Development of stealth transgenes for gene therapy : evaluation of cis-acting inhibitors of antigen presentation

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Chapter 1

Introduction

Part 1

Aim and outline of this thesis

Part I

Aim and outline of this thesis

The aim of the studies presented in this thesis is to develop and evaluate a technique to make transgene products used in gene-therapy applications "invisible" (i.e. stealthed) to the immune system. In addition to the use of previously described Gly-Ala repeat (GAr) domain of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) we identified and studied new inhibitors, from EBNA-1 itself, and from another herpes-virus protein.

In **Chapter 1**, **part 2.1**, we give a general overview of gene therapy and cancer gene therapy. We briefly discuss the status of the clinical studies, and the vectors and genes used in these studies. One of the hurdles in gene therapy is the immune system. In **part 1.2.2** we discuss immune responses against one of the most frequently used vectors for gene therapy, the adenoviral vector. In addition, we evaluate the solutions that have been proposed as well as their feasibility. It is not only an immune response to the (viral) vectors that hampers the applicability of them in gene therapy. There is also ample evidence of transgene-product induced immune response. **Part 1.2.3** reviews this problem. It is evident that many viruses have evolved strategies to counteract the presentation of *neo*antigens. Some of their mechanisms are reviewed in part **1.2.4**.

The cellular immune response against transgene-encoded *neo*antigens is a major hurdle in gene therapy applications where long-term expression of transgenes is desired. Therefore new approaches are needed to prevent rapid clearance of transduced cells. We exploited the Gly-Ala repeat (GAr) domain of the EBNA-1, since the GAr prevents cytotoxic T lymphocyte epitope generation. In **Chapter 2**, the first results on this domain are described. Our data show that the fusion proteins retain their activity and we show how the GAr can be used to stealth transgene products.

Upon closer examination of the EBNA-1 gene we found a nested ORF. In this ORF there was a long repeat present, but because of the frame shift, this consisted of the acidic residues glutamine, glutamic acid, and glycine. We therefore named this repeat the GZr. We tested this repeat in the same way as the GAr and could show that also this repeat is capable of inhibiting antigen presentation in vitro. The results are described in **Chapter 3**.

The herpes simplex virus 1 (HSV-1) thymidine kinase (TK) is frequently used as a produg-activating enzyme in experimental gene therapy. However, in some studies a cellular immune response was mounted against this enzyme, thereby thwarting the therapy. **Chapter 4** deals with the modifications we made in the HSV-TK gene to blunt the immune response. First we fused HSV-TK with the GAr. In addition, we introduced modifications, which would

prevent splicing-out of the codons coding for the active site, and we made point mutations that have been described to enhance the affinity for the prodrug gancoclovir (GCV).

Since the GAr works very efficient in preventing a harmful immune response we set out to identify other proteins with similar functions. The kaposi sarcoma herpes virus (KSHV) a.k.a. human herpes virus 8 (HHV-8) has a protein that is, like EBNA-1, involved in episomal maintenance of the virus genome. This protein, the latency-associated nuclear antigen-1 (LANA-1), has a long acidic repeat. Remarkably, the last one-third of the repeat is strongly similar to newly found GZ-repeat protein that is encoded by the EBNA-1 ORF. In **Chapter 5** we show that also LANA-1 can affect the presentation of antigens.

Chapter 1

Introduction

Part 2

1. General introduction on gene therapy & cancer gene therapy

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   Crit. Rev. in Oncol./Hematol. (2004) 50:51-70. Adapted

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Part 2

2.1 General introduction on gene therapy and cancer gene therapy

2.1.1 General gene therapy

Gene therapy is a potentially powerful form of molecular medicine that it broadly applicable. It provides the prospect of treating a wide variety of inherited as well as acquired diseases.

The basic concept of gene therapy is simple: the introduction of genetic information into target cells to alleviate the effects of an inherited or acquired disorder. This may slowdown disease progression or even lead to a cure. To achieve this, a gene has to be efficiently transferred into cells or tissues. Basically, either viral or non-viral gene-transfer vectors can do this. Peter Medawar, the winner of the Nobel prize for Medicine and Physiology in 1960, defined a virus as 'a piece of bad news wrapped up in protein'. This would argue in favor of the development and use of non-viral vectors for gene therapy. Although conceptually safer, non-viral vectors are usually less efficient than viral vectors. This is the main reason why defective viruses are broadly employed as gene-therapy vectors in experimental gene therapy.

Retrovirus-based vectors were the first used clinically for gene therapy. Nowadays many more viruses are clinically evaluated as gene transfer vectors, i.e. adenovirus, herpes virus, adeno-associated virus, and most recently, lentivirus. The most popular vectors used in clinical trials are the retroviruses (27%) and the adenoviruses (26%) (Wiley website, 2005). In these vectors viral genes are removed to cripple the viruses and to provide space for inserting the therapeutic transgene.

Integration into the host genome might be necessary in those cases where a life-long expression of the transgene is needed. However, there is a risk of disrupting the expression of essential genes associated with vector integration into the host genome. The so-called insertional mutagenesis by vector DNA is a potential hazard to the patient participating in gene therapy studies. The integration of the vectors was long thought to be random. Recent studies however, have revealed that different retroviruses have quite different preferences for integration in human chromosomes. Human immunodeficiency virus (HIV) strongly favors active genes (Schroder et al., 2002), whereas murine leukemia virus (MLV) favors integrating near transcription start sites and only weakly favors active genes (Mitchell et al., 2004). This suggests that integration site selection is affected by different interactions at the integration site. Tethering through protein-protein interactions may play a role in target-site selection.

This offers the possibility to steer integration to a safe site within the genome by generating fusion proteins that tether the integrase to a specific target sequence (Bushman, 2003). An example of a clinical study where retroviral vectors have been used for a hereditary disease is given below.

