and stored at –80°C in 5 ml aliquots.

Transduction
Non-tissue coated 24-well plates were coated with 1 mg/ml retronectin (Takara Biochemicals, Shiga, Japan) overnight. The wells were coated with retroviral supernatant. Subsequently peripheral blood T-cells stimulated with Phytohemagglutinin (1 µg/ml) for 3 days and IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, 100 U/ml) for 1 day were added. Cells were incubated for 2 days on the virus-coated plate in the presence of 100 U/ml IL-2. Then T-cells were transferred to a tissue-culture treated plate, facilitated by cell-dissociation medium (Sigma, MO).

Luciferase assay
PHA and IL-2 stimulated T-cells were first transduced with transient supernatants containing vectors coding for pNFKB->Luc. Twenty-four hours after transduction cells were divided and transduced again with supernatant containing vector coding for 14g2a-ζ, 14g2a-CD28-ζ, 14g2a-OX40-ζ or 14g2a-CD28-OX40-ζ. T-cells were rested for 5-7 days post-transduction without IL-2. Next, T-cells were stimulated at a 1:1 ratio with LAN-1 cells. Forty-eight hours later, the cells were harvested and a protein lysate from an equal number of T-cells was made. 20 µL of protein lysate was mixed with fire-fly luciferase substrate (Promega, WI) and the luminosity measured (monolight 3010, Pharmingen).

Cell-sorting
Sorting the CD8+ fraction of T-cells was performed using anti-CD8 conjugated paramagnetic beads in the CD8+ T-cell isolation kit (Miltenyi biotech, Auburn, CA) and following the manufacturer's instructions.

Co-cultures
After transduction, cells were rested in RPMI 10% FCS 5mMol L-glutamine, without IL-2, for 5-7 days. On the day of first stimulation, the cells were washed counted and plated at $1x10^6$ per well of a 24 well plate at a ratio of 1:1 with 80 Gy irradiated LAN-1 cells. Co-cultures were performed in the absence of IL-2 and in the presence of low-dose IL-2 (20 U/ml).

Cytokine assays
Supernatant was stored at –80°C and the concentration of IFN-γ, IL-2, IL-4, IL-5, IL-10 and TNF-α in CTL culture supernatants was measured using the Human Th1/Th2 cytokine cytometric Bead Array (BD Pharmingen, San Jose, CA) and analyzed with a FACSscan flow cytometer (Becton Dickinson, CA).

Cytotoxicity assay
The cytotoxic activity of transduced and non-transduced PHA blasts and CTL was evaluated in a standard 4-hour ^{51}Cr release assay, as previously described.[35] Target cells incubated in complete medium or in 1% Triton X-100 (Sigma, MO) were used to determine spontaneous and maximum ^{51}Cr release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release - spontaneous release)/(maximal release - spontaneous release).

Statistical analysis
ANOVA was used to evaluate if a significant difference existed between differently transduced cells. Subsequently Student's t-test was used to calculate p-values when comparing two different conditions.

Results

Expression of chimeric TCR constructs in primary T-cells.
The ectodomain, or antigen-recognizing domain of the cTCR used throughout this study, is a single-chain variable Fragment (scFv), derived from GD-2 recognizing antibody 14g2a (Figure 1a). A short spacer derived from IgG1 hinge region connects this scFv to the transmembrane domain. Endodomains used were derived from CD3ζ, CD28 endodomain fused to that of CD3-ζ, endodomain of OX40 fused to that of CD3-ζ or all three endodomains fused in the order CD28, OX40, CD3-ζ. The junctions are illustrated in Figure 1c. These chimeric receptors were expressed in human T-cells using retroviral vectors. All cTCRs migrated at the predicted size as determined by Western blots probed with an anti-CD3ζ antibody (Figure 2a). Under non-reducing conditions, these TCRs exist as homodimers (data not shown). Surface expression of each cTCR on primary T-cells was confirmed by detection of the single chain variable fragment (scFv), using a polyclonal goat anti-mouse-Fab antibody. Constructs containing the CD28 trans-membrane domain had the brightest expression (mean MFI 342) while expression from those containing OX40-ζ and CD3-ζ domains alone had mean MFI of 244 and 198 respectively (Figure 2b). This expression was stable for over 6 weeks.

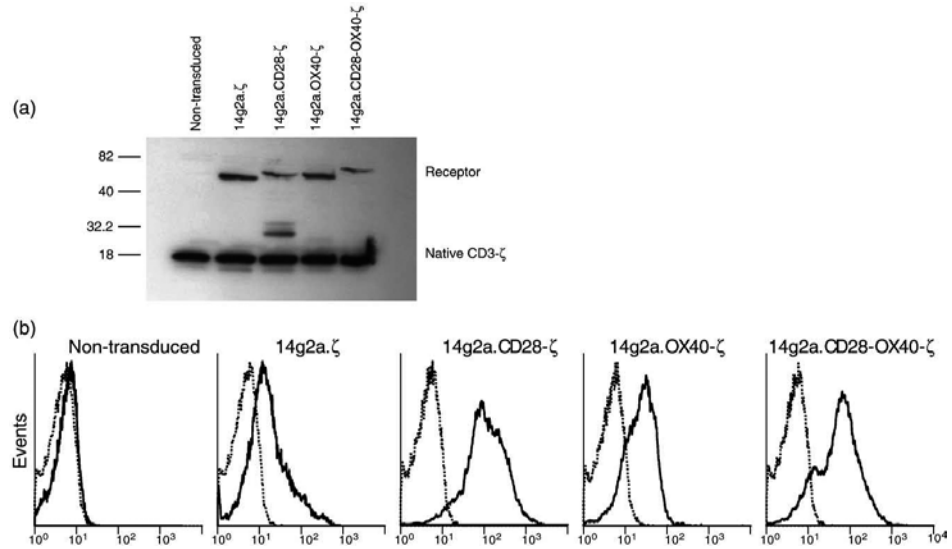

Figure 2. Expression of chimeric receptors in T-cells.
(a) *Jurkat T-cells were transduced with different constructs. Receptor-bright cells were selected using biotin-polyclonal anti-murine F(ab)' antibodies in conjunction with streptavidin-ferromagnetic beads. Western blot was performed under reducing conditions with an antibody detecting CD3ζ. Native CD3ζ is blotted for simultaneously.* **(b)** *Expression of cTCRs in primary T-cells. Peripheral blood T-cells were stimulated with PHA and IL-2 and transduced with different constructs. T-cells were stained with Cy-5 conjugated polyclonal anti-murine F(ab') and analyzed by flow-cytometry.*

OX40 as part of the TCR endodomain signals through its native mediators.
Since the cTCR sandwiches the relatively small intracellular domain of OX40 between the CD3-ζ and CD28 domains, we determined whether it retained the ability to interact with its downstream adaptor molecule TRAF2. We successfully co-immunoprecipitated TRAF2 with TCRs containing the OX40 domain (Figure 3a) in transfected 293T-cells, demonstrating sufficient access of TRAF2 to its binding site in the endodomain of the cTCRs. As OX40

triggers NFkB activity via its intermediate TRAF2, we next compared NFkB activation after stimulation of each of the cTCRs we had constructed and transduced into primary T-cells. We prepared a retroviral vector in which Luciferase expression was driven from a NFkB response element. To prevent read-through from the 5'LTR we used a vector with a non-functional 3'LTR U3 region, so that the 5' and 3' LTR were silenced following retroviral integration. Primary T-cells were first transduced with this reporter construct, and the cell cultures then split and transduced with vector encoding each receptor. Transduced T-cells were harvested on day 2 after stimulation with tumor cells, and cell lysates analyzed for luciferase activity. NFκB activity was more than an order of magnitude higher in T-cells transduced with 14g2a.CD28-OX40-ζ compared to 14g2a.CD28-ζ transductants (Figure 3b; p<0.001). These data indicate that OX40 incorporated in an cTCR is able to bind TRAF-2 to transmit its downstream signal, and potently induces NFκB activity when a CD28 signal is transmitted simultaneously.

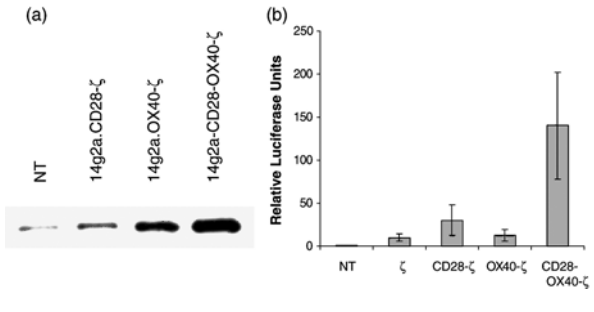

Figure 3. Down-stream signaling of chimeric receptors.
(a) *co-ip of FLAG-tagged TRAF2 with V5-tagged receptors in transfected 293T-cells.* **(b)** *NFkB-Luciferase response in primary T-cells 2 days after stimulation with LAN-1 cells. Mean and standard deviation of 3 separate experiments are shown.*

Retention of CD3ζ activation for tumor cell cytolysis by multi-domain cTCR cytoplasmic domains

We compared the initial capacity of T-cells transduced with each cTCR construct to lyse tumor cells. Receptor positive T-cells were depleted of CD56-positive cells to exclude lysis mediated by an NK cell subpopulation. T-cells transduced with all cTCR constructs killed GD2-positive neuroblastoma cells (LAN-1) whereas no lysis of the GD2-negative rhabdomyosarcoma cells (A204) was observed (Figure 4a). This killing was essentially identical regardless of the endo-domain, so that CD3ζ signaling was not compromised even when it was located deeper in the cytosol due to the presence of one or two membrane–proximal co-stimulatory domains.

14g2a-CD28-OX40-ζ construct signaling induces sustained clonal expansion.

Although killing mediated by GD2 ligation was identical regardless of the constructs used, the consequences of differential intracellular signaling mediated by the three endodomain complexes become readily apparent in experiments designed to measure cytokine secretion and long-term growth. We cultured T-cells transgenic for each construct with equal numbers of irradiated LAN-1 cells and measured the resultant T-cell clonal expansion after 7 days. There was no increase in cell numbers by non-transduced or 14g2a-ζ transduced T-cells. 14G2a-OX40-ζ transduced T-cells showed a minimal increase above baseline (1.6 fold, range 0.9-3), while the combination of CD3ζ with CD28 resulted in a 5.2 fold (range: 1.6-7.2) expansion. With the combination of OX40, CD28 and CD3ζ, however, there was a 10.7 fold (range: 4-15.5) expansion (Figure 5a; p<0.001). This hierarchy of stimulation was confirmed in transduced cells labeled with CFSE, to analyze their clonal expansion in response to GD2+ and GD2-ve target cells (Figure 5b). Only T-cells expressing 14g2a-CD28-OX40-ζ were able to fully complete their expansion program. This is also evident in photomicrographs of T-cells on day 3 post

stimulation (Figure 5c). This finding became far more pronounced after weekly re-stimulation with LAN-1 cells, when 14g2a-CD28-ζ-expressing T-cells were unable to sustain clonal expansion but that of 14G2a-CD28-OX40-ζ transduced T-cells continued beyond the third stimulation. Low dose of IL-2 (20 U/ml) extended the expansion of 14g2a-CD28-ζ transduced cells to 3 weeks, but 14g2a-CD28-OX40-ζ cells were then able to expand beyond 5 weeks.

This increased capacity for sustained proliferation correlated with a sustained capacity to kill tumor target cells. 14g2a-CD28-OX40-ζ transduced cells were still able to kill GD2-positive LAN-1 cells after 6 weeks expansion (Figure 5d), at a time when no other transductants had survived. This sustained growth is not autonomous: proliferation of 14g2a-CD28-OX40-ζ remained fully dependent on the continued presence of stimulation with specific antigen.

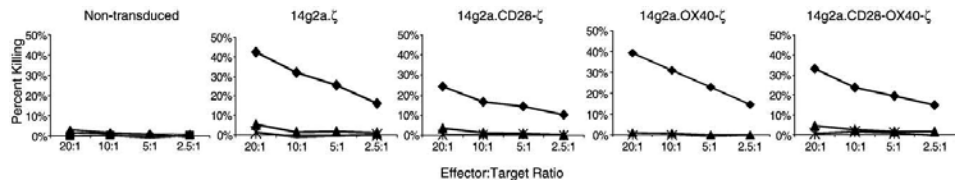

Figure 4. cTCR mediated killing of Target cells.
Chromium release assay using transduced CD56-depleted transduced peripheral blood T-cells as effectors. LAN-1 (♦) a GD-2 positive neuroblastoma cell-line is killed by all constructs but not by non-transduced T-cells. A204 (x) is a GD-2 negative rhadomyosarcoma cell line and is not killed by transduced cells.

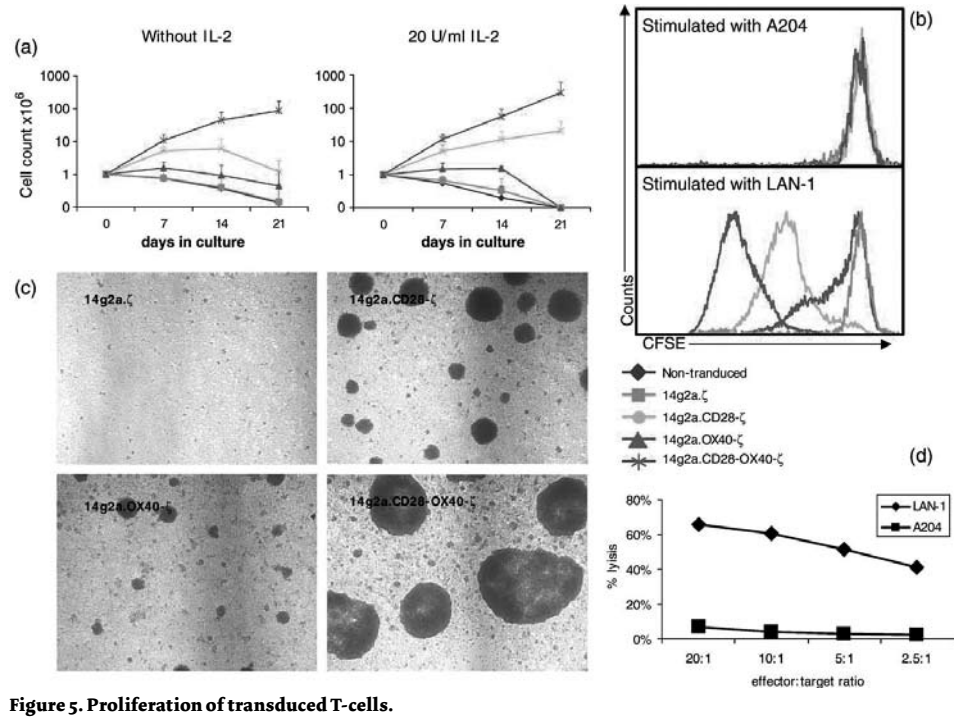

Figure 5. Proliferation of transduced T-cells.
(a) *Growth curves with weekly stimulation with LAN-1 cells without and with IL-2. Mean and standard deviation of 5 separate experiments with primary T-cells from 5 different donors are shown.* **(b)** *CFSE staining of transduced cells stimulated with LAN-1 or A204 cells.* **(c)** *Photomicrograph of cells three days after stimulation with LAN-1 cells.* **(d)** *Chromium release assay 14g2a-CD28-OX40-ζ transduced T-cells after 50 days expansion with weekly stimulation by LAN-1 cells.*

Hence, incorporation of CD28 alone is sufficient for a short-term proliferative signal, but an additional OX40 signal is required to fully develop and maintain T-cell expansion upon activation through the cTCR.

While CD8+ T-cells may not express OX40, they do express adaptor molecules (e.g. TRAF-2) required to transmit OX40 signals. CD8+ T-cells transduced with 14g2a.28-OX40-ζ should be able to proliferate in response to GD2 positive targets independently of CD4+ cells. We demonstrated this, by isolating CD8+ transduced T-cells with magnetic beads, and comparing their expansion with unsorted transduced cells (supplementary data figure 1).

14g2a-CD28-OX40-ζ transduced T-cells produce IL-2 upon activation

A key mechanism of cTCR mediated survival and proliferation is likely the result of cTCR-regulated production of the T-cell autocrine/paracrine growth factor IL-2. We compared the pattern of cytokine release induced by the different constructs by measuring the concentration of IFN-γ, IL-2, TNF-α, IL-4, IL-5 and IL-10 at 72 hours after antigenic stimulation (Figure 6). Cells transduced with the 14g2a.CD28-OX40-ζ construct secreted approximately 10-fold more IL-2 compared to cells transduced with 14g2a.CD28-ζ ($p < 0.01$). TNF-α was 5-fold increased in cells expressing the 14g2a.CD28-OX40-ζ construct ($p < 0.01$). None of the cTCR constructs tested resulted in secretion of the Th2 cytokines IL-10 or IL-4 release greater than 100 pg/ml.

