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

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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 anti-

gens 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.

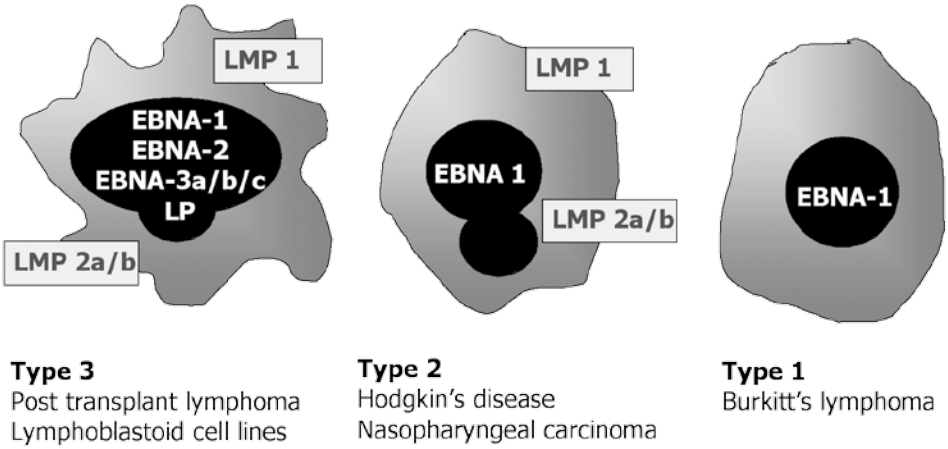

Figure 1. EBV antigen expression in different tumor types

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]

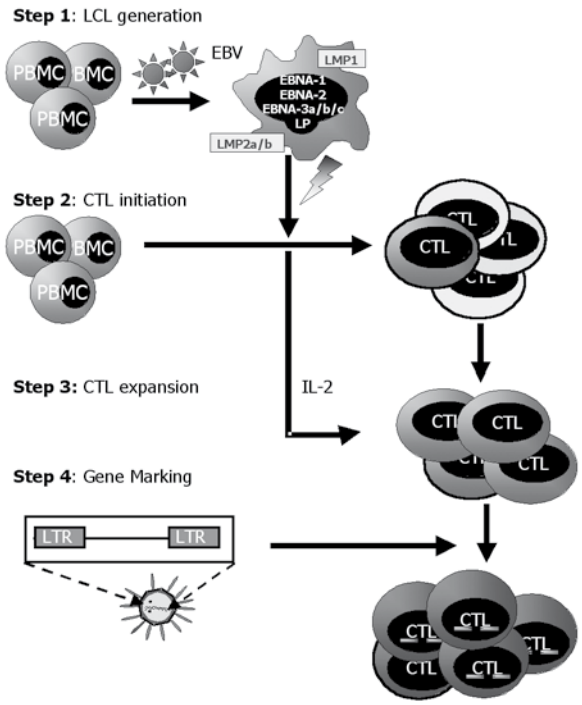

Figure 2. The generation of EBV-specific CTL lines
Step 1: PBMCs are isolated by centrifugation on a ficoll gradient from 30-50 mL of patient blood. A small number of the obtained PBMC (up to 5x10⁶) are infected with a laboratory strain of EBV (B95-8) in the presence of cyclosporin to establish an EBV-transformed 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.

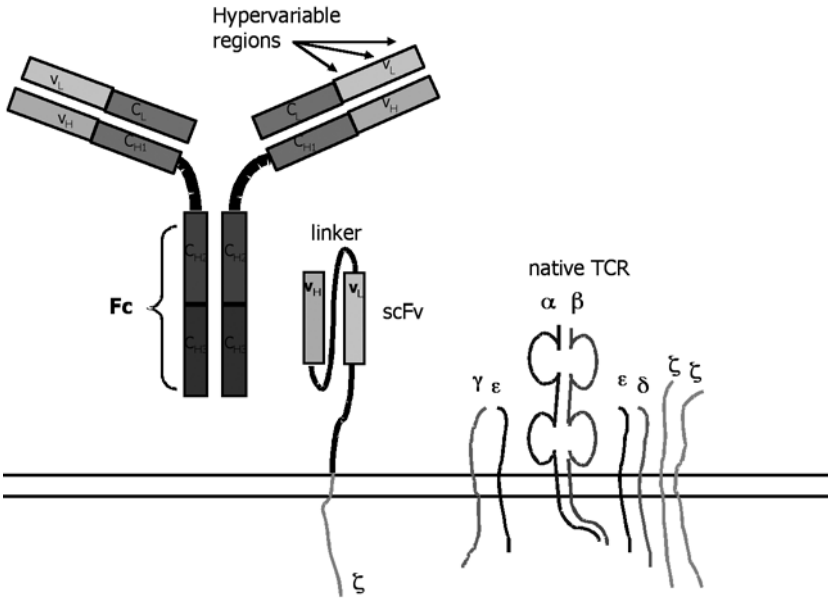

Figure 3. Native and chimeric TCRs for gene transfer

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-re-

sponsiveness 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

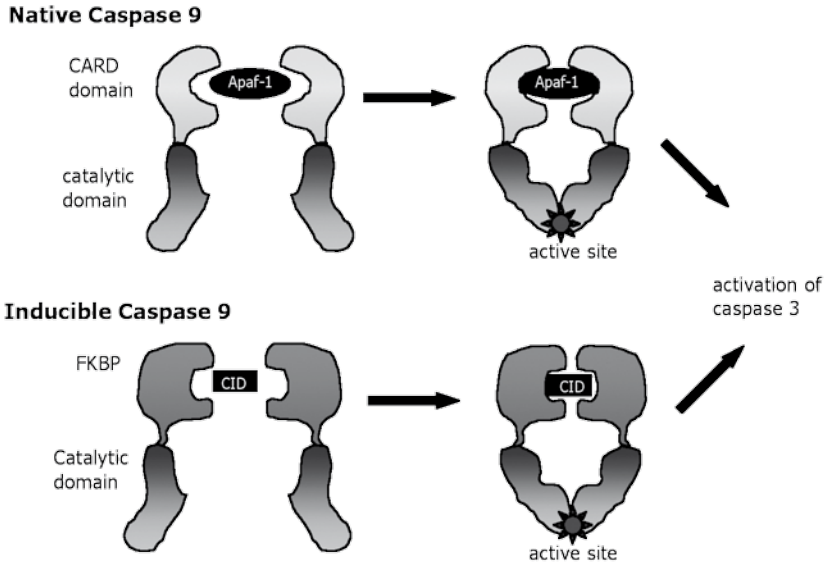

Figure 4. Activation of native and inducible caspase 9

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-ζ, CD3-ζ 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.

Parts of this introduction are adapted from:

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