The most successful trial in gene therapy was done by Alain Fischer and collaborators in children suffering from X-linked SCID-X1 (Severe Combined ImmunoDeficiency). In this hereditary disorder, there is a block in the development of T and natural killer (NK) cells due to a mutation in the gene for the γc cytokine receptor subunit. Stem cells from the hematopoietic system were harvested from patients, stimulated and transduced ex vivo with an MLV-based retroviral vector, expressing the γc cytokine receptor subunit. Then the cells were re-infused into the young patients (Cavazzana-Calvo et al., 2000). The patients were closely followed for 10 months and during this period they were expressing the γc receptors on their T and NK cells. Moreover the cell counts and function were comparable to age-matched controls. Unfortunately, three years after gene transfer three of the children developed T-cell leukemia (Hacein-Bey-Abina et al., 2003a). Upon examination of blood samples it became clear that the leukemia cells contained a single intact copy of a retroviral vector that had integrated in or near the LMO2 oncogene on chromosome 11 (Hacein-Bey-Abina et al., 2003b). This gene is originally identified as a break-point of a translocation that causes a type of T-cell leukemia. It appears that the retroviral insertion caused increased expression of the gene. The insertion was already detectable well before the children showed any clinical symptoms. This is a major setback, for this approach. However, one has to keep in mind that the alternative treatment option for the children having SCID-X1 is bone marrow transplantation. Whereas this has an almost 100% success rate if the donor is a perfect match, the success rate drops to less than 80% for partially matched donors and recipients. In the case of partial matching there are also long term problems, including incomplete B-cell function and possible Graft-versus-Host disease (GVHD). For the patients enrolled in this study no HLA-matched donors could be identified.

The adenoviral vectors have a quality that can be favored over retroviruses. Whereas oncoretroviruses need dividing cells to integrate for transgene expression, adenoviruses can also transduce quiescent cells. There are over 50 different human adenoviral serotypes, but so far most vectors have been derived form the serotypes 2 and 5, the most common serotypes (Horwitz, 1996). This can of course be detrimental to gene transfer and expression due to pre-existing immunity. These problems are overviewed in detail in Chapter 1, part 2.2.

The most illustrious trial conducted with adenoviral vectors is, without a doubt, the trial that resulted in the tragic death of Jesse Gelsinger. It was a phase-I trial aimed at the correction of the ornithine transcarbamylase deficiency (Batshaw et al., 1999). The trial was designed to test the safety of an E1/E4-deleted recombinant adenoviral vector. Jesse participated in the study and received the highest dose. Four days later he died of an unexpected extreme reaction of his innate immunity to the vector. Although it was known that

adenoviral vectors trigger immune response at high doses, the extent of the immune response had not been predicted from earlier experiments. Analysis of the vector distribution also revealed that this was not limited to the intended target organ, the liver, but was also present in the spleen, lymph node and bone marrow. The transduction efficiencies for all these organs were even comparable. This event prompted a temporary halt on gene therapy trials with adenovirus vectors. This result and the circumstances in which this trial had been organized and was carried out led to much debate in the aftermath of this incident.

2.1.2 Cancer gene therapy

The two examples above of gene therapy trials both addressed hereditary (monogeneic) diseases, which consist of only 9% of the indications. Statistics however, shows that the most frequent use of gene therapy is in cancers (66%) (Wiley website, 2005). Here the gene therapy tries to selectively eliminate cancer cells. A major problem here is to achieve sufficiently high transduction and expression efficiencies of the transgenes specifically in the cancerous cells while leaving the patient's normal cells unharmed.

A most obvious way to target growth regulation in cancer cells is to introduce tumor suppressor genes. Some cancers are a direct result of loss of tumor suppressors. The most well know tumor suppressor gene is p53. A number of different viral vectors have been made for transfer and expression of the p53 gene. Especially adenoviral vectors have been evaluated frequently and these have been shown to increase p53 amounts in p53-deficient cells. This resulted in cells growth inhibition and apoptosis in synergy with chemotherapeutic agents (Yen et al., 2000; Horio et al., 2000). Other tumor-directed strategies are the introduction of dominant-negative genes, induction of apoptosis, use of tumor-specific viruses, tumor-specific gene expression, or introducing agents that sensitize tumors to radiation and chemotherapy (Gottesman, 2003).

One of these strategies is the use of oncolytic viruses, i.e. viruses that preferentially replicate in tumor cells. The results are reason for optimism. It began with the discovery that adenovirus lacking the E1B 55K gene (called ONYX-015) would replicate inefficiently in normal cells, and is more efficient in p53-deficient cells (Heise et al., 1999; Biederer et al., 2002; Yoon et al., 2001). Other viruses that are currently evaluated are Newcastle Disease Virus, (Pecora et al., 2002), and reovirus (Etoh et al., 2003; Kilani et al., 2003; Shah et al., 2003; Norman et al., 2004).

Also by prodrug-activation of suicide gene therapy researchers try to achieve specific lysis of tumor cells. Best known in this respect is the herpes simplex virus 1 (HSV1) thymidine-kinase (TK) gene. HSV-TK is the archetypical enzyme used in gene-directed enzyme prodrug therapies (GDEPT). Its capacity to convert the antiherpetic nucleoside analogues ganciclovir and aciclovir to toxic nucleotides has been used effectively in gene therapy protocols to eradicate tumor cells and lymphocytes that expressed the HSV-TK

transgene upon virus-mediated gene transfer. Although effective, in some applications the use of the HSV-TK is limited by the induction of a cellular immune response against the HSV-TK protein. Since HSV-TK is not restricted to tumor cells, the specificity of this approach has to come either from tumor-specific transduction or from tumor-cell specific expression of the transgene.

Tumor-specific gene expression of suicide genes or tumor suppressor genes is mostly achieved by the use of tumor cell-specific transcription regulatory units. Two cancer types where the expression is indeed quite specific make use of vectors carrying pancreatic cancer-specific (Yoshida et al., 2002; Wesseling et al., 2001) and prostate cancer-specific (DeWeese et al., 2001; Martiniello-Wilks et al., 2002) transcription units.

There are three main strategies tested for host-directed cancer gene therapy. Targeting the tumor angiogenesis pathway, protecting the normal tissue, especially bone marrow, from the toxic effects of chemotherapy, and by activating the immune system.

Angiogenesis can be inhibited constitutive expression of inhibitors of angiogenesis like angiostatin and endostatin. However, the expression has to be limited to the tumor site. A drawback of anti-angiogenesis treatments is the requirement of long-term treatment, at least until regression or apoptosis deprive tumor cells from their vasculature.

Protecting bone marrow cells by introducing protecting genes has not been possible yet, because of the low transduction frequency of the hematopoietic stem cells. However, progress is made here and this might help in the protection.