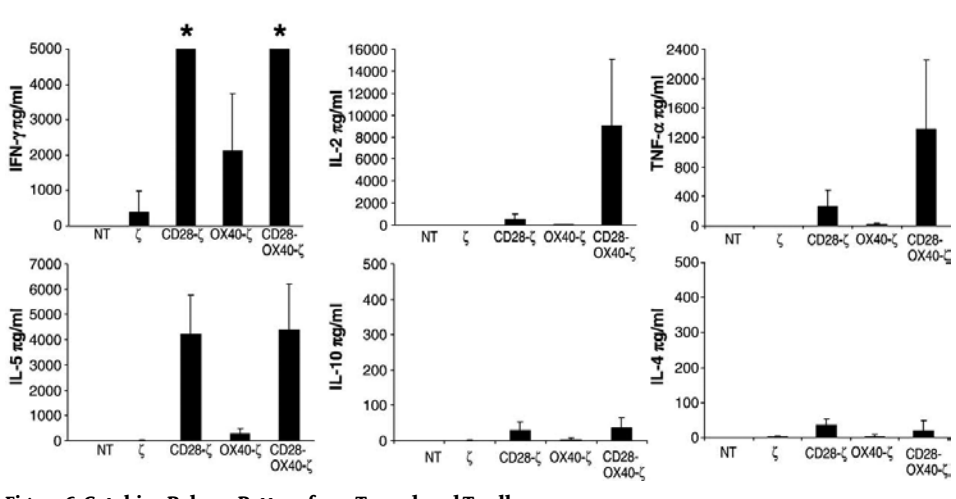

Figure 6. Cytokine Release Pattern from Transduced T-cells.

*Concentration of Th1 and Th2 cytokines in the cell supernatant was measured 3 days after stimulation with either LAN-1 cells or A204 cells. Mean and standard deviation of 5 separate experiments with primary T-cells from 5 different donors are shown. * = >5000 pg/ml.*

Discussion

We have generated antigen-specific receptors that signal through artificial endodomains composed of combinations of intracellular elements of CD3ζ, CD28 and OX40. These constructs were compared with our original cTCR 14g2a-ζ[26], that contains the antigen binding domain of the anti-GD2a antibody 14g2a and the intracellular segment of CD3-ζ. Constructs 14g2a-CD28-ζ, 14g2a-OX40-ζ and 14g2a-CD28-OX40-ζ were stably expressed by primary T-cells after retroviral transduction. All constructs triggered effective killing of GD-2 expressing target cells but only the combination of OX40 and CD28 and CD3-ζ signals *in cis* resulted in maximal NFκB activation associated with increased and prolonged proliferation and augmented cytokine release. Importantly, 14g2a-CD28-OX40-ζ transduced T-cells maintained their proliferative capacity and cytolytic function after multiple encounters with tumor cells. Supplying these three signals from one receptor overcomes the major defect of our current GD-2 targeting cTCR, which may result in improved persistence of transduced T-cells , and better function in the tumor microenvironment.

Although successful in some animal models[27-29], cTCRs containing solely the CD3ζ endodomains, may not be capable of mounting an effective anti-tumor response, particularly when targeting tumors lacking co-stimulatory molecules. For example, T-cells from mice transgenic for a CD3ζ-based receptor fail to proliferate in response to cTCR ligation[11], while in humans, T-cells expressing HIV-*env* specific CD4-ζ receptors did not appear to proliferate, and had no easily discernible clinical benefit.[6-8] Two approaches to circumvent this limitation have been proposed. First, antigen-specific T-cells can be selected and expanded and then transduced with cTCRs to generate bi-specific T-cells. After administration, repeated exposure of the native T-cell receptor to antigen and co-stimulator molecules on APC will supply the necessary signals to expand and maintain activity against the target antigens recognized by the cTCR[24,30]. Alternatively, the signaling domain of the chimeric receptor itself can be altered. Hence, the addition of a CD28 endodomain allows primary T-cells to release IL-2 and to proliferate in response to chimeric receptor engagement.[13,14] cTCRs with a CD28 component have been tested [31], and demonstrate improved function[32].

However, while CD28 activation may initiate clonal expansion and IL-2 release from naïve T-cells, it is now clear that additional signals cooperate in sustaining long-term T-cell growth, activity and survival: in the absence of additional signals, most T-cells rapidly undergo apoptosis[12]. We sought to improve signaling further by incorporation of another domain – OX40, and show *in vitro* data of the consequences. OX40 appears to be one of the principle additional co-stimulatory molecules. In the absence of OX40 , only a small number of antigen-reactive T-cells accumulate in response to antigen[33], and mice that are OX40-deficient or are receiving neutralizing antibodies to OX40L show lower susceptibility to T-cell mediated pathology, including EAE and GVHD.[34-36] Since tumor "rejection" likely needs the expansion and persistence of tumor reactive T-cells, we reasoned that the incorporation of an OX40 signaling domain in a chimeric receptor molecule might favor the development of these properties[37], an expectation confirmed by our data. Although both OX40 and CD28 activate NFκB this occurs via different mediators (Vav and P13K for CD28 and TRAF2 for OX40) and distinct additional signals may be transmitted (eg Akt and AP1) independently of FκB.[38,39] The combination of CD28 and OX40 signaling domains within a single receptor led to proliferation that appeared to mimic the physiologic expansion of naive T-cells following exposure to antigen on APC.

We constructed 14g2a-CD28-OX40-ζ receptor with endodomain components linked in the following order: CD28 followed by OX40, followed by CD3ζ. Previous reports have documented that constructs in which CD28 is moved from its normal membrane-proximal location are non-functional.[13] Hence we did not test constructs with the orientation OX40-CD28-ζ or OX40-ζ-CD28. Constructs with the orientation CD28-ζ-OX40 could not be stably expressed even when we introduced different length spacers between the carboxy-terminal of ζ and the OX40 endodomain (data not shown). The level of surface expression of chimeric TCR appeared to be dictated by the domain spanning the cell membrane. Transduction with cTCRs with CD28 transmembrane domain (14g2a-CD28-ζ and 14g2a.CD28-OX40-ζ) results in the brightest expression, with construct anchored by the OX40 transmembrane domain (14g2a-OX40-ζ) having intermediate expression and CD3-ζ transmembrane domain (14g2a-ζ) having the dimmest. We did not use spacers to link endodomains since these may increase the immunogenicity of the expressed constructs, leading to immune destruction of the transduced T-cells [40]. The efficacy of 14g2a-CD28-OX40-ζ endodomain suggests that any steric hindrance is limited in effect. Although it might in principle be possible to provide the endodomains as separate sequences in *trans*, they would have to be conveyed in separate vectors necessitating multiple transductions of T-cells, and thereby greatly increasing the risk of an unwanted consequence of pro-viral integration.

There are clear concerns with combining a transgene transmitting a proliferative signal and retroviral integration. Two patients with X-linked severe combined immunodeficiency who received hemopoietic stem cells engineered to constitutively express the common gamma-chain receptor developed LMO-2 overexpression due to retroviral integration, and T-cell acute lymphoblastic leukemia followed.[41] However, in our study the transduced cells are mature, memory T lymphocytes, which have an absolute dependence on antigen stimulation and IL-2 supplementation. This continued dependence on external signals to maintain cell survival alleviates concerns about the use of such transduced T-cells, particularly in the intended population of patients with advanced cancer. Certainly, prolonged follow up of patients receiving retrovirally gene modified T-cells has revealed no evidence of lymphoproliferation. Use of self-inactivating retroviral vectors may increase safety. Additionally, toxicity may also result from chimeric T-cell destruction of normal tissue also expressing the target antigen. Co-expression of the cTCR with a suitable suicide gene may help manage this possibility.[42]

Our modification to the cTCR receptor endodomain provides T-cells with panoply of required signals that would otherwise be lacking on tumor cells that supports helper independent clonal expansion. The resulting improvement in proliferation, function and survival may have considerable clinical utility.

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Chapter 7

An Inducible Caspase 9 Safety Switch for T-Cell Therapy

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Blood, 105:4247-54, 2005.

Abstract

The efficacy of adoptive T-cell therapy as treatment for malignancies may be enhanced by genetic modification of infused cells. However, oncogenic events due to vector/transgene integration, and toxicities due to the infused cells themselves have tempered enthusiasm. A safe and efficient means of removing aberrant cells would ameliorate these concerns. We describe a "safety switch" that can be stably and efficiently expressed in human T-cells without impairing phenotype, function or antigen specificity. This reagent is based on a modified human caspase 9 fused to a human FK506 binding protein (FKBP) to allow conditional dimerization using a small molecule pharmaceutical. A single 10 nM dose of synthetic dimerizer drug induces apoptosis in 99% of transduced cells selected for high transgene expression *in vitro* and *in vivo*. This system has several advantages over currently available suicide genes. First, it consists of human gene products with low potential immunogenicity. Second, administration of dimerizer drug has no effects other than the selective elimination of transduced T-cells. Third, inducible caspase 9 maintains function in T-cells over-expressing anti-apoptotic molecules. These characteristics favor incorporation of inducible caspase 9 as a safety feature in human T-cell therapies.

Introduction

Cellular therapies hold great promise for the treatment of human disease, and this promise may be extended if the cells are first genetically modified to alter or augment function. Unfortunately, significant toxicities from the cells themselves or from their transgene products have hampered clinical investigation. There is considerable interest in developing means by which infused cells may be ablated should problems arise from their use. Most experience with safety-switch genes to date has been in T-lymphocytes since immunotherapy with these cells has proved efficacious as treatment for viral infections and malignancies.[1-4]

The herpes simplex virus I-derived thymidine kinase (HSV-TK) gene has been used as a suicide switch in donor T-cell infusions to treat recurrent malignancy and Epstein Barr virus (EBV) lymphoproliferation after hemopoietic stem cell transplantation.[5,6] However, destruction of T-cells causing graft-versus-host disease was incomplete, and the use of ganciclovir (or analogs) as a pro-drug to activate HSV-TK precludes administration of ganciclovir as an anti-viral drug for cytomegalovirus infections. Moreover, HSV-TK-directed immune responses have resulted in elimination of HSV-TK-transduced cells, even in immunosuppressed human immunodeficiency virus and bone marrow transplant patients, compromising the persistence and hence efficacy of the infused T-cells.[5-7] The *E.coli*-derived cytosine deaminase gene has also been used clinically,[8] but as a xenoantigen it is also likely to be immunogenic and thus incompatible with T-cell-based therapies that require long-term persistence.

Transgenic human CD20, which can be activated by a monoclonal chimeric anti-CD20 antibody, has been proposed as a non-immunogenic safety system.[9] However, it results in the unwanted loss of normal B-cells for 6 months or more. An alternative suicide gene strategy is based on human pro-apoptotic molecules fused with an FKBP variant that is optimized to bind a chemical inducer of dimerization (CID)[10], AP1903, a synthetic drug that has proven safe in healthy volunteers.[11] Administration of this small molecule results in cross-linking and activation of the pro-apoptotic target molecules. The application of this inducible system in human T-lymphocytes has been explored using Fas or the Death Effector Domain (DED) of the Fas-associated death domain-containing protein (FADD) as pro-apoptotic molecules. Up to 90% of T-cells transduced with these inducible death molecules underwent apoptosis after administration of CID.[12-16]

While these results are promising, elimination of 90% of transduced cells may be insufficient to ensure safety of genetically modified cells . Moreover, death molecules that act downstream of most apoptosis inhibitors may be effective in a wider range of cells. The activity of membrane proximal apoptosis initiators such as Fas and FADD may be impaired when cellular inhibitors of apoptosis such as c-FLIP, bcl-2 and bcl-xL are upregulated (Figure 1) – a frequent early event in malignant transformation and in the long-term maintenance of memory T-cells.[17,18] Hence, the most deleterious cells may be inadvertently spared.

The efficacy of adoptive immunotherapy may be enhanced by rendering the therapeutic T-cells resistant to immune evasion strategies employed by tumor cells. *In vitro* studies have shown that this can be achieved by transduction with a dominant negative receptor or an immunomodulatory cytokine.[19,20] Moreover, transfer of antigen-specific T-cell receptors allows for the application of T-cell therapy to a broader range of tumors.[21,22] We therefore chose

to develop and test a suicide system for engineered human T-cells to allow their subsequent use in clinical studies. Here we describe how a modification of a late stage apoptosis pathway molecule, caspase 9, can be stably expressed in human T-lymphocytes without compromising their functional and phenotypic characteristics whilst demonstrating exquisite sensitivity to CID, even in T-cells that have up-regulated anti-apoptotic molecules.

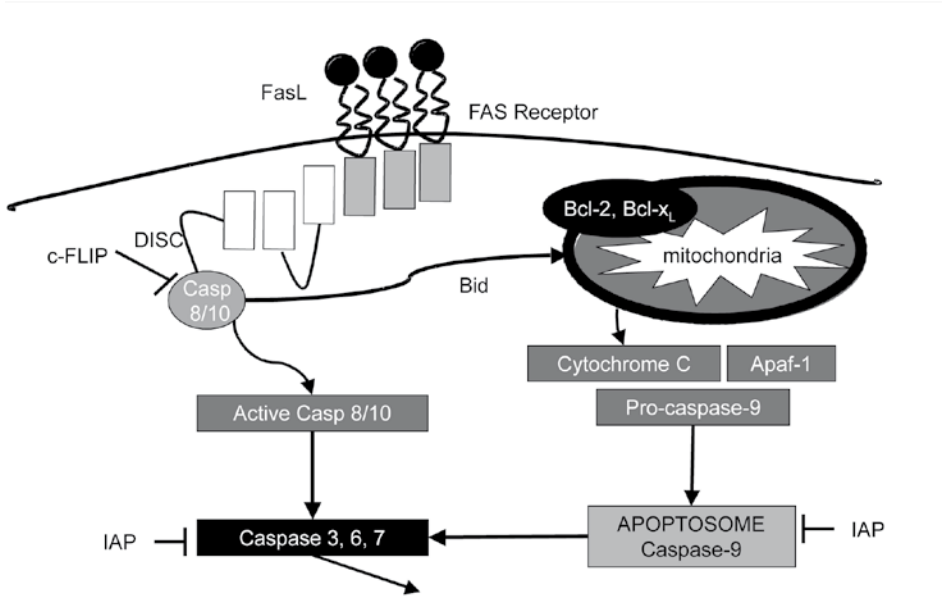


Figure 1. Anti-apoptotic molecules regulate the sensitivity to apoptotic signals
In human lymphocytes apoptosis can be induced through at least 2 pathways. Stimulation of the Fas receptor results in recruitment of the initiator caspase 8, through interaction with the adaptor molecule Fas-associated death domain protein (FADD) by means of its death domains (DD) and death effector domains (DED). In turn, activated caspase 8 activates the effector caspases 3, 6 and 7. Alternatively, disruption of the mitochondrial membrane results in the release of cytochrome c, which activates caspase 9 through interaction with the adaptor molecule, apoptotic protease-activating factor 1 (Apaf-1). Caspase 9 is then able to activate caspase 3. The death receptor-activated extrinsic pathway can crosstalk to the intrinsic mitochondrial pathway through the caspase 8-mediated cleavage of Bid. Inhibitors of apoptosis that engage at different steps of these pathways regulate the balance between apoptosis and survival. FLIP prevents the full activation of caspase 8. Anti-apoptotic bcl-2 family members prevent apoptosis initiated via the mitochondria. Inhibitors of apoptosis proteins (IAPs including X-linked IAP (XIAP)) can either prevent activation of caspases 3, 7 and 9 or inhibit their activated forms.

Material and methods

Plasmids

Full-length inducible caspase 9 (F'F-C-Casp9.I.GFP) consists of full-length caspase 9, including its caspase recruitment domain (CARD)(GenBank NM001229) linked to two 12 kD human FK506 binding proteins (FKBP12, GenBank AH002818) that contain an F36V mutation (Figure 2a).[10] The inducer of dimerization used in this study, AP20187 (generous gift of ARIAD Pharmaceuticals (http://www.ariad.com/regulationkits/reg_kitinfo.html), is a non-toxic synthetic FK506-analogue that has been modified to reduce interactions with endogenous FKBPs, while enhancing binding to this FK506-BP12 variant. Administration of the CID results in the aggregation of inducible caspase 9 molecules, leading to their activation. Caspase 9 will subsequently activate downstream effector caspases, such as caspase 3, and ultimately induce apoptosis (Figure 1). Silent mutations in the third base of multiple codons have been introduced into the first FKBP segment to prevent homologous recombination between the coding sequences of the two linked FKBPs in our retroviral system[12] and a short Ser-Gly-Gly-Gly-Ser linker connects the FKBPs and caspase 9 to enhance flexibility.[14] Inducible Fas consists of the extracellular and transmembrane domains of human low-affinity nerve growth factor receptor (ΔNGFR), 2 $FKBP12_{V36}$s and the cytoplasmic domains of human Fas as described by Thomis *et al*.[12] The cDNA for the p40 and p35 subunit of human IL-12 connected with a flexible linker was cloned from pcDNA3.1, a kind gift from Robert Anderson and Grant Prentice, Royal Free Hospital, London. [23] All constructs were cloned into the retroviral vector MSCV.IRES.GFP.

Western Blot

Transfected 293T-cells were resuspended in lysis buffer (50% Tris/Gly, 10% SDS, 4% beta-mercaptoethanol, 10% glycerol, 12% water, 4% bromophenol blue at 0.5%) containing aprotinin, leupeptin and phenylmethylsulfonyl fluoride (Boehringer, Ingelheim, Germany) and incubated for 30 minutes on ice. After 30-minute centrifugation supernatant was harvested, mixed 1:2 with Laemmli-buffer (Biorad, Hercules, CA), boiled and loaded on a 10% SDS-Polyacrylamide gel. The membrane was probed with rabbit anti-caspase 9 (amino acid residues 299-318) immunoglobulin G (IgG) (Affinity BioReagents, Golden, CO, 1:500) and with mouse anti-GFP IgG (Covance, Berkeley, CA, 1:25,000). Blots were then exposed to appropriate peroxidase-coupled secondary antibodies and protein expression was detected with ECL (Amersham, Arlington Heights, IL, USA). The membrane was then stripped and re-probed with goat polyclonal anti-actin (Santa Cruz Biotechnology at 1:500) to check equality of loading.