The immune system of patients has proved to be a potentially useful target. Mostly, immune effector cells like dendritic cells (DCs), NK cells, and CD8+ cells are targeted. Currently, treatments under development in this category are introduction of cytokines exposure of these cells to cloned antigens to amplify their immune reactivity (Dallal and Lotze, 2001; Reyes-Sandoval and Ertl, 2001).

However, it is clear that although the concept is elegant and straightforward, gene therapy in general, and cancer gene therapies in particular, are difficult to develop in clinically efficacious treatments. There are many pitfalls and major challenges lie ahead in further understanding the limitations of the current procedures and their solutions. Nevertheless, the field of cancer gene therapy has delivered proof-of-concept and will move forward from bench to bedside.

2.2 Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion

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Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion

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Abstract

Human adenoviruses have been adopted as attractive vectors for in vivo gene therapy since they have a well-characterized genomic organization, can be grown to high titres and efficiently transduce a wide spectrum of dividing and non-dividing cells. However, the first-generation of adenoviral (Ad) vectors yielded only transient expression of the transgene in most immunocompetent mice. This constituted a major limitation of this early vector type. In contrast, persistent transgene expression can be established in immunodeficient mice. This suggests that the immunogenicity of adenoviral vectors limits the effective period of adenovirus-based gene therapy. Much effort has been put in devising strategies to circumvent the limitations imposed onto gene therapy by the immune system. Improvements in vector design have significantly improved the performance of the adenovirus vectors. Based on these results it is reasonable to anticipate that new modifications of the vectors will overcome some of the immunological barriers and will further expand the applicability of adenovirus-derived vectors.

Keywords: Gene therapy; Adenovirus; Immunology; Viral vectors

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1. Immune responses against adenoviral vectors

1.1. The immune responses against adenovirus vectors limit their applicability

In the field of gene therapy, the first-generation adenoviral (Ad) vectors are the nowadays best-studied Ad vectors. They lack the E1 region and sometimes the E3 region (Fig. 1). Deletion of the E1 genes increases the space for transgenes, severely diminishes the expression of residual viral genes and renders the recombinant adenovirus replication-defective. However, deletion of E1 is insufficient to completely abrogate viral gene expression. Several studies demonstrated a low expression level of both early and late genes, which resulted in the activation of Ad-specific cytotoxic T lymphocytes (CTLs) [1–8]. The cellular immune response eliminates the Ad vector-transduced cells and thereby extinguishes transgene expression. A cellular immune response can also be induced by the transgene expression. However, the contribution of the transgene expression to the elimination of

transduced cells is still controversial and seems dependent on the recipient, the route of administration and the origin of the transgene [1,8–12].

In general, the cellular immune response towards adenovirus antigens is activated by antigen-presenting cells (APCs). After the uptake of the Ad particle, viral proteins and transgene products are processed into small oligopeptides, which are presented by the major histocompatibility complex (MHC) class-I molecules at the cell surface. It is noteworthy that the de novo synthesis of viral proteins does not appear to be required for antigen presentation, since psoralen-treated, UV-cross-linked, inactive adenovirus vectors still cause activation of a cellular immune response [13]. The binding of $CD8^+$ T cells to this peptide–major histocompatibility complex (MHC) class-I initiates the formation of Ad-specific or transgene-product-specific CTLs (see Fig. 2). The interaction between CD28 and B7 plays a co-stimulatory role in this activation [14]. The cellular immune response is further stimulated by $CD4^+$ helper cells primarily belonging to the Th1 subset [7,11,15]. In contrast to the $CD8^+$ T cells, these $CD4^+$ helper cells are activated by epitopes from the input virions, which are presented by MHC class-II molecules at the surface of APCs (Fig. 2).This activation triggers the Th1 cells to secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ). These cytokines, in turn, induce the differentiation of $CD8^+$ T cells into CTLs [16,17]. In addition, IFN-γ causes the up regulation of MHC-I expression in Ad-transduced cells and consequently facilitates their recognition by CTLs [11,15]. Moreover, activated $CD4^+$ helper cells have also been suggested to destroy Ad-transduced cells themselves, resembling in this way primary CTLs [18].

Characteristics of the various types of adenovirus vectors

'Generation'	Modification
First	E1-deleted (± E3 deletion)
Second	E1-deleted + *ts*E2A (± E3 deletion)
Third	E1-deleted + E4, E2A or E2B deleted (± E3 deletion)
Helper-dependent *	All Ad protein-coding genes deleted

** Also known as: 'gutted', 'gutless', 'minimal', and 'high-capacity' Ads*

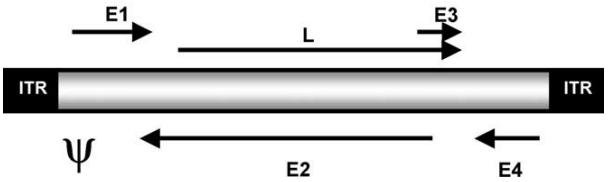

Fig. 1. Schematic outline of the various types of adenovirus vectors. The graph depicts the location of the early gene clusters E1–E4, the late transcription unit (L), and the inverted terminal repeats (ITR). The ψ represents the position of the encapsidation signals.

Apart from the cellular immune response, the adaptive immune system also includes a humoral component, which constitutes a second hurdle to persistent transgene expression. This humoral immune response is initiated by the binding of adenovirus particles to the surface immunoglobulin of B cells [19]. After internalization and processing of the virus, the

adenovirus-derived epitopes are presented at the surface of the B cell by MHC-II molecules (Fig. 2). The resulting antigen–MHC-II complex can be recognized by activated T helper cells of the Th2 subset [11,20]. This specific $CD4^+$ helper cell subset releases cytokines, like IL-4, IL-5, IL-6 and IL-10, which provide indispensable signals for the B cells to differentiate into plasma cells [20]. As a result, the plasma cells secrete antibodies (Abs), which are directed towards the adenoviral capsid. Although T helper cells of the Th1 subset are poor initiators of the humoral immune response, they do play a role in Ab-isotype switching [21]. Whereas Th2 cells control the production of the Ab isotypes IgG1, IgG2b, IgA and IgE by cytokines, such as IL-4, Th1 cells control the switch to IgG2a or IgG3 by means of IFN-γ secretion [20,22,23].

The development of Ad-specific antibodies does not contribute to the elimination of Ad-transduced cells and hence does not affect the persistence of transgene expression [12]. However, Ad-specific Abs will bind the Ad vector and thereby prevent cell entry and promote opsonization by macrophages. Consequently, Ad-specific Abs hamper the efficacy of repeated administrations of the Ad vector, which would be required to keep the transgene expression at the desired level. Thus, although repeated administrations of the Ad vector can prolong expression of the transgene in immunodeficient recipients, the efficiency is dramatically reduced in immunocompetent recipients [1,7,24–28]. Moreover, a large proportion of the human population harbours humoral immunity to Ad vectors as a result of previous infections [24]. Consequently, effective adenovirus-mediated gene transfer in humans may be frustrated even at the first administration of an Ad vector. It is noteworthy that vector-specific immunity does not prevent vector-specific activation of the innate immune system that occurs at very-high vector doses [29].

Apart from Ad-specific Abs, neutralizing Abs might also be generated against the transgene product. These transgene-product-specific Abs can neutralize the trangene product once it enters the circulation and thereby abrogate the effect of gene transfer, irrespectively of persistence of transgene expression [9,10,30]. Moreover, several studies have indicated that, instead of the immunogenicity of adenoviral proteins, the immunogenicity of foreign transgene-encoded proteins is a primary determinant of the persistence of transgene expression [9,10,30–33]. The contribution of foreign transgene products to the observed immune responses is not unexpected, but it represents a substantial hurdle for gene therapy of hereditary diseases. The immune response to the transgene product may be dependent on the nature of the mutation that affects the endogenous gene. Such correlation has been found in hemophilia patients transfused with blood-clotting factor VIII. Inhibitory antibodies develop in a proportion of patients with hemophilia A following replacement therapy. In the patients with severe molecular defects (viz. intron-22 inversions, large deletions, and stop mutations) about one in every three patients develop an inhibitor response. In contrast, inhibitors occur in only 1 in 20 patients with small deletions and antigen (i.e. clotting factor IX in an murine

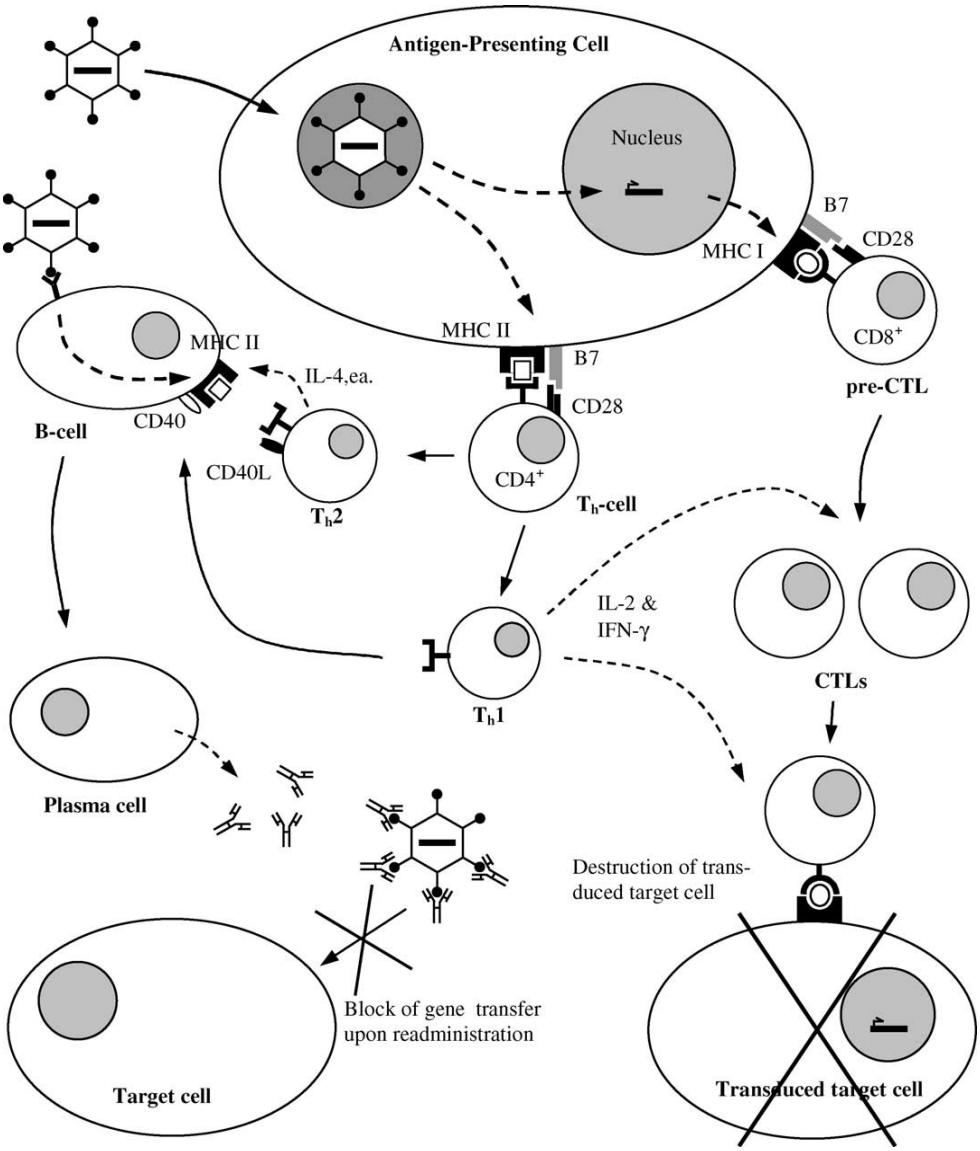

Fig. 2. Activation of the host's immune system upon adenoviral gene transfer. Antigen-presenting cells (APCs) process de novo synthesized viral proteins or transgene products and present these to $CD8^+$ T cells by means of MHC class-I molecules. This causes the $CD8^+$ T cells to form cytotoxic T lymphocytes (CTL), which specifically destroy the transduced target cells. The proliferation of CTLs is further stimulated by $CD4^+$ helper cells of the Th1 subset. Their activation is triggered by epitopes from the input virus, which are presented by MHC class-II molecules on the cell surface of APCs. Apart from the cellular immune response, $CD4^+$ T helper cells also participate in the activation of the humoral immune response. Binding of the Ad vector to B cells and interaction with an activated T helper cell induce B cells to differentiate into plasma cells. The subsequent production of Ad-specific neutralizing antibodies limits the beneficial effect of a repetitive administration of the same Ad vector by blocking its cellular entry.

hemophilia B model) may even induce tolerance to the therapeutic antigen [36]. The immunogenicity of the transgene may be depend on the synthesis of the neoantigen in the antigen-presenting cells (see below) [37]. Hence, it will be essential to monitor immune responses to the transgene products in all patients enrolled in gene-therapy studies.