Cell lines

B95-8 EBV transformed B cell lines (LCL), Jurkat and MT-2 cells (kindly provided by Dr S. Marriott, Baylor College of Medicine, Houston) were cultured in RPMI 1640 (Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS) (Hyclone). Polyclonal EBV-specific T-cell lines were cultured in 45% RPMI/45% Clicks (Irvine Scientific, Santa Ana, CA)/10% FBS and generated as previously reported.[2] Briefly, peripheral blood mononuclear cells (2 x 106 per well of a 24 well plate) were stimulated with autologous LCL irradiated at 4000 rads at a responder: stimulator (R:S) ratio of 40:1. After 9-12 days, viable cells were restimulated with irradiated LCL at a R:S ratio of 4:1. Subsequently, cytotoxic T-cells (CTL) were expanded by weekly restimulation with LCL in the presence of recombinant human interleukin-2 (rhIL-2, Proleukin, Chiron Corporation, Emeryville, CA) (40-100 U/ml).

Retrovirus transduction
For the transient production of retrovirus, 293T-cells were transfected with iCasp9/iFas constructs, along with plasmids encoding gag-pol and RD114 envelope using GeneJuice™ transfection reagent (Novagen, Madison, WI). Virus was harvested 48-72 hours post transfection, snap frozen and stored at –800C until use. A stable FLYRD18-derived retroviral producer line was generated by multiple transductions with VSV-G pseudotyped transient retroviral supernatant.[24] FLYRD18 cells with highest transgene expression were single-cell sorted, and the clone that produced the highest virus titer was expanded and used to produce virus for lymphocyte transduction. The transgene expression, function and retroviral titer of this clone was maintained during continuous culture for over 8 weeks. For transduction of human lymphocytes, a non-tissue-culture treated 24-well plate (Becton Dickinson, San Jose, CA), was coated with recombinant fibronectin fragment (FN CH-296) (Retronectin™, Takara Shuzo, Otsu, Japan, 4 μg/ml in PBS, overnight at 4°C) and incubated twice with 0.5 ml retrovirus/well for 30 minutes at 37°C. Subsequently 3-5x10^5 T-cells per well were transduced for 48-72 hours using 1 ml virus/well in the presence of 100 U/ml IL-2. Transduction efficiency was determined by analysis of expression of the co-expressed marker gene GFP on a FACScan flow cytometer (Becton Dickinson). For functional studies, transduced CTL were either non-selected or segregated into populations with low, intermediate or high GFP expression using a MoFlo® cytometer (Dako Cytomation, Ft Collins, CO) as indicated.

Induction and analysis of apoptosis
CID (AP20187, ARIAD Pharmaceuticals, Cambridge, MA) at indicated concentrations was added to transfected 293T-cells or transduced CTLs and at the time points shown. Adherent and non-adherent-cells were harvested and washed with Annexin binding buffer (BD Pharmingen, San Jose, CA). Cells were stained with Annexin-V and 7-amino-actinomycin D (7-AAD) for 15 minutes according to manufactures instructions (BD Pharmingen, San Jose CA). Within 1-hour post staining, cells were analyzed by flow cytometry using CellQuest software (Becton Dickinson, San Jose CA).

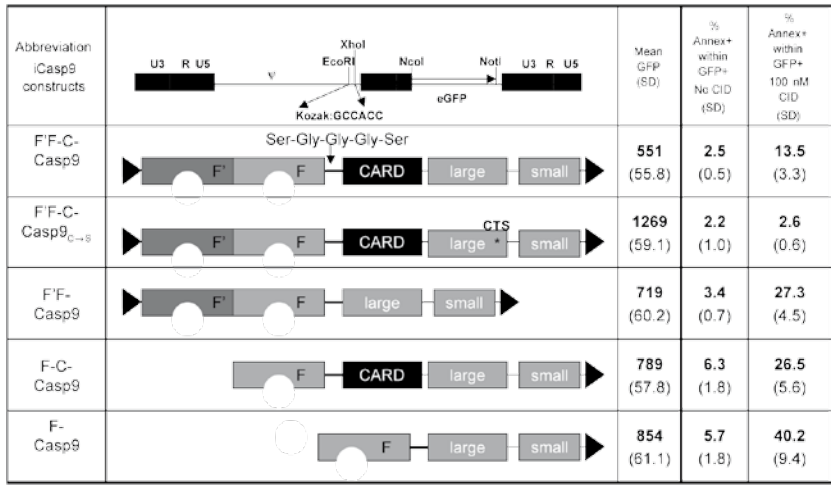
Cytotoxicity assay
The cytotoxic activity of each CTL line was evaluated in a standard 4-hour ^{51}Cr release assay, as previously described.[25] Target cells included autologous LCL, human leukocyte antigen-class I-mismatched LCL and the lymphokine-activated killer cell-sensitive T-cell lymphoma line HSB-2. Target cells incubated in complete medium or 1% Triton X-100 (Sigma) were used to determine spontaneous and maximum ^{51}Cr release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release – spontaneous release)/(maximal release – spontaneous release).

Phenotyping
Cell surface phenotype was investigated using the following monoclonal antibodies: CD3, CD4, CD8, (Becton Dickinson, San Jose, CA) and CD56 and TCR-α/β (Immunotech, Miami, FL). ΔNGFR-iFas was detected using anti-NGFR antibody (Chromaprobe, Aptos, CA). Appropriate matched isotype controls (Becton Dickinson, San Jose, CA) were used in each experiment. Cells were analyzed with a FACSscan flow cytometer (Becton Dickinson).

Analysis of cytokine production
The concentration of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, IL-5, IL-10 and tumor necrosis factor-α (TNFα) in CTL culture supernatants was measured using the Human Th1/Th2 cytokine cytometric Bead Array (BD Pharmingen, San Jose, CA) and the concentration of IL-12 in the culture supernatants was measured by ELISA (R&D system, Minneapolis, MN) according to the instructions of the manufacturer.

experiments
NOD/SCID mice, 6-8 weeks of age, were irradiated (250 rad) and injected s.c. in the right flank with 10-15x10^6 LCL resuspended in Matrigel (BD Bioscience, Bedford, MA). Two weeks later mice bearing tumors that were approximately 0.5 cm diameter were injected in the tail vein with a 1:1 mixture of non-transduced and iCasp9.I.GFP$_{high}$-transduced EBV-CTL (total 15x10^6). Four-six hours prior and 3 days post CTL infusion, mice were injected i.p. with recombinant hIL-2 (2000 U, Proleukin, Chiron Corporation, Emeryville, CA). On day 4, the mice were randomly segregated in 2 groups: 1 group received CID (50 μg AP20187 i.p.) and one group received carrier only (16.7% propanediol, 22.5% PEG400 and 1.25% Tween 80 i.p.). On day 7, all mice were sacrificed. Tumors were homogenized and stained with anti-human CD3 (BD Pharmingen, San Jose, CA). By FACS analysis, the number of GFP+ cells within the gated CD3+ population was evaluated. Tumors from a control group of mice that received only non-transduced CTL (total 15x10^6) were used as a negative control in the analysis of CD3+/GFP+ cells.

a



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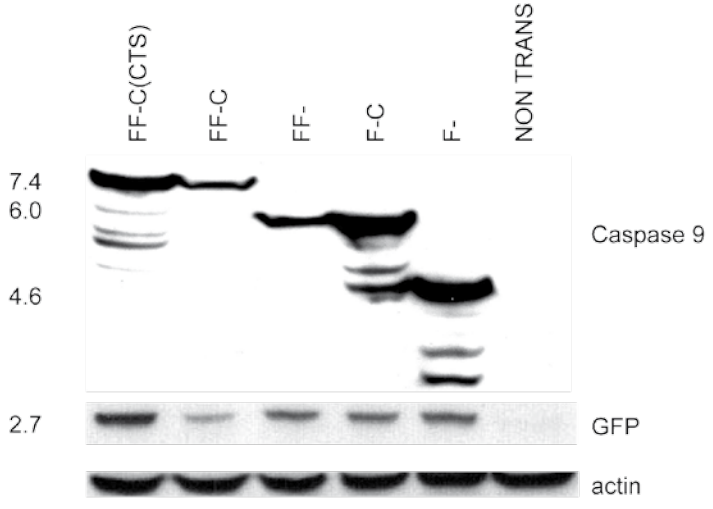


Figure 2. Modifications of full-length inducible caspase 9

(a) *The full-length inducible caspase 9 molecule (F'-F-C-Casp9) consists of 2 FK506 binding proteins (FKBPs) linked with a Ser-Gly-Gly-Gly-Ser linker to the small and large subunit of the caspase molecule. The amino acid sequence of one of the FKBPs (F') is codon-wobbled to prevent homologous recombination when expressed in a retrovirus. F'F-C-Casp9$_{C>S}$ contains a cysteine>serine mutation at position 287 that disrupts its activation site. In constructs F'F-Casp9, F-C-Casp9 and F'-Cas p9, either the caspase activation domain (CARD), one FKBP or both were deleted respectively. All constructs were cloned into MSCV.IRES.GFP as EcoRI-XhoI fragments. 293T-cells were transfected with each of these constructs and 48 hours post transduction expression of the marker gene GFP was analyzed by flow cytometry. In addition, 24 hours post transfection, 293T-cells were incubated overnight with 100 nM CID and subsequently stained with the apoptosis marker Annexin-V. The mean and standard deviation of transgene expression level (mean GFP) and number of apoptotic cells before and after exposure to CID (% Annexin V within GFP+ cells) from 4 separate experiments are shown.*

(b) *Co-expression of the inducible caspase 9 constructs of the expected size with the marker gene GFP in transfected 293T was demonstrated by western blot using a caspase 9 antibody specific for amino acid residues 299-318 present both in the full-length and truncated caspase molecules as well a GFP-specific antibody. Additional smaller size bands likely represent degradation products. Degradation products for the F'F-C-Casp9 and F'F-Casp9 constructs may not be detected due to a lower expression level of these constructs as a result of their basal activity. Equal loading was confirmed by blotting for actin.*

Results

Optimization of expression and function of inducible caspase 9

We initially screened previously described caspases 3, 7 and 9 for their suitability as inducible safety-switch molecules both in transfected 293T-cells and in transduced human T-cells.[14] Only inducible caspase 9 (iCasp9) could be expressed at levels sufficient to confer sensitivity to CID (data not shown). However, even the initially tested iCasp9 could not be maintained stably at high levels in T-cells, and we hypothesized that basal activity of the transgene was eliminating transduced cells. The CARD domain is responsible for physiological dimerization of caspase 9 molecules, by a cytochrome C and adenosine triphosphate (ATP) driven interaction with apoptotic protease-activating factor 1 (Apaf-1). Hence the CARD domain appears superfluous in this context and its removal might reduce basal activity. Given that only dimerization rather than multimerization is required for activation of caspase 9, we also reasoned that a single FKBP domain might be adequate to effect activation. We therefore compared the activity of iCasp9 derivatives in which either the CARD domain, or one of the two FKBP domains or both had been removed. A construct with a disrupted activation site, F'F-C-Casp9$_{C>S}$, provided a non-functional control (Figure 2a). All constructs were cloned into the retroviral vector MSCV[26] in which retroviral long terminal repeats (LTRs) direct transgene expression and enhanced Green Fluorescent Protein (GFP) is co-expressed from the same mRNA by utilization of an internal ribosomal entry site (IRES). In transfected 293T-cells expression of all inducible caspase 9 constructs at the expected size as well as co-expression of GFP was demonstrated by western blot (Figure 2b). Protein expression (estimated by mean fluorescence of GFP and visualized on western blot) was highest in the non-functional construct F'F-C-Casp9$_{C>S}$ and greatly diminished in the full-length construct F'F-C-Casp9. Removal of the CARD (F'F-Casp9), one FKBP (F-C-Casp9) or both (F-Casp9) resulted in progressively higher expression of both inducible caspase 9 and GFP, and correspondingly enhanced sensitivity to CID (Figure 2a). Based on these results, the F-Casp9 construct (henceforth referred to as iCasp9$_M$) was used for further study in human T-lymphocytes.

Stable expression of iCasp9$_M$ in human T-lymphocytes

The long-term stability of suicide gene expression is of utmost importance, since suicide genes must be expressed for as long as the genetically engineered cells persist. For T-cell transduction, a FLYRD18-derived retroviral producer clone that produces high-titer RD114-pseudotyped virus was generated to facilitate the transduction of T-cells.[24] We evaluated iCasp9$_M$ expression in EBV-specific CTL lines (EBV-CTL), since these have well-characterized function and specificity and are already being used as therapy for prevention and treatment of EBV-associated malignancies.[2,27] Consistent transduction efficiencies of EBV-CTL of >70% (mean: 75.3%, range: 71.4-83.0% in 5 different donors) were obtained after a single transduction with retrovirus. The expression of iCasp9$_M$ in EBV-CTL was stable for at least 4 weeks post transduction without selection or loss of transgene function (data not shown).

iCasp9M does not alter transduced T-cell characteristics

To ensure that expression of iCasp9$_M$ did not alter T-cell characteristics, we compared the phenotype, antigen-specificity, proliferative potential and function of non-transduced, or non-functional iCasp9-transduced EBV-CTL, with that of iCasp9$_M$-transduced EBV-CTL. In four separate donors, transduced and non-transduced CTL consisted of equal numbers of CD4$^+$, CD8$^+$, CD56$^+$ and TCR$\alpha\beta^+$ cells (Figure 3a). Similarly, production of cytokines includ-

ing IFN-γ, TNF-α, IL-10, IL-4, IL-5 and IL-2 was unaltered by iCasp9$_M$ expression (Figure 3b). iCasp9M-transduced EBV-CTL specifically lysed autologous LCL comparable to non-transduced and control-transduced CTL (Figure 3c). Expression of iCasp9$_M$ did not affect the growth characteristics of exponentially growing CTL, and importantly, dependence on antigen and IL-2 for proliferation was preserved (Figure 3d).

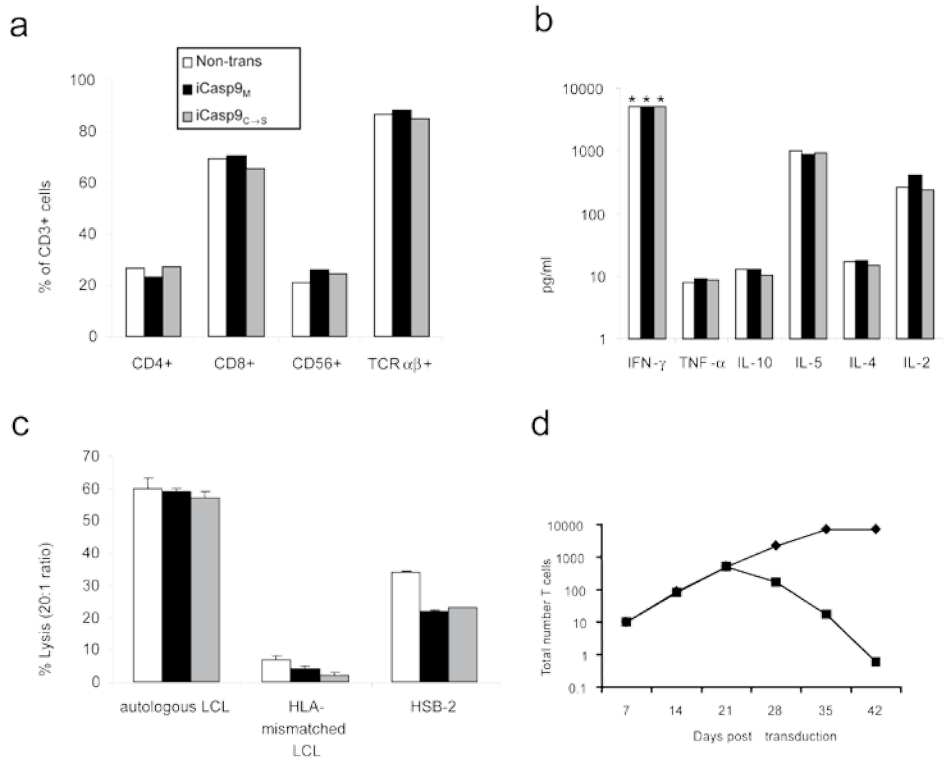
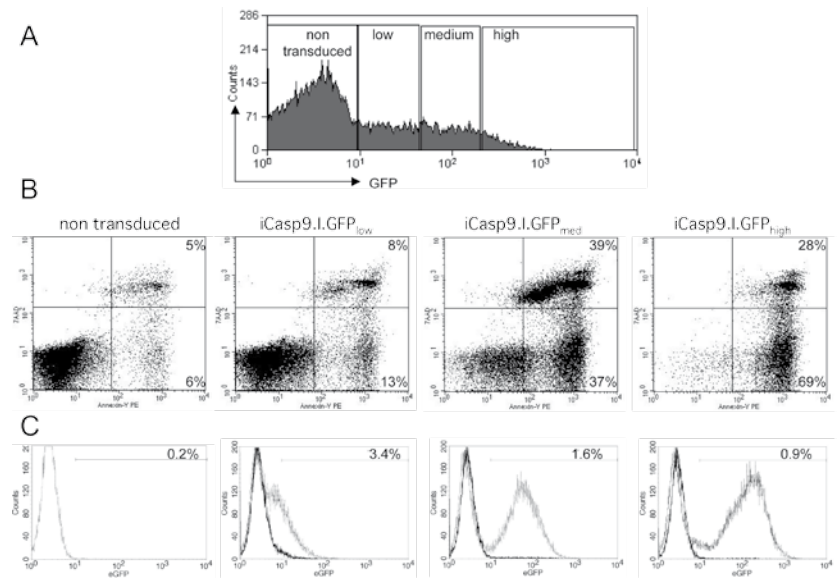


Figure 3. Expression of iCasp9M does not affect the phenotype or function of EBV- CTLs
(a) *Phenotype,* **(b)** *secretion of Th1 and Th2-type cytokines upon antigen stimulation, and* **(c)** *cytolytic activity against autologous LCL, HLA-mismatched LCL and HSB-2, a LAK cell target were compared in non-transduced, F-Casp9$_M$ transduced and F'F-C-Casp9$_{C→S}$ transduced EBV on day 15-18 post transduction (two antigenic stimulations post transduction). Examples of experiments using EBV-CTL from 4 different donors are shown. * = > 5000 pg/mL. (d) On day 21 post transduction the normal weekly antigenic stimulation with autologous LCL and IL-2 was continued (♦) or discontinued (■) to evaluate the antigen-dependence of iCasp9$_M$ transduced CTL.*

Elimination of >99% of T-lymphocytes selected for high transgene expression in vitro

To provide an effective safety switch, suicide gene induction should eliminate all gene-modified cells. Therefore, iCasp9$_M$ proficiency in CTLs was tested by monitoring loss of GFP expressing cells after administration of CID; 91.3% (range: 89.5–92.6% in 5 different donors) of GFP$^+$ cells were eliminated after a single 10 nM dose of CID (Figure 4a). Similar results were obtained regardless of exposure time to CID (range 1 hour – continuous) (data not shown). In all experiments, CTLs that survived CID treatment had low transgene expression with a 70% (range 55-82%) reduction in mean fluorescence intensity of GFP post CID. No further elimination of the surviving GFP$^+$ T-cells could be obtained by an antigenic stimulation followed by a second 10 nM dose of CID (data not shown).