Obviously, strategies to prevent the cellular and humoral immune responses, e.g. adaptive immunity towards the adenovirus vector and to the transgene product may lead to significant improvement of gene therapy of hereditary diseases. Hence, research is mainly focused on these components of the host's immune response. However, the innate immune responses, as a first line of defence also influence vector persistence. The non-specific innate immune response acts rapidly after viral entry [38]. The early phase of the host's immune response is predominantly brought about by neutrophils, macrophages and natural killer (NK) cells and lasts about 4 days, until the adaptive immune response is fully activated [39]. The activation of the adaptive immune response is much more rapid upon re-infection of the same pathogen, which is due to the so-called immunologic memory.

The contribution of the early immunity to the clearance of adenoviral vectors was initially reported by Worgall et al. [40]. After intravenous administration of a first-generation Ad vector to immunocompetent and immunodeficient mice, 90% of the viral genome was eliminated from the liver within the first 24 h. Similar vector elimination was seen after intratracheal administration, although in this case the vector loss was 70% within 24 h [41]. After the fatal incident in a clinical trial for treatment of patients with a deficiency in the liver enzyme ornithine transcarbamylase (OTC) [42], it became evident that the innate immune system is highly activated by intravascular administration of high doses of Ad vectors [43]. Subsequent animal experiments could reproduce the events albeit with higher vector doses. In mice, an acute cytokine response is mediated by macrophages and dendritic cells. Within 6 h high amounts of IL-6, IL-12 and TNF-α are released. Depletion of DCs and macrophages blocked the production of these inflammatory cytokines. In addition, this prevented the CTL response against the transgene-expressing cells in the liver. These data indicated that DCs and macrophages are essential for both innate and adaptive immunity [43,44]. Similar findings were also reported in a study on the activation of innate immunity in non-human primates [43,44]. In the OTC clinical trial, the production of IL-6, but not TNF-α was first detected at 2 h and peaked at 8 h after vector administration. In all but one patient, the IL-6 levels returned to base line by 24 h. In the other patient, the IL-6 response was severely prolonged and did not return to baseline. This was associated with an acute and lethal systemic inflammatory response and multi-organ failure [42–44]. Remarkably, immunity to Ad does not prevent the vector-induced toxicity [29]. So, besides modulation of the adaptive immune response, the modulation of the early immune responses might also be required to facilitate effective gene transfer in vivo with Ad vectors.

1.2. Attenuation of the early immunity

1.2.1. Feasibility of macrophage depletion

Upon administration, a major fraction of the Ads is eliminated within the first 24 h. This rapid clearance has directed research towards modulation of the non-specific, early immunity

[40,41]. Macrophages and Kupffer cells are important components of the innate immune system and, consequently, are an interesting target for the attenuation of the early clearance of adenoviral vectors. The dose–response after intravenous administration of adenovirus vectors is essentially non-linear. Low doses of adenovirus vectors are taken up by Kupffer cells and macrophages and quickly degraded. Only after saturating these cells, the adenoviruses transduce the hepatocytes [43,45]. To assess the contribution of macrophages to the clearance in an in vivo situation, these cells were transiently depleted by means of a dichloromethylene bisphosphate (Cl_2MBP) or gadolinium chloride ($GdCl_3$) treatment [41,46–48]. In mouse lung, the depletion of alveolar macrophages significantly attenuated vector elimination [41]. Twenty-four hours after intratracheal administration, the lungs of macrophage-depleted mice contained two times more viral DNA than control mice. Depletion of Kupffer cells from the liver by $GdCl_3$ or Cl_2MBP revealed a similar effect on the viral genome level after intravenous administration [46,48].

Besides the effect on the innate immune response, Kupffer-cell depletion also resulted in a prolonged persistence of vector DNA and transgene expression. Recently, long-term expression of blood-clotting factor VIII has been achieved after macrophage and Kupffer-cell depletion in factor VIII knock-out mice [49,50]. Possibly, elimination of these cells affects the adaptive immune system, which can be attributed to their function as APCs. Nevertheless, the macrophage-depleting effect of $GdCl_3$ or Cl_2MBP does not counteract the rapid vector elimination completely. This implies that adenoviral degradation by macrophages is only partially responsible for the observed vector elimination within 24 h after administration.

These data are extremely important for our understanding of the precise roles of the macrophages and Kupffer cells in the elimination of vector DNA. However, it is not likely that strategies involving depletion of the entire macrophage or Kupffer-cell populations will become applicable in a clinical gene-therapy setting.

1.2.2. Possible vector modifications

Although Kupffer-cell depleting agents have a beneficial effect on the efficacy of gene transfer, their clinical application might be hampered by pharmacological side effects and an increased susceptibility to those diseases, which are normally eliminated by the innate immune system [48]. Hence, adaptations of the vector are preferred to the immunomodulation of the recipient. The short time lag and the non-specific action of the innate immune response, however, severely complicate the applicability of vector modification. The expression cassette and vector dose do not affect this type of early vector clearance [40]. In addition, second-generation Ad vectors are eliminated with similar efficiency, which suggests that the innate immune response is dependent on the vector capsid components [51]. Hitherto, no adenoviral vectors that evade the innate immune system have been reported.

Since the non-specific adenoviral uptake into macrophages seems not to be influenced by

modifications of the Ad vector genome, the attenuation of the early vector clearance should be focused on other early-induced, antiviral defence mechanisms. Kuzmin et al. [46] suggested two different vector-elimination routes. Within 24 h after administration, they observed degradation of viral DNA in hepatocytes and vector secretion into the bile, which accounted for a 2-log decrease in viral DNA levels in the liver of BALB/c mice. The relevance of these Ad vector elimination routes is further supported by the observation that hepatic uptake of more than 90% of the administered virus dose is a specific, receptor-mediated phenomenon [52]. This leaves a minor role for the non-specific uptake of virus in the hepatic sinusoids and suggests that modification of the adenoviral tropism might have some potential for diminishing the early clearance of adenoviral vectors.

The success of a re-targeting approach is dependent on the possibility to circumvent NF-κB activation, which is seen as the key regulator of early, antiviral immune responses. This is based on the capacity of NF-κB to activate the expression of several cytokines, such as TNF-α and interferons, which induce vector clearance via cytolysis and inflammatory responses [47,53]. NF-κB is activated within 20 min after vector administration and might be induced by binding of the viral fibre to the adenovirus receptor. However, penton–integrin interaction or endosome rupture might also trigger NF-κB activation, which renders the effect of re-targeting on the early immune responses uncertain [47]. Presumably, the direct reduction of NF-κB or cytokine function might provide better leads for vector adaptations. This was supported by observations on bcl-2 transgenic mice, which were intravenously injected with an adenoviral vector encoding the IκBα super suppressor, IκBM [54]. Suppression of NF-κB by IκBM and Bcl-2 improved the vector persistence. Expression of IκBM alone was, however, insufficient to prevent the clearance of vector DNA.

The inhibition of the TNF-α function forms another possibility to diminish the early immune responses. One way to accomplish this might be provided by adenovirus itself. The E3-encoded proteins E3-14.7K and E3-10.4K/14.5K have been shown to inhibit the TNF-α-mediated apoptosis of virally-infected cells [55–58]. In addition, NF-κB and TNF-α both activate the E3 promoter, which suggests an evolutionary benefit of the E3-encoded proteins at high levels of NF-κB and TNF-α [59]. Since the E3-region is not essential for viral replication in tissue culture, this domain usually deleted from adenoviral vectors to increase the space for transgenes. However, the performance of adenoviral vectors, which retained the E3-region proved better in terms of transgene expression and reduction of the adaptive immune response [60,61]. Whether these positive effects can be attributed to a diminution of the early immune responses is unclear, but it seems unlikely that Ad vector-mediated E3-14.7K and E3-10.4K/14.5K expression might completely abolish the clearance of vector DNA observed within 24 h post-administration.

1.3. Circumventing the adaptive immune response by adaptations of the patient

1.3.1. Immunomodulation

The lack of long-term transgene expression with Ad vectors is generally attributed to the cytotoxic and the humoral immune responses, which are strongly induced by Ad vectors. To increase the persistence of the transgenes, various studies have aimed at creating a temporary unresponsiveness of the host immune system. Some success has been obtained with immunosuppressive agents like cyclosporin A, cyclophosphamide, deoxypergualin and FK506, which have shown to attenuate the cellular and, in some cases, humoral immune responses [25,62–67]. A similar effect can be established with cytoablative regimens like anti-CD4, anti-CD8 or anti-T cell receptor antibodies. The anti-CD8 or anti-T cell receptor Abs cause the depletion of CTLs and extend transgene expression [61,68]. Apart from the attenuation of the CTL response, the transient depletion of $CD4^+$ T cells by anti-CD4 Abs also prevented the development of anti-Ad neutralizing Abs. Although it was not unequivocally demonstrated [69,70], this permitted in several cases repeated administrations of the Ad vector [61,71–73]. Nevertheless, application of these regimens is hampered generally by limited efficacy, potential toxicity, and impairment of pre-existing immunity.

To evade these disadvantageous effects, an alternative strategy is preferred, which attenuates the host's immune response by a blockade of co-stimulatory interactions between APCs, T cells and B cells. Such a co-stimulatory signal is provided by the interaction between B7 and CD28, which is required for the activation of $CD4^+$ and $CD8^+$ T cells by APCs (Fig. 2). Elimination of the B7–CD28 co-stimulation can be established by CTLA4Ig, a recombinant molecule, which binds to B7 and thereby blocks the interaction with CD28. Systemic co-administration of CTLA4Ig and an Ad vector resulted in prolonged transgene expression, correlating with reduced T cell activation [69,74,75]. Although the B7–CD28 interaction also affects the humoral branch of the immune apparatus, a secondary administration of the adenoviral vector was inefficient, unless the CTLA4Ig was locally produced by means of an Ad vector with a CTLA4Ig expression cassette [76,77].

An alternative co-stimulatory signal, which is used to modulate the host immune response, is mediated by CD40 and CD40 ligand (CD40L). The interaction of CD40L on activated $CD4^+$ T cells and CD40 on B cells stimulates the humoral immune response. The interruption of this interaction can be established by an anti-CD40 ligand Ab, which was shown to inhibit the production of Ad-specific neutralizing Abs and facilitate a repeated administration of an Ad vector in mouse lung and liver [78–80]. In addition, the cellular immune response was also affected, which caused a prolonged persistence of transgene expression. Similar results were obtained in the lungs of a non-human primate model [81]. Since the interventions in co-stimulatory interactions primarily acted on separate arms of the immune response, a more pronounced effect was anticipated if CTLA4Ig and anti-CD40L Ab were combined. Indeed, transient inhibition of both co-stimulatory pathways induced a prolonged transgene

expression in mouse liver (>180 days) and alveoli (>90 days). Additionally, it permitted secondary transduction upon re-administration of the same Ad vector [82,83].

In spite of the extended effect of gene transfer by means of immunosuppressants, the above-mentioned agents exert their effect via a non-specific attenuation of the host immune response. This entails a risk for the patient in case of simultaneous infections with other microorganisms at the time of treatment. Moreover, the immunosuppressants are associated with various potential side effects. Therefore, in general immunosuppression is not a preferred approach in clinical gene therapy.