Supplementary data:
Correlation between transgene expression level and function of iCasp9$_M$
(a) *To determine the correlation between transgene expression and function of iCasp9$_M$ iCasp9$_M$.IRES.GFP-transduced EBV-CTL were selected for low (mean 21), intermediate (mean 80) and high (mean 189) GFP (expression. (b) Selected T-cells were incubated overnight with 10 nM CID and subsequently stained with Annexin V and 7-AAD. Indicated are the percentages of Annexin V$^+$/7-AAD$^-$ and Annexin V$^+$/7-AAD$^+$ T-cells. (c) Selected T-cells were mixed 1:1 with non-transduced T-cells and incubated with 10 nM CID following antigenic stimulation. Indicated is the percentage of residual GFP-positive T-cells on day 7.*

Therefore, the non-responding CTLs most likely expressed insufficient iCasp9$_M$ for functional activation by CID. To test this hypothesis CTLs were sorted for low, intermediate and high expression of the linked marker gene GFP and mixed 1:1 with non-transduced CTLs from the same donor to allow for an accurate quantitation of the number of transduced T-cells responding to CID-induced apoptosis. The number of transduced T-cells eliminated increased with the level of GFP transgene expression (supplementary data). For GFP$_{high}$ selected cells, 10 nM CID led to deletion of 99.1% (range: 98.7% – 99.4%) of transduced cells (Figure 4a). Rapid induction of apoptosis in these GFP$_{high}$-selected cells is demonstrated by apoptotic characteristics such as cell shrinkage and fragmentation within 14 hours of CID administration (Figure 4b). Of these T-cells, 64% (range: 59-69%) had an apoptotic (Annexin-V+/7-AAD-) and 30% (range: 26-32%) had a necrotic (Annexin-V+/7-AAD+) phenotype (Figure 4c). In contrast, the induction of apoptosis was significantly lower in T-cells selected for intermediate or low GFP expression (supplementary data). For clinical applications therefore, transduced cells may have to be sorted for sufficient transgene expression before administration. CID-induced apoptosis was inhibited by the pan-caspase inhibitor zVAD-fmk (100 μM for one hour prior to adding CID) (data not shown). Titration of CID showed that 1 nM CID was sufficient to obtain the maximal deletion effect (Figure 4d). This dose response remained unchanged for at least 4 weeks post transduction (data not shown).

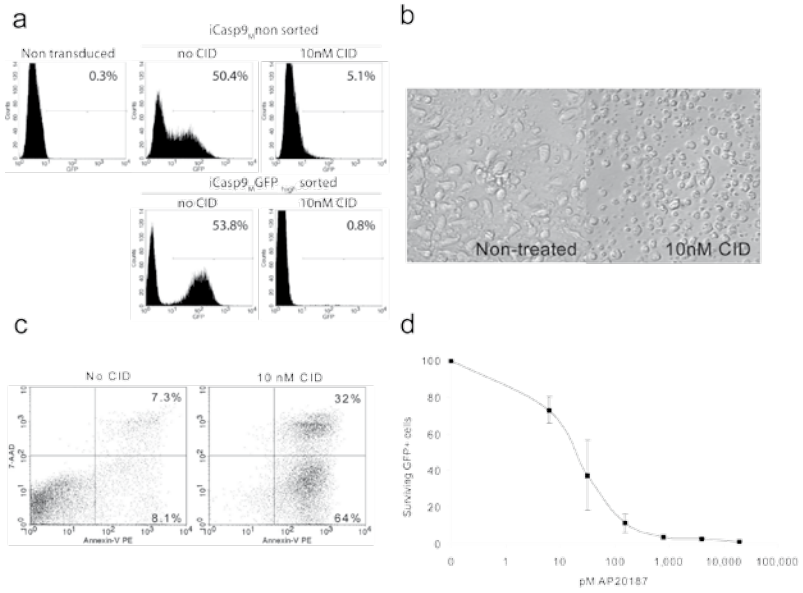


Figure 4. Administration of CID eliminates iCasp9$_M$-expressing T-cells
(a) *On the day of antigen stimulation, F-Casp9$_M$.I.GFP transduced CTL were either untreated or treated with 10 nM of CID. Seven days later, the response to CID was measured by flow cytometry for GFP. The percentage of transduced T-cells was adjusted to 50% to allow for an accurate measurement of residual GFP$^+$ cells post CID treatment. The responses to CID in unselected (top panel) and GFP$_{high}$ selected CTL (lower panel) was compared.* **(b-c)** *After overnight incubation with 10 nM CID F-Casp9$_M$.I.GFP$_{high}$ transduced T-cells had apoptotic characteristics such as cell shrinkage and fragmentation by microscopic evaluation and staining with markers of apoptosis showed that 64% of T-cells had an apoptotic phenotype (Annexin-V$^+$, 7-AAD$^-$) and 32% a necrotic phenotype (Annexin V$^+$, 7-AAD$^+$). A representative example of 3 separate experiments is shown.* **(d)** *A dose-response curve using the indicated amounts of CID (AP20187) shows the sensitivity of F-Casp9$_M$.I.GFP$_{high}$ to CID. Survival of GFP$^+$ cells is measured on day 7 after administration of the indicated amount of CID. Shown are mean and standard deviation. Similar results were obtained using AP1903, which has proven safe in a clinical trial in healthy volunteers.*[11]

iCasp9$_M$ is functional in malignant cells that express anti-apoptotic molecules

We had selected caspase 9 as an inducible pro-apoptotic molecule for clinical use rather than previously described iFas[12] and iFADD[16], since caspase 9 acts relatively late in apoptosis signaling and should be less susceptible to inhibition by apoptosis inhibitors. Thus suicide function should be preserved not only in malignant, transformed T-cell lines that express anti-apoptotic molecules,[28,29] but also in subpopulations of normal T-cells that express elevated anti-apoptotic molecules as part of the process to ensure long-term preservation of memory cells.[18,30] To test this hypothesis we first compared the function of iCasp9$_M$ and iFas in EBV-CTL. Like iCasp9, inducible Fas[12] was expressed by the MSCV.IRES.GFP vector. For these experiments both ΔNGFR.iFas.I.GFP and iCasp9$_M$.I.GFP-transduced CTLs were sorted for GFP$_{high}$ expression and mixed with non transduced CTLs at a 1:1 ratio to obtain cell populations that expressed either iFas or iCasp9$_M$ at equal proportions and at similar levels (Figure 5a). Elimination of GFP$^+$ cells after administration of 10 nM CID was more rapid and more efficient in iCasp9$_M$ than in iFas-transduced CTL (99.2% ± 0.14% of iCasp9M-transduced cells compared to 89.3% ± 4.9% of iFas-transduced cells at day 7 post CID, $p<0.05$) (Figure 5b).

Second, we compared the function of iCasp9$_M$ and iFas in two malignant T-cell lines: Jurkat, an apoptosis-sensitive T-cell leukemia line, and MT-2, an apoptosis-resistant T-cell line, due to c-FLIP, and bcl-x$_L$ expression.[31,32] Jurkat cells and MT-2 cells were transduced with iFas and iCasp9$_M$ with similar efficiencies (92% vs. 84% in Jurkat, 76% vs. 70% in MT-2) and were cultured in the presence of 10 nM of CID for 8 hours. Annexin-V staining showed that although iFas and iCasp9$_M$ induced apoptosis in an equivalent number of Jurkat cells (56.4% ± 15.6% and 57.2% ± 18.9% respectively), only activation of iCasp9$_M$ resulted in apoptosis of MT-2 cells (19.3 % ± 8.4% and 57.9% ± 11.9% for iFas and iCasp9$_M$ respectively) (Figure 5c). These results demonstrate that in T-cells over-expressing apoptosis-inhibiting molecules, the function of iFas can be blocked, while iCasp9M can still effectively induce apoptosis.

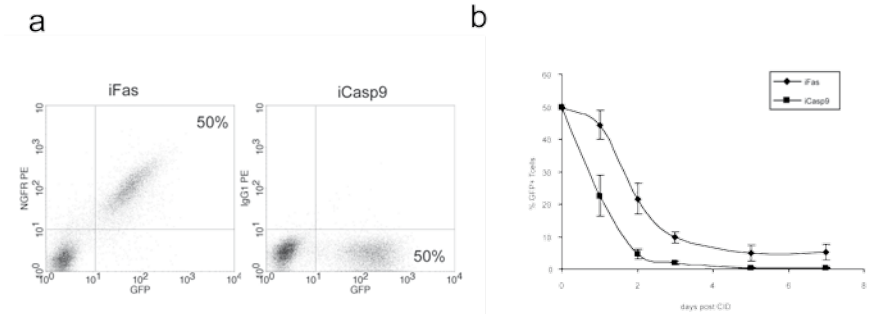


Figure 5. Comparison of functionality of iFas and iCasp9$_M$ in T-cells
(a) *EBV-CTL were transduced with ΔNGFR-iFas.I.GFP or iCasp9$_M$.I.GFP and sorted for high GFP expression. Transduced CTL were then mixed 1:1 with non-transduced CTL.* **(b)** *On the day of LCL stimulation, 10 nM CID was administrated, and GFP was measured at the time points indicated to determine the response to CID. Mean and standard deviation of three experiments are shown.* **(c)** *The human T-cells lines Jurkat and MT-2 were transduced with ΔNGFR-iFas.I.GFP or iCasp9$_M$.I.GFP. An equal percentage of T-cells was transduced with each of the suicide genes: 92% for ΔNGFR-iFas.I.GFP vs. 84% for iCasp9$_M$.I.GFP in Jurkat, and 76% for ΔNGFR-iFas.I.GFP vs. 70% for iCasp9M.I.GFP in MT-2 (data not shown). T-cells were either non-treated or incubated with 10 nM CID. Eight hours after exposure to CID, apoptosis was measured by staining for Annexin-V and 7-AAD. Representative example of three experiments is shown.*

iCasp9$_M$-mediated elimination of T-cells expressing an immunomodulatory transgene

To determine whether iCasp9$_M$ could effectively destroy cells genetically modified to express an active transgene product, we measured the ability of iCasp9$_M$ to eliminate EBV-CTL stably expressing IL-12 to enhance their anti-tumor activity.[20] While IL-12 was undetectable in the supernatant of non-transduced and iCasp9$_M$.IRES.GFP-transduced CTL, the supernatant of iCasp9$_M$.IRES.IL-12 transduced cells contained 324-762 pg/mL of IL-12. After administration of 10 nM CID, however, the IL-12 in the supernatant fell to undetectable levels (less

than 7.8 pg/mL). Hence, even without prior sorting for high transgene-expressing cells, activation of iCasp9$_M$ is sufficient to completely eliminate all T-cells producing biologically relevant levels of IL-12 (Figure 6).

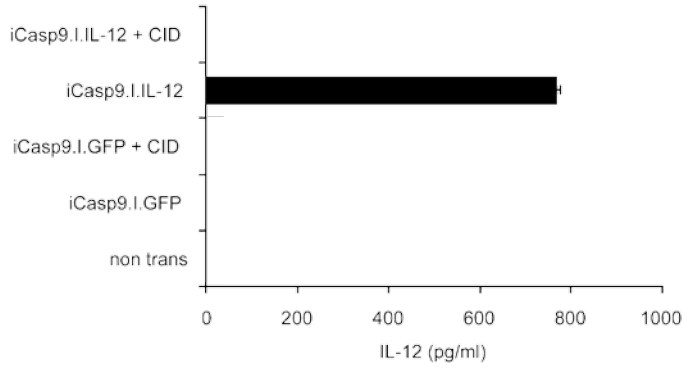


Figure 6. Function of iCasp9$_M$ when co-expressed with IL-12
The marker gene GFP in the iCasp9$_M$.I.GFP constructs was replaced by flexi IL-12, encoding the p40 and p35 subunit of human IL-12. iCasp9$_M$.I.GFP and iCasp9$_M$.I.IL-12 transduced EBV-CTL were stimulated with LCL, and then left un-treated or exposed to 10 nM CID. Three days after a second antigenic stimulation, IL-12 in the culture supernatant was measured by IL-12 ELISA (detection limit of this assay is 7.8 pg/mL). Results of 1 of 2 experiments with CTL from 2 different donors are shown.

Elimination of >99% of T-cells selected for high transgene expression
Finally, to evaluate the function of iCasp9$_M$ in transduced EBV-CTL , we used a SCID mouse-human xenograft model for adoptive immunotherapy.[33] After i.v. infusion of a 1:1 mixture of non-transduced and iCasp9$_M$.IRES.GFP$_{high}$-transduced CTL into SCID mice bearing an autologous LCL xenograft, mice were treated either with a single dose of CID or carrier only. Three days after CID/carrier administration, tumors were analyzed for human CD3$^+$/GFP$^+$ cells. Detection of the non-transduced component of the infusion product using human anti-CD3 antibodies, confirmed the success of the tail vein infusion in mice that received CID. In mice treated with CID, there was a greater than 99% reduction in the number of human CD3$^+$/GFP$^+$ T-cells, compared with infused mice treated with carrier alone, demonstrating equally high sensitivity of iCasp9M transduced T-cells *in vivo* and *in vitro* (Figure 7).

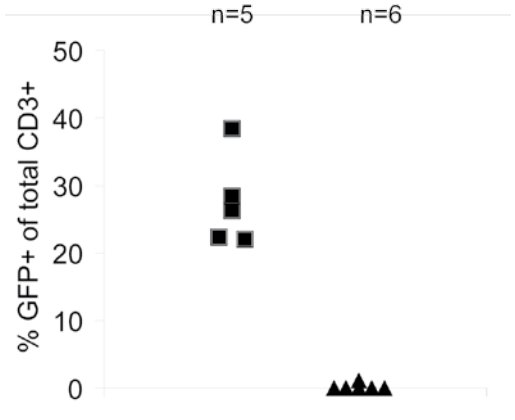


Figure 7. Function of iCasp9$_M$
NOD/SCID mice were irradiated and injected s.c. with 10-15x10^6 LCL. After 14 days, mice bearing tumors of 0.5 cm in diameter received a total of 15x10^6 EBV-CTL. (50% of these cells were non-transduced and 50% were transduced with iCasp9$_M$.I.GFP and sorted for high GFP expression.) On day 3 post CTL administration mice received either CID (50 µg AP20187) (▲, n=6) or carrier only (■, n=5) and on day 6 the presence of human CD3+/GFP+ T-cells in the tumors was analyzed. Human CD3+ T-cells isolated from the tumors of a control group of mice that received only non-transduced CTL (15x10^6 CTL) (n= 4) were used as a negative control for the analysis of CD3+/GFP+ T-cells within the tumors.

Discussion

A suicide gene designed to eliminate gene-modified T-cells ideally should be co-expressed stably in all cells carrying the modifying gene, at levels high enough to elicit cell death. Thus, it must have low basal activity along with high specific activity, together with minimal susceptibility to endogenous anti-apoptotic molecules. We have developed an inducible caspase 9, iCasp9$_M$, which has low basal activity allowing stable expression for more than four weeks in human T-cells. A single nM dose of CID is sufficient to kill over 99% of iCasp9$_M$-transduced cells selected for high transgene expression both *in vitro* and *in vivo*. Moreover, when co-expressed with the pivotal Th1 cytokine IL-12, activation of iCasp9$_M$ eliminated all detectable IL-12 producing cells, even without selection for high transgene expression. As caspase 9 acts downstream of most anti-apoptotic molecules, a high sensitivity to CID is preserved regardless of the presence of increased levels of anti-apoptotic molecules of the bcl-2 family. Thus iCasp9$_M$ should induce destruction even of transformed T-cells and memory T-cells that are relatively resistant to apoptosis.

Recent insights into caspase 9 activation allow us to propose a molecular mechanism of iCasp9$_M$ activation.[34] In contrast to other caspase molecules, proteolysis appears to be insufficient and unnecessary for activation of caspase 9.[35,36] Crystallographic and functional data indicate that dimerization of inactive caspase 9 monomers leads to conformational change induced activation.[37] In a physiological setting the concentration of pro-caspase 9 is in the 20 nM range[35] – well below dimerization threshold. According to the proposed model the energetic barrier to dimerization is overcome by homophilic interactions between the CARD domains of Apaf-1 and caspase 9, driven by cytochrome C and ATP.[37] Over-expression of caspase 9 joined to two FKBPs results in a situation where spontaneous dimerization might occur and account for the observed toxicity of the initial construct. Removal of one FKBP resulted in increased gene expression probably by reducing spontaneous dimerization and hence toxicity. While multimerization is required for activation of surface death receptors, this model predicts that dimerization should be sufficient to mediate activation of caspase 9. Indeed iCasp9 constructs with a single FKBP function as effectively as those with two FKBPs. Increased sensitivity to dimerizer by removal of the CARD domain probably represents a reduction in the energetic threshold of dimerization upon CID binding. In short, our final construct simply represents replacement of one dimerization/activation module (CARD) with another (FKBP12).

Unwanted immune responses against cells expressing virus or bacteria-derived lethal genes such as HSV-TK and cytosine deaminase can impair their persistence.[5-7] The FKBPs and pro-apoptotic molecules that form the components of iCasp9$_M$ are human-derived molecules and are therefore less likely to induce an immune response. Although the linker between FKBP and caspase 9 and the single point mutation in the FKBP domain introduce novel amino acid sequences, the latter was not immunologically recognized by macaque recipients of iFas-transduced T-cells.[15] Moreover, unlike virus-derived proteins such as HSV-TK, no memory T-cells specific for these junctional sequences should be present, reducing the risk of immune response-mediated elimination of iCasp9$_M$-transduced T-cells.

Elimination of all cells expressing the therapeutic transgene is a prerequisite for a safety-switch for clinical applications. Previous studies using inducible Fas or DED of FADD,

showed that approximately 10% of transduced cells were unresponsive to activation of the destructive gene.[12,15,16] One explanation for unresponsiveness to CID is low expression of the transgene: both iCasp9$_M$-transduced T-cells in our study and iFas-transduced T-cells in studies by others[12,16] that survived after CID administration had low levels of transgene expression. We interpreted this as a retroviral "positional effect" and attempted to achieve more homogeneous expression of transgene by flanking retroviral integrants with the chicken beta-globin chromatin insulator.[38] This modification dramatically increased the homogeneity of expression in transduced 293T-cells, but had no significant effect in transduced primary T-cells (data not shown). Selection of T-cells with high expression levels minimized variability of response to the dimerizer. Over 99% of transduced T-cells sorted for high GFP expression were eliminated after a single 1 nM CID dose. This demonstration supports the hypothesis that cells expressing high levels of suicide gene can be isolated using a selectable marker. Although a very small number of residual cells may cause resurgence of toxicity, a deletion efficiency of up to two logs will significantly decrease this possibility. For clinical use, co-expression with a non-immunogenic selectable marker such as truncated human NGFR, CD20 or CD34 instead of GFP will allow for selection of high transgene expressing T-cells.[39-41] Co-expression of such a selectable marker can either be obtained using an IRES or by post-translational modification of a fusion protein containing a self-cleaving (e.g. 2A)-sequence.[42] In contrast, in situations where the sole safety concern is the transgene (e.g. artificial T-cell receptors, cytokines)-mediated toxicity, this selection step may be unnecessary, as tight linkage between iCasp9$_M$ and transgene expression ensures elimination of those cells that are expressing biologically relevant levels of the therapeutic transgene. We demonstrated this by co-expressing iCasp9$_M$ with IL-12; activation of iCasp9$_M$ completely abolished measurable IL-12 production. However, this may depended on the function and the activity of the transgene.

The other explanation for unresponsiveness to CID is that high levels of apoptosis inhibitors may attenuate CID-mediated apoptosis. These include c-FLIP, bcl-2 family members, and inhibitors of apoptosis proteins (IAPs), which normally regulate the balance between apoptosis and survival. For instance, upregulation of c-FLIP and bcl-2 render a subpopulation of T-cells, destined to establish the memory pool, resistant to activation-induced cell death in response to cognate target or antigen-presenting cells.[18,30] In several T-lymphoid tumors, the physiological balance between apoptosis and survival is disrupted in favor of cell survival.[28,29] A suicide gene should delete all transduced T-cells including memory and malignantly transformed cells. Thus, to ensure safety, preserved function of the inducible suicide gene in the presence of increased levels of anti-apoptotic molecules is critical. The apical location of iFas (or iFADD) in the apoptosis signaling pathway may leave it especially vulnerable to inhibitors of apoptosis and these molecules are therefore less suited to being the key component of an apoptotic safety switch. While Caspase 3 or 7 as terminal effector molecules, appear to be ideal candidates, we were unable to express either in primary human T-cells at functional levels (data not shown). One possible explanation is that caspase 3 and 7, unlike caspase 9, make poor substrates for themselves and thus require prohibitively high cellular concentrations for cleavage.[14] We therefore chose caspase 9 that bypasses the inhibitory effects of c-FLIP and anti-apoptotic bcl-2 family members and could be expressed stably at functional levels. Although X-linked Inhibitor of Apoptosis (XIAP) could in theory reduce spontaneous caspase 9 activation (Figure 1),[43] the high affinity of AP20187 (or AP1903) for FKBP$_{V36}$ likely displaces this non-covalently associated XIAP. Indeed, in contrast to iFas, iCasp9$_M$ remained functional in a transformed T-cell line that over expresses anti-apoptotic molecules, including bcl-x$_L$.

We have described a new inducible safety switch, designed specifically for expression from an oncoretroviral vector by human T-cells. iCasp9$_M$ can be activated by AP1903 (or analogs), a small chemical inducer of dimerization that has proven safe at the required dose for optimum deletional effect,[11] and unlike ganciclovir or Rituximab has no other biological effects . Therefore, expression of this suicide gene in T-cells for adoptive transfer will increase safety and hence broaden the scope of clinical applications.

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Chapter 8

Discussion

Cancer immunotherapy aims to destroy malignant cells by triggering an immunological response similar to the highly effective and specific elimination of virus-infected cells. One means of doing this is by adoptive transfer of tumor-specific T-cells. This strategy is particularly attractive as it allows for *ex vivo* generation, characterization and modification of a therapeutic product in the absence of tumor-related inhibitory factors. EBV-associated tumors provide an excellent model to develop and study this approach as the tumor associated viral antigens are immunogenic and the T-cell responses against these can be reactivated using established protocols. Homing, persistence and tumor-protective effects of adoptively transferred EBV-specific T-cells have previously been demonstrated for highly immunogenic EBV-positive tumors developing in immunocompromised hosts.[1-3] Here, we studied the feasibility and efficacy of this approach for EBV-positive NPC, a tumor that expresses subdominant viral antigens and can arise despite pre-existing EBV-immunity. Further, to enable a broader application of T-cell therapy to inherently less immunogenic tumors, we explored the transgenic expression of tumor-specific TCRs as a method to obtain T-cells specific for non-viral tumor-associated antigens. Finally, to address potential unwanted side effects of T-cells genetically modified to redirect their specificity or to enhance their function, we developed an inducible safety switch with desirable characteristics for use in T-cell therapy. Here, the results of these efforts are summarized and their implications for adoptive T-cell therapy are discussed.

Adoptive T-cell therapy as treatment for EBV type II latency tumors

Our phase I clinical trial of adoptive T-cell therapy as treatment for advanced NPC showed that CTL infusion is safe, although caution is required when treating patients with bulky tumors in close relation to viral organs, and provides the first indications of anti-tumor activity with clinical responses in 3 of 6 patients treated with refractory/relapsed disease (Chapter 3). This study addressed a number of important questions regarding the generation, characterization and efficacy of the autologous EBV-specific T-cells used for adoptive transfer. The first question was if CTL specific for the viral antigens expressed on this tumor could be reactivated and expanded from the peripheral blood of patients with advanced NPC as was previously demonstrated for healthy bone marrow donors.[2] To date, we successfully generated autologous EBV-specific CTL lines for 15 of 15 patients. These CTL lines expanded at similar rates as compared to CTL lines from healthy donors. This is in contrast to CTL lines from patients with relapsed EBV-positive Hodgkin's disease that required additional mitogenic stimulation to obtain sufficient expansion.[4,5] The normal growth rate despite the previous exposure to radiation and chemotherapy demonstrated the feasibility of this approach for this patient group.

The next question was if the CTL generated from patient PBMC had the desired phenotype and function. Secretion of cytokines such as IL-10 by the NPC tumor cells[6] may switch patient T-cells to a Th 2 phenotype, compromising their cytolytic activity. Further, impaired perforin expression and IFN-γ secretion in PBMC from NPC patients has been reported.[7] Nevertheless, all CTL lines, derived from autologous PBMC, lysed autologous LCL without reactivity towards autologous PHA blasts. Although the majority of T-cells in the CTL lines were CD8+, all contained a small number of CD4+ cells which has proved important for long term survival of infused T-cells in previous studies.[1,8] Thus, despite reported inherent defects of T-cells from NPC patients, autologous T-cells with the desirable cytolytic activity and phenotype could be reactivated and expanded *ex vivo*.

The availability of autologous APC poses a major block to the expansion of polyclonal antigen-specific T-cells. While non-specific methods of T-cell expansion, such as CD3/28 beads can expand T-cell clones without loss of specificity, this method is not effective for polyclonal T-cells.[9] Artificial APCs have been successfully used to expand T-cells in an antigen specific manner,[10,11] but a broad panel of such clinical-grade reagents is required to accommodate their use for antigen presentation through all HLA alleles. We chose to use LCL for reactivation and expansion of EBV-specific T-cells from PBMC, as these are readily generated from all patients using a minimal amount of blood. Moreover, they express costimulatory and cell adhesion molecules, which makes them excellent APC. However, a concern was whether LMP2-specific T-cells could be reactivated from the low number of precursors predicted to circulate in the peripheral blood of patients with LMP2-positive tumors,[12,13] using LCL that express immunodominant latent and early lytic cycle antigens.[14] By screening with a panel of peptides spanning the entire LMP2 sequence, we demonstrated that in the majority of CTL lines from patients with NPC and other EBV latency type II malignancies LMP2-specific T-cells were present (Chapter 2). As expected, the number of T-cells specific for LMP2 epitopes was low compared to the frequency of immunodominant antigen-derived epitopes, but nevertheless functional LMP2-specific T-cells could be reactivated and expanded using LCL.

This screening method further allowed us to characterize novel LMP2 epitopes recognized by these CTL. Hence, in addition to responses to previously described epitopes, 8 new epitopes were identified. The latter were restricted through HLA alleles for which no LMP2 epitopes were yet available. The majority of these and previously identified epitopes are conserved between the prototype B95-8 strain used to establish the LCL and viral strains from different geographical origin.[15] Thus, LCL-reactivated T-cells are expected to recognize LMP2-epitopes expressed on the tumor.

LMP2 peptides representing these epitopes and tetramers-derived from these provide valuable reagents to study the effects of immunotherapy. Monitoring the expansion and persistence of adoptively transferred T-cells is crucial for evaluating and improving T-cell therapy, and ultimately for understanding the interaction between the immune system and growing tumors. Using ELISPOT and tetramer assays we monitored the LMP2-specific T-cells populations, as identified in the infusion product, in the peripheral blood prior and following CTL infusion. In 4 of 8 patients with detectable LMP2-specificity in the infusion product a transient increase in the number of LMP2 specific T-cells was found. This indicates a limited expansion and limited persistence of the infused T-cells . However, it is unclear if the number of LMP2-specific T-cells in the peripheral blood reflects the anti-tumor activity of the infused T-cells at the tumor site. Further, despite the absence of a consistent and persistent increase in LMP2-specific immunity in the peripheral blood, clinical responses were observed in 3 of 6 patients with refractory disease. In future studies, the interpretation of the obtained results can be greatly facilitated by introducing a transgene in the infusion product that allows for discrimination from endogenous T-cells and tracking of adoptively transferred T-cells. In conclusion, this clinical study demonstrated feasibility and safety and the first hints of efficacy, which initiated investigation to further refine and enhance the potential of CTL therapy.

Strategies to improve T-cell therapy

A number of parameters could be responsible for the suboptimal efficacy of CTL therapy in the current setting. First, although the majority of the infused CTL lines were shown to contain LMP2-specific T-cells their number may be insufficient to eradiate all tumor cells. We therefore developed a protocol based on LMP2-expressing DCs for reactivation and LMP2-overexpressing LCLs for subsequent expansion of LMP2-specific T-cells. As both DCs and LCLs can be transduced with the chimeric Ad5Ad35 vector the production of only one clinical-grade vector is required. Using these LMP2-overexpressing APCs the specificity within the CTL lines was efficiently enriched for LMP2 specific T-cells (Chapter 4). This protocol is now being used in a clinical study of adoptive T-cell therapy for EBV-positive Hodgkin's disease.

An alternative strategy is the upregulated expression of immunodominant EBV antigens in the NPC tumor cells. A number of chemotherapeutic antigens, including cisplatinum and 5-fluorouracil, both of which are included in the standard treatment regimen for NPC, are shown to induce the switch from the latent to the lytic form of EBV infection in NPC tumor cells.[16] As a subpopulation of LCL expresses early lytic EBV antigens, LCL-reactivated CTL lines generally contain significant numbers of lytic antigen-specific T-cells as was shown for the CTL lines generated for the NPC patients (Chapter 3). Some of these chemotherapeutics also increase the expression of LMP1 in tumor cells, providing additional target antigen enhancement prior to CTL infusion.[17] Chemotherapy may therefore, in addition to its direct anti-tumor effect, sensitize the tumor for EBV-specific T-cell therapy.

A second immune evasion strategy used by tumor cells is the production of a variety of cytokines and chemokines that can negatively affect the maturation and function of immune cells. These include vascular endothelial growth factor, prostaglandin E_2, TGF-β and IL-10. For NPC in particular, enhanced levels of IL-10 at the tumor site and systemically[6] may inhibit antigen presentation and the induction of T helper type I responses.[18] Rendering T-cells resistant to these inhibitory factors may increase their efficacy. Transduction of EBV-specific T-cells with a dominant negative TGF-β receptor makes them insensitive to the anti-proliferative and anti-cytolytic effects of TGF-β.[19] Transgenic expression of IL-12 in CTL was shown to overcome the inhibitory effects of a T helper 2 environment while avoiding the systemic toxicity of recombinant IL-12.[20] These *in vitro* data suggests that harnessing EBV-specific CTL against one or more inhibitory factors present at the tumor site may enhance their efficacy and the value of this approach is currently being investigated in *in vivo* models.

The anti-tumor activity of adoptively transferred T-cells may further be inhibited by T-cell populations with a regulatory function. CD4+CD25+ T-cells are present in the peripheral blood and malignant effusions in patients with various types of cancer.[21,22] *In vitro* experiments have shown that these regulatory T-cells can inhibit T-cell responses in an antigen-specific or non-specific manner.[23,24] Such regulatory T-cells may contribute to the outgrowth of the potentially immunogenic tumor cells of NPC and may explain the lack of an efficient anti-tumor response in immunocompetent hosts. Further, it was demonstrated that regulatory T-cells could reverse the inhibition of tumor growth induced by adoptive transfer of tumor-specific T-cells.[25] This observation suggests that prior depletion of regulatory T-cell populations can enhance the efficacy of immunotherapeutic interventions. Indeed,

removal of CD4+CD25+ cells resulted in enhanced anti-tumor activity of a melanoma vaccine in mice.[26] Similarly, depletion of T-cells with an inhibitory effect prior to CTL infusion may result in improved efficacy. However, ideally, this approach would target a marker that is unique to regulatory T-cells. CD25 is a good candidate, although it is also expressed on activated T-cells and hence targeting this molecule may inadvertently deplete pre-existing tumor-specific T-cells. The desired effect may alternatively be obtained by general lymphodepletion if tumor-specific T-cells can be infused after lymphodepletion.

Finally, incomplete tumor responses post CTL therapy may be due in part to the failure of infused T lymphocytes to undergo adequate expansion. Only those T-cells that proliferate can entry into the memory pool and establish long-term anti-tumor protection.[27] However, the size of the T-cell compartment is maintained at a steady state by a number of potent homeostatic mechanisms involving cytokines or ligands, counting mechanisms, and the availability of space.[28] While these mechanisms are bypassed during immune responses, with increases in the size of spleen, lymph nodes and blood, there is a rapid return to a steady state when antigen stimulation subsides. Because of these homeostatic mechanisms, expansion of infused T-cells is likely to be limited in a steady state situation. In the context of a T-cell deficit however, mature T-cells proliferate to restore the steady state.[29] This may explain why, in the recipients of T-cell-depleted stem cell transplants, infused EBV-specific T-cells expand by 4 logs or more.[1,2] In contrast, when EBV-specific T-cells are given to NPC patients, in whom the T-cell compartment is already replete, expansion of adoptively transferred T lymphocytes is much lower or absent. Lymphoid depletion as a strategy to improve the efficacy of adoptively transferred has already shown evidence of success. When melanoma patients received cyclophosphamide and fludarabine prior to the adoptive transfer of large numbers of highly-activated melanoma-specific tumor infiltrating T-cells, peripheral repopulation and proliferation of the transferred cells was observed, as well as clinical responses (> 50% tumor reduction) in 18 of 35 patients treated.[30,31] Instead of chemotherapeutic agents, monoclonal antibodies specific for mature lymphocytes may provide a more specific means of obtaining the desired lymphodepletion. The mAb of choice should have low toxicity, a short half-life, high specificity and be available as a clinical grade reagent. Although the pan T-cell antibody anti-CD3 may have optimal specificity, its long half life and induction of tolerance reduce its attraction for this purpose.[32] mAbs specific for CD45, a pan lymphocyte marker, can profoundly deplete lymphocytes in peripheral blood and lymphoid organs, whilst sparing hematopoietic progenitor cells.[33,34] The pharmacokinetics of CD45 MAbs, with a half life of circa 8 hours are ideal for this purpose since they allow CTL infusion shortly after CD45 MAb administration and provide a window of several weeks for adoptively transferred CTL to proliferate prior to recovery of endogenous lymphocytes. We have implemented this method of lymphodepletion in our current study of CTL therapy for NPC.