1.3.2. Specific immune tolerance to the adenoviral vector

In order to limit the risks that are associated with systemic immunosuppression, the adaptation of the host immune response should be Ad-specific. In mice, a state of immunologic unresponsiveness to the Ad vector can be obtained by means of an intrathymic inoculation [84,85]. Transplantation of adenovirus-infected pancreatic islets into the thymus of adult mice or a direct intrathymic injection of the Ad vector in neonates impaired the Ad-specific cellular immune response, which prolonged hepatic transgene expression upon intravenous administration of Ad vectors. In mice, the intrathymic inoculation did not affect the humoral immune response, which prevented a repetitive administration of Ad vectors. In Gunn rats, however, these strategies also abrogated the humoral immune response to Ad vectors. Administration of the Ad vector during the neonatal period or direct intrathymic inoculation of the Ad antigens inhibited the development of Ad-specific CTLs and anti-Ad neutralizing Abs [86,87]. This tolerization allowed a prolonged transgene expression by repetitive administrations of the Ad vector. Moreover, the induced tolerance did not include wild-type (*wt*) adenovirus. Injection of *wt* Ad5 into tolerized animals mounted a *wt* Ad-specific CTL response.

Despite its Ad specificity, the feasibility of these tolerization methods for clinical application is uncertain. Although many inherited disorders can be diagnosed early in childhood or even prenatally, the human immune system is at birth at a higher developmental stage than that of rodents. This might affect the result of the tolerization. In addition, the human thymus involutes during life, which limits the applicability period of the intrathymic administration and excludes a large part of the human population. Moreover, it is still unknown whether the induced tolerance is permanent or requires a regularly repeated intrathymic injection. Apart from the impact on the patient, the lack of permanent tolerance will obviously impair the utility of the intrathymic tolerization even further.

In this context, the utility of oral tolerization will have more potential for future human application. As was demonstrated by Ilan et al. [88], Gunn rats could be tolerized to the Ad vector by an oral administration of the main adenoviral capsid proteins. Subsequent administration of an Ad vector showed a reduced development of anti-Ad-specific CTLs and

neutralizing Abs. This permitted a prolonged transgene expression by repetitive injections of the Ad vector. Tolerance to an immunogenic transgene product might be induced in a similar way [89,90]. Thus, oral tolerization seems a potential and specific method to overcome a major hurdle for Ad-mediated gene therapy. Interestingly, pre-existing immunity to adenovirus did not hamper the effect of oral tolerization to an Ad vector [91]. However, it still has to be investigated whether oral tolerance to an Ad vector excludes *wt* adenovirus. In view of the morbidity and mortality associated with an adenovirus infection in immunocompromised patients, the absence of tolerance to *wt* adenovirus is of major importance to the safety of this tolerization method [92,93].

1.4. Circumventing the specific immune response by adapting the adenoviral vector

1.4.1. Multiple-deleted vectors

Preferably, abrogation of the Ad-induced immune response should be accomplished without any kind of immune suppression with its consequent risk for the patient. This implies that the immunogenicity of the adenoviral vector itself should be diminished in order to evade the host immune response. The most generally applied Ad vector is the first-generation E1-deleted vector, which, in many cases, also lacks the non-essential E3 region. As has been demonstrated, this vector elicits an intense immune response, which is, at least in part, caused by a residual expression of viral antigens [1–8]. Based on the assumption that reduction of the adenoviral genome would lead to a further limitation or complete elimination of early and late viral gene expression, initial attention was focussed on the additional deletion of the E2A or E4 region [94–99]. The E2A gene encodes a DNA-binding protein (DBP), which is essential for the initiation and elongation of viral DNA synthesis, for the modulation of E4 transcription and for the expression of the late viral genes via activation of the major late promoter (MLP) [4,100]. The E4 region, which contains seven open reading frames (ORFs), is essential for viral DNA replication, late gene expression, efficient assembly of the virus particle and inhibition of host-cell protein synthesis [94,101,102].

Initial studies on the effect of doubly defective vectors were performed with constructs that carried the H5*ts125* mutation in the E2A gene, in addition to the E1-and E3-deletions. This temperature sensitive (*ts*) mutant is defective at the non-permissive temperature (39 °C), while its function is not affected at the permissive temperature (32 °C). Such a conditional effect has the concomitant advantage that it does not require the generation of a new helper cell line, which complements the lack of E1 and E2A function. In comparison to a first-generation Ad vector, the ΔE1/*ts*E2A Ad vector exhibited a reduction in late virus-gene expression, a diminished infiltration of $CD8^+$ T cells and a prolonged transgene expression in several animal models [2,3,5,103]. In contrast, Fang et al. [104] observed no improved performance with a similar ΔE1/*ts*E2A vector in Balb/c mice and haemophilia B dogs. This

inconsistency might be caused by a variation of the transgene, promoter, recipient or administered dose. In the latter study, however, the performance of the vector was correlated to the serum level of transgene product. Due to a humoral immune response to the transgene product or a silencing of the trans-gene promoter, the detected transgene product might deviate considerably from the persistence of the vector genome and consequently mask the effect of the E2A mutation.

Nevertheless, irrespective of the actual improvement brought about by the *ts*E2A mutation, the persistence of the ΔE1/*ts*E2A vector in the liver of immunocompetent mice (>70 days) was clearly inferior to that of a first-generation virus in immunodeficient animals (>300 days) [3,25]. Although several factors might be involved, it is generally accepted that, in vivo, at a body temperature of 37 °C, mutant DBP is not completely inactivated and might diminish the effect on vector persistence [3,5,95,103]. The elimination of this residual effect of mutant DBP is easily accomplished by the deletion of the E2A region from the vector. Apart from the guaranteed absence of DBP, it will also reduce the risk of RCA formation during Ad vector propagation and additionally prevent a possible reversion of the *ts* mutation to the wild-type phenotype. Accordingly, E1-and E2A-deleted (ΔE1/ΔE2A) vectors and their complementing cell lines were generated [95,97,98]. As anticipated, with these ΔE1/ΔE2A vectors neither viral DNA replication, nor late-protein synthesis in human cells was discernable in the infected cells [4,95,97]. However, in vivo, the ΔE1/ΔE2A Ad vector did not reveal significantly lower vector toxicity than a ΔE1 Ad vector [105,106]. Moreover, no differences were observed in Ad-specific CTL activity and the development of anti-Ad Abs, resulting in a similar persistence of their viral DNA in liver and lung of immunocompetent mice [4]. So, in general, the ΔE1/ΔE2A vector did not meet the expectations that were raised by the ΔE1/tsE2A vector, although the deletion of E2A showed a more pronounced effect on late gene expression than the *ts*E2A mutation [95].