Encouragingly, the clinical responses induced by adoptive transfer of melanoma-specific T-cells in lymphodepleted hosts were strongly associated with the level of persistence of the infused T-cells.[35] Lymphodepletion may have promoted the persistence of infused T-cells by enhancing the access to cytokines such as IL-7 and IL-15. IL-15 protects T-cells against activation-induced cell death and promotes homeostatic maintenance of memory CD8+ T-cells.[36] Further, in a lymphopenic environment the infused T-cells have a drive to proliferate which may allow them to transfer from effector memory to central memory cells and as such

providing long-term tumor protection.[37] Additional strategies to obtain long-lived function of adoptively transferred T-cells include their transduction with the required cytokines: the tumor-specific T-cells then locally provide the required cytokines thereby avoiding the adverse effects of systemic cytokine administration.[38] Further, vaccination strategies may improve persistence of infused T-cells. For example, DC vaccination induced cytokine production, enhanced proliferation, increased tumor infiltration and a more robust tumor response of adoptively transferred T-cells.[39] In the setting of EBV-associated malignancies irradiated LCL could be used for boosting of tumor-specific T-cells by vaccination. Clinical studies of these and other approaches are being initiated in our and other institutions to test their attributive effect to improved efficacy of T-cell therapy.

Strategies to broaden application of T-cell therapy

While EBV-related malignancies are elegant models to test general ideas about immunotherapy of cancer, unfortunately for most malignancies immunogenic and well-defined antigens are not yet available. Most tumor antigens for non-viral malignancies are either over-expressed differentiation antigens or aberrantly expressed fetal antigens. If adoptive immunotherapy for these forms of cancer is to be successful some new approaches must be undertaken. We must overcome the barrier of easily generating and expanding sufficient numbers of tumor-specific T-cells that are available for all patients regardless of HLA phenotype. Although methods usually relying on DCs as powerful antigen presenting cells are being developed these may still be impractical or not applicable for all antigens. For example, although HA-1 specific T-cells can be reactivated and expanded using peptide-pulsed DCs[40] this strategy is not successful in all cases. An attractive solution is TCR transfer: grafting T-cells with TCRs with the desired specificity. Although seemingly simple this approach has not been practical mainly for technical reasons including inefficient transduction of T-cells, poor expression or assembly of the transgenic TCR and the use of separate vectors for expression of the TCRα and β chains. We attempted to solve these problems using RD114 envelope-pseudotyped virus to obtain high transduction efficiency,[41] a retroviral vector that accommodates high transgene expression in T-cells (splicing SFG)[42] and a foot-and-mouth disease-derived 2A sequence to obtain equal expression of the TCR α and β chain from a single vector.[43] Using this expression system an HA-1 tetramer-positive population was detectable after a single transduction of EBV-specific CTL (Chapter 5). The HA-1 TCR transduced CTL had dual specificity for HA-1 and EBV-antigens and, importantly, cytolytic activity against hematopoietic cells natively expressing HA-1 was demonstrated.

We hypothesize that the main limitation to further enhance expression of the transgenic TCR is promiscuous cross pairing of transgenic TCRα and β chains with native TCRβ and α chains respectively. This leads to lower expression, lower function and potentially novel specificities. As other groups, we attempted to generate single-chain receptors,[44,45] but failed to detect any surface expression or function in this form. Currently, we are trying to introduce leucine zipper motifs that will force correct pairing of the transgenic TCRα and β chains.[46] The risk of TCRs with auto-immune reactivity as a results of cross-paring with native TCRs may be further reduced by transduction of antigen-specific CTL instead of polyclonal T-cells with unselected specificity.[47]

Once we have developed a strategy for readily achievable, high level functional transgenic TCR expression, this approach should be transferable to any TCR. However, as high avidity CTL are required for optimal anti-tumor activity,[48,49] the main challenge will be to obtain high avidity CTL clones specific for tumor antigens. HA-1 provides an ideal target in that the allo-nature of this response likely increases the presence of high affinity receptors. The majority of tumor associated antigens, however, are self antigens, resulting in a peripheral T-cell repertoire that is devoid of high avidity antigen-specific T-cells, due to self tolerance.[50] A number of strategies have been developed to circumvent tolerance to self-antigens to obtain high affinity tumor-specific TCRs. These include the induction of allo-restricted antigen-specific T-cells[51,52] and vaccination of HLA-transgenic mice with peptides representing self antigen epitopes[50,53]. Alternatively, low affinity TCRs isolated from a tolerized environment can be engineered to enhance their affinity.[54,55] Further, as CD4+ T-cells play an important role in the induction and persistence of tumor immunity, it is desirable to extend this approach to HLA class II-restricted TCRs. So far only a limited number of HLA class II-restricted tumor antigen-derived epitopes have been identified. Grafting CD4+ T-cells with MHC class I-restricted TCRs provides an alternative means to obtain tumor-specific CD4+ cells. Willemsen et al demonstrated this approach is feasible when the CD8α is co-expressed with the transgenic MHC class I TCR.[56] Finally, rapid screening techniques (as proposed in Chapter 5) need to be developed to obtain a large array of high affinity tumor-antigen specific TCRs restricted through different HLA alleles to allow application of this strategy to a broad patient group.

Chimeric TCRs are very attractive since they graft a surface specificity. Unlike native TCR they are not HLA restricted, nor are they susceptible to downregulation of HLA molecules and defects in antigen processing. Moreover, T-cell mediated effector functions are more likely to result in tumor cell lysis than humoral immune response alone. Cytokine secretion upon T-cell activation by tumor antigen will result in the recruitment of additional components of the immune system, amplifying the anti-tumor immune response. The main problem with the chimeric TCR approach is the number of truly tumor-specific target antigens available. In the absence of these, markers of non-essential tissue such as CD19 and CD20 (B cells) or prostate surface proteins may be used as alternatives.

The clinical benefits obtained so far with chimeric TCRs based on a CD3-ζ have been limited, at least in part because of incomplete T-cell activation by the tumor cells, which do not provide the required co-stimulatory signals. Indeed, incorporation of a CD28 signaling domain clearly improved efficacy in animal models,[57,58] but remains limited to tumor growth inhibition. The recently proposed concept that different co-stimulatory molecules are required to provide proliferative and survival signals suggested that incorporation of addition costimulatory signals in artificial TCRs is necessary for optimal function.[59] Incorporation of OX40 in our model chimeric receptor allowed for extensive and prolonged expansion of redirected T-cells upon stimulation with tumor cells even in the absence of exogenous IL-2 (Chapter 6). This increased expansion may be the result of a stronger proliferative signal but more likely results from protection against apoptosis as OX40 has been shown to be instrumental in maintaining high expression of anti-apoptotic molecules.[60] The lack of increased activity of T-cells transduced with a TCR containing OX40-ζ in the absence of CD28, is in line with the observation that the physiological expression of both OX40 and OX40L requires CD28-B7 interactions.[60] Thus, the combined incorporation of CD3-ζ, CD28 and OX40

in the endodomain of the tumor-specific TCR ensures the presence of essential signals for target cell lysis, clonal activation and proliferation as well as early and sustained survival signals resulting in a potent anti-tumor response *in vitro*. It remains to be determined if this triple domain can further enhance the anti-tumor activity obtained with CD28.CD3-ζ based TCRs . Animal studies have already shown the first indication that OX40 engagement can enhance anti-tumor immunity.[61,62] Incorporation of OX40 in the endodomain of a tumor-specific TCR may provide a similar beneficial effect while circumventing the risk of the induction of auto-immunity when using an OX40 activating antibody.[63]

Safety using genetically modified T-cells

Genetic modification of T-cells used for adoptive transfer is likely to be the next incremental step in the development of adoptive T-cell therapy. Strategies of rendering T-cells resistant to tumor evasion mechanisms or to provide them with cytokines that enhance their survival have shown promising results *in vitro*.[19,20] In addition, grafting T-cells with the desired specificity will greatly facilitate the generation of tumor-specific T-cells to a broader range of antigens. However, these genetically modified T-cells may have unwanted toxicity caused by the transgene or by targeting antigens that are not solely expressed on tumor cells.[35,64,65] These concerns can be addressed by incorporation of a safety switch that allows for elimination of transduced cells in the event of toxicity. We developed an inducible caspase 9 that in contrast to other safety mechanisms has low immunogenic potential and selectively and virtually completely eliminates transduced cells (Chapter 7). In contrast to inducible death molecules that act as initiators of the apoptosis pathway, inducible caspase remains functional in the presence of most inhibitors of apoptosis. This is important to ensure function in T-cells that are relatively apoptosis-resistant including memory T-cells and malignantly transformed T-cells. The optimal function of inducible caspase 9 requires selection for T-cells with bright transgene expression. This can be achieved by co-expression of non-immunogenic marker genes such as CD20, truncated CD34 or truncated NGFR.[66-68] The development of retroviral vectors that allow for reliable co-expression of multiple gene makes the expression of a transgene, a suicide gene and a marker gene from a single vector a feasible possibility/goal.[69]

In the majority of studies depending on gene transfer to T-cells, oncoretroviral vectors that rely on integration in the host genome for transgene expression were used. Murine retroviruses have been associated with the induction of leukemia in two children receiving corrective therapy for X-linked severe combined immunodeficiency.[70] In this study patients received CD34+ bone marrow stem cells transduced with the common gamma chain – a critical proliferative signaling protein. It is difficult to extrapolate this potential danger to studies of transduced peripheral blood T-cells especially when bearing in mind that over 100 patients have received retrovirally transduced T-cells without subsequent leukemogenesis. Nevertheless, vector designs that reduce the risk of insertional mutagenesis are warranted. Self-inactivating (SIN) retroviral vectors in which the LTRs become inactivated upon integration reduce the risk of vector driven overexpression of genes in close proximity to the unemployed 3' LTR. Flanking the transgenic promoter-enhancer in the retroviral cassette with a chromatin insulator provides and additional safety mechanism.[71] The use of SIN vectors was so far inhibited by the difficulty of obtaining high titer virus. This limitation may now be overcome as RD114-pseudotyped transient transfection produced vector affords us high titer virus that facilitated efficient transduction of T-cells (Chapter 5-7).

Future of adoptive T-cell therapy

From the clinical studies of adoptive T-cell therapy including the study described in this thesis, valuable expertise has been gained with the generation, characterization and monitoring of tumor-specific T-cells. With improved knowledge of the interaction between the immune system and malignant cells insights are emerging how to enhance efficacy. Improved vector design, improved transduction protocols and a suicide switch allow for the clinical implementation of T-cells genetically modified to obtain the desired specificity and enhance potency. Well designed clinical trials are now required to evaluate the effect of modifications on expansion, persistence and function of adoptively transferred T-cells.

Crucial in this is the monitoring of grafting and homing of the infused T-cells. Monitoring the number of tumor-specific T-cells using ELISPOT or tetramer assays does not discriminate between those T-cells that were adoptively transferred and a population arising de novo in the host. Similarly, the TCR-Vβ spectratype of the infusion product is a surrogate indicator.[72] Genetic modification provides a unique marker that permits infused T-cells to be distinguished from a newly arising population of identical specificity and phenotype.[73] Slightly different vectors can be used to mark different cell population allowing differential tracking.[74] Further, as a single transduced cell and its progeny have a unique site of integration of the retroviral vector this "signature" can be used to follow individual T-cell clones over time. Highly valuable information regarding expansion, persistence and homing of infused T-cells has been obtained using gene marked virus and tumor-specific T-cells in adoptive T-cell therapy studies.[1,3,5,75,76] During the clinical study described in this thesis gene marking was not available due to safety concerns following the two cases of leukemia in children receiving retrovirally modified stem cells.[70] Also for the immediate future at least, it is unlikely that retroviral transduction of adoptively transferred cells purely for marking purposes will be pursued. However, studies involving genetically improved T-cells also provide gene marking. Here, the potential benefit of the modification would outweigh the risks of insertional mutagenesis, in particular when used to treat cancer patients with relapsed/refractory disease.

Although monitoring the fate of adoptively transferred T-cells provides essential information, only a combination of improved anti-tumor immunity and clinical response can demonstrate the effect of immunotherapy. This requires sensitive tumor markers and imaging techniques in particular when treating patients with minimal residual disease. For NPC, the concentration of cell-free EBV-DNA as detected in plasma is emerging as a reliable marker for diagnosis and response to treatment.[77] However, the sensitivity of this method, in other words what size tumor results in a detectable level of EBV-DNA in plasma, still needs to be determined. Further, in our clinical study, evaluation of tumor responses proved difficult in a number of cases as MRI and CT scan did allow for discrimination between radiation-induced fibrosis and residual NPC.[78] We therefore implemented the more sensitive PET-scan as method of choice in our current clinical studies. Combined imaging of the adoptively transferred T-cells and the targeted tumor would provide highly valuable insight in the efficacy of adoptive T-cell therapy strategies. MRI imaging of tumor-specific T-cells labeled with physiologically inert nanoparticles has been shown a sensitive real-life imaging technique to study T-cell migration and homing in mice.[79] Transgenic expression of a reporter that can be visualized by PET scan (e.g. sodium–iodine-symporter) may become available as a non-invasive imaging technique for long-term monitoring of the infused cells.[80]

Key to the rapid implementation of new insights in cancer immunotherapy is successful interaction with industry and regulatory agents.[81] Reluctance by industry to make their products (e.g. cytokines) available for clinical study forces the use of less optimal reagents. Agreements to share liability and financial risk between industry and government could address this issue. Regulatory rules set down by government agencies for phase I clinical studies represent a tremendous burden to the clinical researcher. Standards applicable to the pharmaceutical industry are often mandated. Extremely expensive testing and re-testing of clinical products drains grant money, as does the requirement for extensive infrastructures for quality assurance and data monitoring. While such standards are reasonable for drugs developed for use by a large number of patients for non-life-threatening disorders, the regulatory burden for even the simplest study acts a powerful deterrent to investigators. To allow for rapid execution of novel clinical studies, simplification of requirements should be implemented. Government agencies should create centralized facilities for generating vectors and cell products, since high throughput of production and testing can greatly reduce the costs of staffing and facilities and concentrate expertise. Specialized committees that include members working in the immunotherapy field may be best able to address issues relevant to biological agents. Such changes would foster an effective relationship between regulatory agencies and clinical investigators with the mutual goal of moving the field forward in safe and efficient manner.

In conclusion, this thesis describes incremental advances made in overcoming barriers to the successful use of adoptive T-cell therapy. Our studies and others have demonstrated the feasibility of generating sufficient autologous tumor-specific T-cells from patients with advanced malignancy for clinical use. Improved reactivation and expansion methods are now becoming available for selection of T-cell specific for less immunogenic tumor antigens.[82-84] Our protocol to generate LMP2-specific CTL has already been implemented in an ongoing clinical study. For non-immunogenic tumor antigens, or antigens for which tolerance exits, native TCR transfer using modern retroviral vector techniques, may soon allow any TCR specificity to be grafted onto autologous T-cells in a clinically feasible manner. Harnessing T-cells with costimulatory molecules or other immune modifiers is a promising strategy to overcome immunosuppressive strategies employed by tumor cells to prevent their eradication, the value of which in now being evaluated in animal models. Inducible caspase 9 provides a non-immunogenic highly effective suicide gene that will allow many of these new approaches to be implemented in clinical protocols. Together, engineering work on T-cell selection and genetic modification, such as presented in this thesis, may well allow us to develop adoptive T-cell therapy into an effective non-toxic treatment modality for cancer.

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Summary

Nasopharyngeal carcinoma (NPC) is a cancer arising from the nasal cavities and throat. Although rare in Europe and in North-America this is one of the 10 most common cancers in Southeast Asia. The vast majority of these cancers are associated with Epstein-Barr Virus (EBV) – a ubiquitous virus infecting most people by the age of 18. It is felt that this virus plays an important role in causing this cancer along with other known risk factors - the consumption of salted fish and environmental factors. When diagnosed at an early stage NPC can be effectively treated with radiotherapy and chemotherapy. However, as this cancer gives rise to few symptoms at an early stage, disease is often advanced at diagnosis. At this advanced stage treatment options are limited. Further, radiotherapy, a cornerstone of treatment, causes significant and unpleasant side effects when applied to this delicate region of the body. For these reasons the development of new treatments that selectively destroy cancer cells without affecting normal tissues are badly needed. One such strategy is immunotherapy which utilizes the immune system to selectively target and destroy tumor cells.

The immune system protects the body against invading pathogens such as bacteria and viruses. It consists of different components that each have their own function but are mutually interactive. The innate immune system provides a first barrier against potentially harmful invaders. Following activation of the innate immune system, the acquired immune system generates a response specially targeted towards the invading pathogen. This response is mediated by two types of white blood cells: B-cells and T-cells. These cells use their receptors to distinguish between normal healthy cells and virally infected cells. B-cell receptors recognize foreign proteins directly, while in contrast, T-cells recognize small protein fragments (epitopes) that are processed within the cells and presented on the cell surface by a specialized family of proteins referred to as the major histocompatibility complex (MHC). The type of MHC used varies for each individual so that the virus epitopes recognized vary from person to person. Antigen recognition by B-cells results in the production of antigen-specific antibodies which bind pathogens and trigger their immunological destruction. When viral epitopes are recognized by T-cells two types of T-cells are activated: cytotoxic T-cells that actively destroy the infected cells and helper T-cells that coordinate the immune response. Selecting the T- and B-cells that appropriately recognize invading pathogens takes time – but some of these immune cells survive for decades, ready to be quickly activated when the same pathogen is encountered again, in this way forming immunological “memory”.

It is thought that the immune system can distinguish cancer cells, like virus-infected cells, from their healthy counterparts. For example, certain proteins are expressed in a mutated form or at a higher level on cancer cells as compared to healthy tissue. Other proteins are only present in tumor cells and embryonic tissue but are absent in normal differentiated cells. A subgroup of cancers expresses viral proteins such as EBV proteins in NPC and in certain lymph node cancers and human papilloma virus (HPV) proteins in cervical cancer. Despite these differences between normal cells and cancers cells cancer can develop even in individuals with a normal functioning immune system. It is well known that certain

viruses can survive in the body of a healthy individual in a latent (or quiescent) state. These viruses have co-existed for centuries with the human immune system and developed strategies to hide or subvert the immune system. Tumors are likely equipped with similar strategies to inhibit the players of the immune system and hamper their response.

Convincing the immune system to initiate a response against antigens present on cancer cells may overcome these barriers and provide an effective form of treatment. Initiation of such a response can be achieved in two different ways: (1) stimulating the immune system with the tumor-specific antigen and encouraging it to elicit an immune response, a strategy similar to vaccination as protection against infectious diseases or (2) collecting blood from the patient to select and expand those T-cells that can recognize the tumor cells and subsequently give these selected cells back to the patient. This last strategy, commonly referred to as adoptive T-cell therapy has the advantage that the tumor antigen can be presented to the T-cells in an ideal context (on professional antigen presenting cells) and in an ideal environment (in the presence of stimulating cytokines and away from inhibiting factors secreted by the tumor cells). For these reasons adoptive T-cell therapy was our strategy of choice to develop a novel treatment for NPC.

The viral proteins expressed by NPC provide target antigens for the immune system which makes it an ideal candidate to develop immunotherapy. Most people have encountered EBV before adolescence and therefore their blood contains T-cells that specifically recognize EBV antigens. Previous work in our laboratory has demonstrated that a large number of EBV-specific T-cells can be expanded from a small amount of blood using B-cells infected with a laboratory strain of EBV as stimulator cells. After these T-cells have undergone vigorous testing including tests for specificity and sterility they can be given back to the patient. This strategy has already proven successful as prophylaxis and treatment for EBV-associated lymphomas that can develop in patients that underwent bone marrow transplantation. These lymphomas express a broad range of EBV antigens including EBV nuclear antigen (EBNA)-3a, -3b and -3c that elicit a strong T-cell response (latency type 1, chapter 1, figure 1). For this reason this type of lymphoma can only arise in patients with a suppressed immune system such as bone marrow transplant recipients and HIV-infected individuals. Other EBV-associated cancers such as Hodgkin's lymphoma and NPC can develop in individuals with a normal functioning immune system. These tumors express a limited number of EBV antigens including latent membrane protein (LMP)-1 and -2 and EBNA-1 all of which are only weak antigens (EBV latency type 2). Nevertheless these viral antigens do provide tumor target antigens to develop immunotherapeutic strategies for NPC.

The first part of this thesis describes the initial steps towards adoptive T-cell therapy for EBV latency type 2 cancers, in particular NPC. First the feasibility of generating EBV-specific T-cells from the blood of patients with advanced cancer was evaluated (Chapter 2). Despite previous treatment with radiation and chemotherapy EBV-specific T-cells were successfully generated for all patients. EBV-infected B-cells were used to select and expand T-cells that are specific for EBV antigens including LMP2. A screening procedure using pools of LMP2 protein fragments demonstrated that all patient T-cell lines generated contained T-cells specific for LMP2. For each patient T-cell line it was determined which particular LMP2 epitopes were recognized. So far LMP2 epitopes had only been identified for HLA types common in Caucasian populations. However, NPC is more common in other ethnic populations for which no epitopes were yet available. Using the T-cell lines generated for our North-American patient group, epitopes were identified for HLA types common in the Asian and Hispanic populations. With this new information we can now monitor the number of LMP2-specific T-cells in a broad group of patients and thus gain insight in the effect of therapy.

Subsequently, 10 patients with advanced NPC were treated with EBV-specific T-cells on a phase 1 trial (Chapter 3). Of these 10 patients 4 patients were in remission and 6 patients had a relapsed or residual tumor not responsive to conventional therapy. Adoptive T-cell therapy was without side effects in 9 out of 10 patients. In one patient the administration of the T-cells was associated with a significant swelling at the tumor site. Of the 6 patient with relapsed/residual disease 2 patients did not respond, 1 patient has stable disease, 1 patient had a partial response, and 2 patients are in complete remission. These clinical responses could not be correlated with the number of LMP2-specific T-cells in the peripheral bloodstream. One explanation is that the technique used is not sensitive enough to detect small numbers of LMP2-specific T-cells. An alternative explanation is that the injected T-cells travel to the tumor site and are therefore not detectable in the peripheral blood stream.

Although the results of this study were encouraging, adjustments are required to make this type of therapy successful for the majority of patients. The effect of immunotherapy may be enhanced by increasing the number of T-cells that can recognize antigens expressed by the tumor cells such as LMP2. Chapter 4 describes a method to selectively expand LMP2 specific T-cells instead of expanding T-cells that recognize all latent EBV antigens including those that are not present on NPC. Instead of using EBV-infected B-cells we now used specialized antigen presenting cells, dendritic cells, genetically modified to express large amounts of LMP2. The T-cells reactivated using this method were subsequently expanded using EBV-infected B-cells genetically modified to enhance their expression of LMP2. This technique is implemented in an ongoing clinical trial of adoptive therapy of LMP2-specific T cells as treatment for EBV-positive Hodgkin's lymphoma. Other methods to enhance the efficacy of adoptive T cell therapy are discussed in Chapter 8.

Unfortunately only a small number of cancers express viral antigens that are easy to recognize by the immune system. For application of adoptive immunotherapy to a broad range of tumors a method needs to be developed to generate large numbers of T-cells specific for antigens that normally elicit no or only a weak immune response. This is the aim of the research described in the second part of this thesis.

The antigen specificity of a T-cell is determined by its T-cell receptor (TCR). A TCR consist of a conglomerate of proteins, the extracellular components of which recognize and bind the antigen (Chapter 1, figure 3), while the intracellular components subsequently transmit signals resulting in proliferation of the T-cells, killing of the recognized target cells and the secretion of cytokines. Using gene transfer techniques, the antigen recognizing component of a TCR can be isolated from a T-cell with the particularly desired specificity and then transferred to a large number of other T-cells. These modified T-cells then acquire the same specificity as that from which the TCR originated. Using a similar strategy the specificity of an antibody can be grafted onto a T-cell. The antigen recognition domain of the TCR is replaced by the antigen recognition domain of the antibody. The resulting chimeric TCR

combines the antigen specificity of an antibody with the effector function of a T-cell. Once a T-cell or antibody with the desired specificity has been isolated, this method of grafting specificity onto other cells, allows you to readily obtain a large number of T-cells with the same specificity. Although this technique is attractive so far a number of problems have hindered its application in clinical studies. First, the expression level of the newly introduced transgenic TCR needs to be in a similar range as compared to endogenous expressed TCR as to obtain functional antigen recognition. This requires a technique for the introduction of transgenic genes that is optimized for T-cells.

Second, from previous work by others it is known that antigen recognition alone is not sufficient for the elimination of tumor cells. Additional signals provided by co-stimulation molecules are required for an optimal T-cell mediated immune response. Moreover, in the absence of co-stimulation signals antigen recognition can result in antigen tolerance rather than elimination. Co-stimulation molecules are often absent on tumor cells and this may represent a mechanism by which they can evade an immune response. When built-in co-stimulation signals are provided by the transgenic TCR this escape route may be blocked and an effective anti-tumor response may be induced.

Finally, to be able to use such genetically modified T-cells as treatment in humans a fail-safe system is desirable so that in the event of unwanted side-effects the infused T-cells can be destroyed. Building in a suicide gene which activation leads to cells death provides such a safety system. A suicide gene derived from herpes simplex virus has already been used in clinical studies. Disadvantage of this system is that T-cells transduced with this virus-derived gene are recognized by the immune system as infected and are therefore destroyed. This compromises a long-lived effect of the infused T-cells. A suicide gene that consists solely of human-derived components would overcome this problem.

These steps towards the clinical application adoptive T-cell therapy with genetically modified T-cells form the backbone of the research in the second part of this thesis. Chapter 5 describes how functional expression of a transgenic TCR can be obtained using an optimized method for the introduction of a transgene in human T-cells. For this project, minor histocompatibility antigen HA-1 was used as a model. HA-1 specific T-cells have a proven role in the elimination of relapsed leukemias in bone marrow transplant recipients. However, obtaining HA-1 specific T-cells using standard stimulation and expansion techniques is a difficult, and labor intensive process. Using an optimized gene transfer technique T-cells transduced with a HA-1 specific TCR were obtained that appear to function as well as T-cells natively specific for HA-1. This technique is expected to facilitate the production of large numbers of these therapeutic T-cells.

Subsequent work was done to evaluate if by building in different combinations of co-stimulation molecules in TCRs T-cells can be equipped with the essential signals to mount an effective anti-tumor response (Chapter 6). For this study a chimeric TCR derived from a tumor antigen specific antibody was used. The basic TCR without build-in co-stimulation was sufficient for the elimination of tumor cells. However, T-cell proliferation and secretion of immunomodulatory cytokines, both essentials factors in maintaining an ongoing immune response, were only induced in the presence of co-stimulation molecules CD28 and OX40. These results suggests that by providing CD28 and OX40 signals within the TCR an effective tumor response can be obtained and the absence of these essential signals on the tumor cells can be overcome.

Finally, Chapter 7 describes the development of a suicide gene based on caspase 9, a human molecule that plays an essential role in the process of programmed cell death (apoptosis). Caspase 9 was fused with the binding domain of a synthetic molecule (AP20187) to obtain an inducible form of caspase 9. Administration of AP20187 results in the formation of a complex of two inducible caspase 9 molecules which leads to their activation and initiation of the apoptosis cascade. This inducible caspase 9 molecule can be expressed in human EBV-specific T-cells without interfering with their normal function and specificity. Activation of inducible caspase 9, when expressed above a certain threshold level, resulted in the elimination of all transduced T-cells. This suicide gene accommodates the implementation of newly developed adoptive T-cell therapy strategies, including those proposed in this thesis, in clinical studies in the near future.

Samenvatting

Dit proefschrift beschrijft onderzoek naar adoptieve T-cel therapie als behandeling voor tumoren die geassocieerd zijn met het Epstein Barr virus (EBV) en strategieën om deze vorm van therapie te benutten voor andere tumoren die niet geassocieerd zijn met een virus.

Nasopharynxcarcinoom (NPC) is een tumor van de neusholte. Deze vorm van kanker is zeldzaam in Europa en Noord-Amerika, maar is een van de tien meest voorkomende tumoren in Zuidoost-Azië. Meer dan 90% van de NPCs van het ongedifferentieerde type zijn geassocieerd met het EBV wat suggereert dat infectie met dit virus een rol speelt in het ontstaan van deze tumoren. Daarnaast kunnen genetische factoren en omgevingsfactoren, zoals het eten van gezouten vis, aan dit proces bijdragen. Wanneer NPC wordt ontdekt in een vroeg stadium kan deze tumor effectief worden behandeld met bestraling en chemotherapie. Maar aangezien deze vorm van kanker aanvankelijk weinig of geen klachten geeft, is de ziekte vaak in een gevorderd stadium ten tijde van de diagnose. In deze latere tumorstadia is de genezingskans aanzienlijk geringer. Bovendien heeft bestraling in het nasopharynxgebied vaak ernstige bijwerkingen, waaronder het ontstaan van secundaire tumoren. Om deze redenen wordt gezocht naar een behandelingsstrategie die leidt tot de selectieve vernietiging van tumorcellen zonder aantasting van gezond weefsel. Een van de behandelmogelijkheden is immuuntherapie waarbij het afweersysteem wordt ingezet voor de vernietiging van tumorcellen.

Het afweersysteem heeft tot taak het lichaam te beschermen tegen schadelijke indringers, pathogenen, waaronder bacteriën en virussen. Dit beschermingssysteem bestaat uit verschillende componenten: het aangeboren afweersysteem dat een eerste verdediging vormt tegen pathogenen en het verkregen afweersysteem dat vervolgens een pathogeenspecifieke afweerreactie induceert. De hoofdcomponenten van het verkregen afweersysteem zijn B-cellen en T-cellen. Deze witte bloedcellen kunnen door middel van hun receptoren onderscheid maken tussen geïnfecteerde cellen en gezonde cellen. B-cellen herkennen eiwitten aan het oppervlak van geïnfecteerde cellen en produceren vervolgens antistoffen om deze onschadelijk te maken. T-cellen herkennen eiwitfragmenten (epitopen) die in de cel worden geproduceerd en vervolgens door moleculen van het major histocompitibility complex (MHC) aan het celoppervlak worden gepresenteerd. Deze MHC moleculen verschillen per individu zodat de virusepitopen die worden herkend door de T-cellen niet voor iedereen dezelfden zijn. Herkenning van virusepitopen resulteert in de activatie van twee typen T-cellen: cytotoxische T-cellen die de geinfecteerde cellen doden en helper T-cellen die de immuunrespons coördineren. Een belangrijke eigenschap van dit verkregen afweersysteem is het geheugen wat ervoor zorgt dat, wanneer hetzelfde pathogeen opnieuw het lichaam binnen dringt, het afweersysteem snel en effectief kan ingrijpen.

Er zijn aanwijzigingen dat tumorcellen, net als virusgeinfecteerde cellen, verschillen van gezonde cellen afkomstig uit hetzelfde weefsel. Zo komen bepaalde eiwitten in tumorcellen in een gewijzigde vorm tot expressie. In andere tumoren komen eiwitten voor die normaal alleen in embryonaal weefsel aanwezig zijn. Een subgroep tumoren brengt eiwitten tot ex-

pressie afkomstig van een virus (bijvoorbeeld humaan papillomavirus in baarmoederhalskanker en EBV in bepaalde lymfekliertumoren en NPC). Ondanks deze verschillen tussen gezonde cellen en tumorcellen kunnen tumoren ontstaan en uitgroeien, zelfs in individuen met een normaal functionerend afweersysteem. Dit is vergelijkbaar met virussen die in een latente (slapende) vorm kunnen overleven in immuuncompetente individuen. Deze virussen hebben tijdens hun coëvolutie met het humane afweersysteem een reeks ontsnappingsmechanismen ontwikkeld om te kunnen overleven en zich te kunnen verspreiden. Tumoren beschikken over vergelijkbare mechanismen om herkenning van tumorcellen door het afweersysteem te verhinderen en de functie van de afweercellen te dempen.

Het aansporen van het afweersysteem tot een tumoreiwitspecifieke reactie kan deze barrières mogelijk wegnemen en leiden tot een effectieve vernietiging van de tumor. Activatie van het afweersysteem kan bereikt worden op tenminste twee manieren: (1) door middel van vaccinatie met tumor(geassocieerde)eiwitten, vergelijkbaar met vaccinatie ter bescherming tegen infectieziekten of (2) door buiten het lichaam tumorspecifieke T-cellen te selecteren en te vermenigvuldigen en deze volgens terug te brengen in de bloedbaan van de patiënt. Deze laatste strategie, adoptieve T-cel therapie, heeft als voordeel dat een tumorspecifieke immuunreactie wordt opgewekt in de ideale context (professionele antigeenpresenterende cellen) en in een ideaal milieu (vrij van immuunsuppressieve stoffen die worden uitgescheiden door tumorcellen). Om deze redenen is adoptieve T-cel therapie gekozen als strategie om een nieuwe behandelingsmethode te ontwikkelen voor NPC.

NPC is een ideale kandidaat voor immuuntherapie aangezien de virale eiwitten die door de tumorcellen tot expressie worden gebracht, kunnen dienen als herkenningspunt voor het afweersysteem. Het merendeel van de bevolking is op de kinderleeftijd al in aanraking gekomen met EBV (het virus dat ook de ziekte van Pfeiffer veroorzaakt) en heeft derhalve EBV-specifieke T-cellen in het bloed. Eerder onderzoek in ons laboratorium heeft aangetoond dat EBV-specifieke T-cellen kunnen worden opgekweekt vanuit een kleine hoeveelheid bloed door middel van stimulatie met een B-cellijn die is geinfecteerd met EBV. Nadat de cellen zijn getest voor specificiteit en steriliteit kunnen deze vervolgens worden toegediend aan de patiënt. Deze strategie is al succesvol gebleken als preventie en behandeling voor EBV-geassocieerde lymfomen bij patiënten die een beenmergtransplantatie hebben ondergaan. Deze lympfomen brengen een reeks EBV-eiwitten tot expressie (waaronder EBV nucleus antigenen (EBNA)-3a, -3b en –3c) die een sterke T-cel reactie opwekken (EBV latentie type 3, figuur 1, hoofstuk 1). Deze immunogene tumoren ontstaan dan ook alleen bij patiënten met een verminderde afweer zoals transplantatie- en HIV-geinfecteerde patiënten. In andere EBV-geassocieerde tumoren zoals NPC en Hodgkin lymphoom (HL), die uitgroeien in individuen met een normaal functionerend immuunsysteem is het expressiepatroon van EBV-eiwitten beperkt tot de minder immunogene eiwitten EBNA1 en latent membraaneiwit (LMP)-1 en –2 (EBV latentie type 2). Desalniettemin kunnen deze minder immunogene EBV eiwitten worden gebruikt als tumorspecifieke antigenen voor immuuntherapie.

In het eerste deel van dit proefschrift worden de eerste stappen beschreven naar adoptieve T-cel therapie als behandeling voor EBV latentie type 2 tumoren, waaronder NPC. Allereerst werd de haalbaarheid bestudeerd van het opgroeien van EBV-specifieke T-cellen uit het bloed van patiënten met tumoren in gevorderd stadium (hoofdstuk 2). Ondanks voorafgaande behandeling met bestraling en chemotherapie konden EBV-specifieke T-cellen worden opgekweekt voor alle patiënten. EBV-getransformeerde B-cellen werden gebruikt voor de reactivatie en vermenigvuldiging van T-cellen specifiek voor EBV antigenen waaronder LMP2. Door middel van een screeningstechniek met pools van LMP2 eiwitfragmenten werd aangetoond dat in het merendeel van de T-cellijnen cellen specifiek voor het tumorantigeen LMP2 aanwezig waren. Van de cellijn van elke patiënt werd in kaart gebracht welke LMP2 epitopen werden herkend. Tot op heden waren met name LMP2-epitopen gekarakteriseerd voor HLA-typen die veel voorkomen in West-Europa. Een groot deel van de NPC-patiënten in Noord-Amerika heeft echter een andere ethniciteit en derhalve vaak een HLA-type waarvoor nog geen epitopen beschikbaar zijn. Gebruikmakend van de T-cellijnen van deze patiënten werden LMP2-epitopen geidentificeerd voor HLA-typen die algemeen voorkomen in Azië en Zuid-Amerika. Dit uitgebreide panel van LMP2-epitopen kan nu worden gebruikt om het aantal LMP2-specifieke T-cellen voor en na immuuntherapie te bestuderen bij een brede patiëntengroep en zo inzicht te verkrijgen in het effect van de behandeling.

In een fase 1 klinische trial werden vervolgens 10 patiënten met NPC in een gevorderd stadium (3 of 4) behandeld met EBV-specifieke T-cellen (hoofdstuk 3). Van deze 10 patiënten waren 4 patiënten in remissie en 6 patiënten hadden een recidu of recidief tumor na behandeling met chemotherapie en bestraling. Adoptieve T-cel therapie was zonder bijwerkingen in 9 van de 10 patiënten. In 1 patient was adoptieve T-cel therapie geassocieerd met significante zwelling op de plaats van de tumor. Van de 6 patiënten met recidief/residu tumor tentijde van de T-cel behandelingen was er geen respons in 2 patiënten, stabilisatie van tumorgroei in 1 patiënt, een gedeeltelijke respons in 1 patiënt en een complete respons in 2 patiënten. Deze klinische responsen konden niet worden gecorreleerd met een toegenomen aantal LMP2-specifieke T-cellen in de bloedbaan. Dit kan mogelijk worden verklaard door een te lage gevoeligheid van de gebruikte detectietechniek voor tumorspecifieke T-cellen of doordat de geinjecteerde T-cellen zich in de tumor bevonden en derhalve niet in de bloedbaan meetbaar waren.

Hoewel het resultaat van deze klinische trial bemoedigend was, zijn aanpassingen noodzakelijk om deze strategie succesvol te maken voor het merendeel van de patiënten. De effectiviteit van adoptieve T-cel therapie kan mogelijk worden verhoogd door het aantal geinjecteerde T-cellen dat specifiek is voor een tumorantigeen, zoals LMP2, te vergroten. In hoofdstuk 4 wordt een techniek beschreven waarbij selectief LMP2-specifieke T-cellen worden opgekweekt in plaats van T-cellen die alle latente EBV-antigenen herkennen inclusief antigenen die niet in NPC tot expressie komen. In plaats van EBV-getransformeerde B-cellen werden nu dendritische cellen gebruikt die genetisch gemodificeerd waren zodat deze een hoge expressie hadden van het LMP2 antigeen. De geactiveerde LMP2-specifieke T-cellen werden vervolgens vermenigvuldigd door stimulatie met een EBV-geinfecteerde B-cel lijn die LMP2 tot overexpressie brengt. Deze techniek wordt nu toegepast in een klinische trial voor adoptieve immuuntherapie als behandeling voor EBV-positieve HL. Andere strategieën om de effectiviteit van adoptieve T-celtherapie te verbeteren worden besproken in hoofdstuk 8.

Helaas brengt maar een gering aantal tumoren virusantigenen tot expressie die gemakkelijk herkenbaar zijn voor het immuunsysteem. Om adoptieve immuuntherapie ook toe te kunnen passen op andere niet-virus geassocieerde tumoren is het noodzakelijk om een

ethode te ontwikkelen voor het verkrijgen van een groot aantal T-cellen die specifiek zijn voor een tumorantigeen dat normaal geen of alleen een zwakke afweerreactie opwekt. Dit is het doel van het onderzoek beschreven in het tweede deel van dit proefschrift.

De antigeenspecificiteit van een T-cel wordt bepaald door de T-celreceptor (TCR). Een TCR bestaat uit extracellulaire en een intracellulaire componenten. De extracellulaire component herkent en bindt het antigeen (Figuur 3, hoofdstuk 1). De intracellulaire component zendt vervolgens signalen uit die zorgen dat de T-cel zich vermenigvuldigt, de targetcel doodt en acitiverende stoffen (cytokinen) uitscheidt. Uit een T-cel met de gewenste specificiteit kan de TCR worden geisoleerd en deze kan vervolgens door middel van gentransfer tot expressie worden gebracht in andere T-cellen. Deze gemodificeerde T-cellen verkrijgen dan dezelfde specificiteit als de T-cel waarvan de TCR afkomstig is. Op een vergelijkbare wijze kan de specificiteit van een antilichaam worden overgebracht op een T-cel. Het antigeenherkenningsdomein van een TCR wordt dan vervangen door het antigeenherkenningsdomein van een antilichaam. De resulterende chimère receptor combineert de specificiteit van een antilichaam met de effectorfuncties van een T-cel. Door middel van gentransfer kan dus, wanneer eenmalig een receptor of antilichaam met de gewenste tumorspecificiteit is geisoleerd, een groot aantal tumorspecifieke T-cellen worden verkregen.

Hoewel deze techniek in theorie aantrekkelijk is, zijn er een aantal moeilijkheden die succesvolle toepassing in klinische studies tot nu toe hebben verhinderd. Ten eerste moet het niveau van expressie van de nieuw geintroduceerde (transgene) antigeenreceptor vergelijkbaar of groter zijn dan dat van de TCRs die al op de T-cel aanwezig waren om een functionele herkenning van het gewenste antigeen te verkrijgen. Dit vereist de ontwikkeling van een techniek voor genetische modificatie die is geoptimaliseerd voor toepassing in humane T-cellen.

Ten tweede is reeds aangetoond dat antigeenherkenning door de T-cel alleen niet voldoende is voor eliminatie van tumorcellen. Aanvullende signalen via costimulatie moleculen zijn noodzakelijk voor een effectieve T-cel reactie. In afwezigheid van zulke costimulatiesignalen kan antigeenherkenning leiden tot tolerantie voor het tumorantigeen in plaats van eliminatie van de tumorcellen. Costimulatiemoleculen zijn vaak afwezig op tumorcellen en dit is mogelijk een van de mechanismen waarmee tumoren aan het afweersysteem kunnen ontsnappen. Door costimulatiesignalen in te bouwen in de tumorspecifieke TCR kan deze ontsnappingsroute mogelijk worden geblokkeerd en een effectieve immuunreactie tegen de tumor worden opgewekt.

Tenslotte, om zulke gemodificeerde T-cellen te kunnen gebruiken als behandeling voor patiënten is het gewenst om over een veiligheidssysteem te beschikken. In geval van bijwerkingen kunnen de toegediende cellen dan worden uitgeschakeld. Dit kan worden bereikt door samen met de transgene TCR een veiligheidsgen in te bouwen. In geval van toxiciteit kan dit gen worden geactiveerd. Dit resulteert dan in de zelfdestructie van de toegediende cellen. Een reeds beschikbaar veiligheidsgen is afkomstig van het herpes simplex virus. Dit systeem heeft echter als nadeel dat de cellen getransduceerd met dit viruseiwit als geinfecteerd worden herkend door het afweersysteem. De getransduceerde cellen worden vervolgens opgeruimd door het afweersysteem wat een langdurig effect van immuuntherapie in de weg staat. Het is daarom noodzakelijk om een veiligheidsgen te ontwikkelen dat bestaat uit uitsluitend humane componenten om een afweerreactie gericht tegen de gemodificeerde cellen te voorkomen.

Deze stappen naar de klinische toepassing van transgene TCRs in adoptieve immuuntherapie vormen het uitgangspunt van het tweede deel van dit proefschrift. In hoofdstuk 5 wordt beschreven hoe functionele expressie van een transgene tumorspecifieke TCR kan worden verkregen door middel van een geöptimaliseerde techniek voor het introduceren van een nieuw gen in humane T-cellen. Voor dit onderzoek is het minor histocompatibiliteitsantigeen HA-1 gebruikt als modeltumorantigeen. HA-1 antigeenspecifieke T-cellen hebben een reeds aangetoonde rol in de eliminatie van recidief leukemieën bij patiënten die een beenmergtransplantatie hebben ondergaan. T-cellen getransduceerd met een HA-1 antigeenspecifieke TCR met behulp van deze vernieuwde methode herkenden en doodden niet alleen cellen geladen met het relevante epitoop maar ook B-cellen die het antigeen op natuurlijke wijze tot expressie brengen. Deze techniek voor genetische modificatie vergemakkelijkt de productie van grote aantallen van zulke therapeutische T-cellen.

Vervolgens werd bestudeerd of door het inbouwen van verschillende costimulatiemoleculen in een TCR de gemodificeerde T-cellen kunnen worden voorzien van de noodzakelijke signalen voor een effectieve afweerreactie (hoofdstuk 6). Voor deze studies werd gebruik gemaakt van een chimère TCR afgeleid van een tumorantigeen specifiek antilichaam. De basale TCR zonder ingebouwde costimulatiemoleculen was voldoende voor de doding van de tumorcellen. Vermenigvuldiging van de tumorspecifieke T-cellen en uitscheiding van afweersysteem activerende stoffen, vereist voor een langdurige afweerreactie, kon echter alleen worden geinduceerd in de aanwezigheid van de costimulatiemolekulen CD28 en OX40. Dus door het inbouwen van essentiële activatiemoleculen in de TCR kan een effectieve afweerreactie in gang worden gezet en de afwezigheid van deze factoren op de tumorcel worden omzeild.

Tenslotte is in hoofdstuk 7 de ontwikkeling beschreven van een veiligheidsgen gebaseerd op caspase 9, een humaan molecuul dat een centrale rol speelt in de het proces van celdood (apoptose). Caspase 9 werd gefuseerd met een bindingsdomein voor een synthetisch molecuul (AP20187) zodat een induceerbare vorm van caspase 9 ontstond. Door toediening van AP20187 vormden twee induceerbare caspase 9 moleculen een complex wat leidde tot hun activatie en het in gang zetten van het proces van celdood. Dit veiligheidsmolecuul kon tot expressie worden gebracht in humane EBV-specifieke T-cellen zonder te interfereren met hun normale functie en antigeenspecificiteit. Activatie van induceerbaar caspase 9 leidde, wanneer het tot expressie werd gebracht boven een drempelniveau, tot doding van nagenoeg alle getransduceerde T-cellen. Dit veiligheidsgen kan bijdragen aan een spoedige klinische evaluatie van verbeterde adoptieve immuuntherapiestrategieën, waaronder deze als voorgesteld in dit proefschrift.

List of Abbreviations

(m)Ab	(monoclonal) antibody
APC	antigen presenting cell
APAF-1	apoptotic protease activating factor 1
CARD	caspase-activation-recruitment domain
CID	chemical inducer of dimerization
CTL	cytotoxic T cell
DC	dendritic cell
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
EBNA	Epstein Barr virus nuclear antigen
EBV	Epstein Barr virus
ELISPOT	enzyme linked immunospot
ER	endoplasmatic reticulum
FCS	fetal calf serum
FKBP	FK506 binding protein
GVHD	graft-versus-host disease
HD	Hodgkin's disease
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPV	human papillomavirus
HSV-TK	herpes simplex virus thymidine kinase
iCasp9	inducible caspase 9
iFas	inducible fas
IFN-γ	interferon-γ
(r)IL	(recombinant) interleukin
LCL	EBV-transformed lymphoblastoid B cell line
LMP	latent membrane protein
LTR	long terminal repeat
MHC	major histocompatibility
minor H antigen	minor histocompatibility antigen
NPC	nasopharyngeal carcinoma
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PTLD	post transplant lymphoproliferative disease
scFv	single chain variable fragment
SCT	stem cell transplantation
TAP	transporter associated with antigen processing
TGF-β	transforming growth factor-β
Th	T helper
TIL	tumor infiltrating lymphocyte
TNF-α	tumor necrosis factor-α

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Curriculum Vitae

De auteur van dit proefschrift werd geboren of 11 januari 1974 te Alphen aan den Rijn. In 1992 behaalde zij het gymnasium-β diploma aan het Christelijk Lyceum (later: Groene Hart College) in Alphen aan den Rijn. In datzelfde jaar begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden. Vanaf 1994 combineerde zij deze opleiding met de studie Geneeskunde. In 2000 behaalde zij zowel haar doctoraal bul geneeskunde als biomedische wetenschappen. Voor haar afstudeerstage biomedische wetenschappen onder begeleiding van dr. M.E. Ressing, dr. R. Offringa en prof.dr. C.J.M. Melief op de afdeling tumor immunologie in het LUMC werd haar een LUMC fellowship toegekend. Na het voltooien van haar co-assistentschappen in het LUMC en geaffilieerde ziekenhuizen (1998-2000) en het behalen van het arstexamen (cum laude) was zij van oktober 2000 tot januari 2005 werkzaam in het Center for Cell and Gene Therapy (CAGT), Baylor College of Medicine, Houston in de Verenigde Staten. Onder begeleiding van prof.dr. C.M. Rooney en prof.dr. H.E. Heslop (beiden CAGT) en prof.dr. E.A.J.M. Goulmy en prof.dr. R.M. Egeler (respectievelijk afdeling Immunohematologie en afdeling kindergeneeskunde, LUMC) verrichtte zij het in dit proefschrift beschreven onderzoek. Sinds februari 2005 is zij werkzaam als arts assistent kindergeneeskunde in het opleidingsprogramma van University College London Hospital en Great Ormond Street Hospital in Londen.

Stellingen

Behorende bij het proefschrift van Karin Straathof

Adoptive T-Cell Therapy as Treatment for Epstein Barr Virus-Associated Malignancies - Strategies to Enhance Potential and Broaden Application

1. Ondanks recente behandeling met bestraling en chemotherapie, kunnen vanuit het perifere bloed van patiënten tumorantigeen specifieke T-cellen worden gekweekt.
Dit proefschrift

2. Door gebruik te maken van dendritische cellen voor reactivatie, en van EBV-getransformeerde B-cellen die het tumorantigeen tot overexpressie brengen voor expansie, kan een grote hoeveelheid tumorspecifieke T-cellen worden verkregen vanuit een kleine hoeveelheid bloed.
Gottschalk et al, Blood 101:1905-12 (2003), dit proefschrift

3. Door het incorporeren van een niet-immunogeen suicidegen, kan de veiligheid van adoptieve T-cel therapie worden verhoogd, zonder dat dit interfereert met hun mogelijke therapeutische werking.
Dit proefschrift

4. Een snelle en productieve methode om T-cel epitopen te identificeren, is het screenen van T-cel responsen die gericht zijn tegen reeksen overlappende peptide die het doelantigeen representeren.
Kern et al, J Virol 73:8179-84 (1993), dit proefschrift.

5. Suboptimale T-cel activatie als gevolg van de afwezigheid van costimulatiemoleculen op de target tumorcel kan worden gecompenseerd door costimulatie signaalcomponenten in te bouwen in een transgene T-cel receptor.
Haynes et al, Blood 100:3155-63 (2002), dit proefschrift

6. Adoptieve transfer van tumorspecifieke T-cellen heeft de potentie om niet alleen virusgeassocieerde, maar ook om niet-virus geassocieerde tumoren uit te roeien.
Dudley et al, Science 298:850-54 (2002)

7. Het vermenigvuldigen en in stand houden van in vivo adoptief getransfundeerde T-cellen is essentieel voor hun anti-tumor effect.
Heslop et al, Nat Med 2:551-55 (1996), Robbins et al, J Immunol 173:7125-30 (2004).

8. Het koppelen van antilichaamspecificiteit aan T-cel effectorfunctie, is een aantrekkelijke manier om ontsnapping van de tumor aan het afweersysteem door middel van mutaties in componenten van het antigeenbewerkings- en presentatieproces, te voorkomen.

9. Het verstrekken van patenten voor genen, genmutaties en eiwitten verhindert de bloei van de wetenschap.

10. Het binnen afzienbare tijd genezen van kanker kan alleen worden bereikt door het volgen van een duidelijk geformuleerd plan van actie, onder goede leiding, en met alle benodigde financiële middelen – analoog aan het Marshall plan.
Dr. Sidney Farber, debate National Cancer Act, USA 1971

11. De afwezigheid van een volledig rookverbod in alle horeacagelegenheden in Nederland druist in tegen een belangrijk prinicipe in de gezondheidzorg: voorkomen is beter dan genezen.

12. Roeiers zijn goede wetenschappers; voor beide is teamwerk en een lange adem essentieel.