How the difference in vector persistence between the ΔE1/*ts*E2A and ΔE1/ΔE2A vector can be explained remains unknown. Many factors might be involved, which even include a possible negative effect of the complete lack of DBP. Normally, the transcription of E4 is inhibited by the action of DBP 6 h post-infection [100,107,108]. This implies that E4 expression is prolonged if E2A is deleted, which might affect the immune response [109]. Presumably, the mutant DBP from the ΔE1/*ts*E2A vector exhibit residual activity at 37 °C, which is sufficient for controlling the E4 expression and might contribute to the prolonged persistence of this vector.

Simultaneously with the above-mentioned deletion mutants, E1-and E4-deleted (ΔE1/ΔE4) vectors and their complementing cell lines were being developed [94,96,99]. Like the ΔE1/ΔE2A vector, the ΔE1/ΔE4 vectors induced a significant reduction of late gene expression [4,110–112]. Additionally, absence of the E4 region resulted in a decreased expression of E2A and a block of viral DNA replication [110]. Consequently, the E4 deletion

resulted in a decreased vector toxicity and inflammation profile in vivo [106,111,112]. Its beneficial effect on in vivo vector persistence remains, however, controversial [4,110,112]. Moreover, the expression of the transgene from a ΔE1/ΔE4 vector is sometimes considerably lower than that from a first-generation Ad vector [110,113]. This effect may be explained by the observation that persistent transgene expression from the CMV or RSV promoter is dependent on the availability of E4 proteins [114].

To assess the role of the individual E4 gene products in transgene expression and vector toxicity, a series of different ΔE1/ΔE4 vectors, which retained one or a combination of the various E4 ORFs, was analysed [106,115]. In vitro, expression from the CMV promoter was clearly abolished when E1 and E4 were completely deleted. However, the retention in the vector of ORF3,4 or ORF3,6,7 prevented the decline of expression [115]. In vivo, the requirements for optimal transgene expression are generally the same. However, E4 ORF6,7 is responsible for the elevated toxicity and inflammatory responses of the vector in liver [106,115]. So, although the deletion of E4 is beneficial to vector performance, it will reduce the persistence of transgene expression in context of a CMV or RSV promoter unless E4 ORF3 or E4 ORF3, 4 are retained.

Recently, a novel vector was reported, which combined the profitable effects of the E2A and E4 deletions [109]. This ΔE1/ΔE2A/ΔE4 vector lacked the E1, the E2A and the E4 regions, except E4 ORF3. In comparison to a ΔE1/ΔE2A vector, this vector revealed a further attenuation of immunogenicity and liver toxicity, as well as an elevated transgene expression [109].

1.4.2. Helper virus-dependent vectors

Although the second-generation Ad vectors showed significant reduction of the late viral gene expression and attenuated cytotoxicity, the objective of persistent transgene expression was not reached. Since the ΔE1/ΔE2A/ΔE4 vector resulted in a better performance, further improvement was expected, if all or most viral genes would be removed [109]. This should restrict the immunogenicity of the vector entirely to the injected viral capsid proteins and the transgene product. In addition, its safety profile should be enhanced and its insert capacity increased to its maximum. However, the propagation of such vectors is complicated, because all adenoviral functions should be complemented in *trans*. Basically, this can be accomplished by using a regular E1-complementing cell line in combination with an E1-deleted helper virus, which explains the designation of this generation of Ad vectors as 'helper-dependent' Ad (HD) vectors.

In time, several strategies have been developed to generate HD vectors [116]. In the most straightforward strategy, the transgene-containing expression cassette was cloned within the ITR and packaging signal (ψ) sequences. Although this approach established the production

of infective HD virions, the yield of the HD vector was low and the contamination with helper virus was relatively high (≥1%) [117–119]. A more advanced strategy generated the HD vector in 293 cells by means of the Cre-recombinase, which excised a *loxP*-flanked region of adenovirus genes [120,121]. As demonstrated by Lieber et al., a 25 kb region was efficiently deleted and resulted in formation of infective 9 kb HD virions. Moreover, it could be propagated at high titres with less than 0.5% contamination of undeleted virus. Nevertheless, in mice the persistence of the truncated genome was very short (<5 days).

The most successful and nowadays most frequently applied strategy to generate HD vectors is a combination of both approaches. The HD genome is constructed by cloning, while the Cre-lox system is applied to the helper virus to minimize contamination of the HD vector batch [122]. To that end, loxP-sites were inserted around the packaging signal of the helper virus, so that excision by Cre-recombinase would render the helper unpackagable (Fig. 3). Serial passages of the HD vector in helper virus-infected 293-Cre cells produced a high titre of the HD vector. In addition, the produced HD vector batch contained less than 0.01% contamination with helper virus [122]. However, the efficiency of production is dependent on the actual size of the HD genome, as was demonstrated by Parks et al. [123]. Whereas HD vectors with a genome size of 75–105% of the wild-type genome were efficiently packaged, HD vectors were inefficiently packaged and prone to rearrangements, if their genome size was less than 75% of the wild-type. Whether this also explains the observed instability of the 9 kb HD vector is unclear. However, all analysed HD vectors with a genome within the specified limits showed prolonged persistence [124–126].

In many cases, the preferred HD-genome size is obtained by the supplemental use of "stuffer" DNA. The impact of the stuffer is however not limited to the discussed size constraints. The origin and nature of the stuffer DNA also affect the performance of the HD vector [127,128]. While HD vectors with prokaryotic stuffer DNA (bacteriophage λ DNA) induced the development of stuffer-directed CTLs, the stuffer DNA obtained from the human hypoxanthine–guanine phosphoribosyltransferase (HPRT) gene showed no such CTL response and enhanced transgene expression [127]. The distinction between these two fragments might be due to differences base composition. Nevertheless, the elimination rate of both vectors from mouse liver was similar. Irrespective of its beneficial human origin, the presence of several Alu repeats, a MAR and two retroviral long terminal repeats (LTR) in the HPRT stuffer might interfere with vector propagation and stability, as well [128]. To optimize the production and efficacy of an HD vector, stuffer DNA should lack known signals and repeat units. With these criteria in mind, two human DNA fragments, termed HSU and AFO, were selected. Insertion of these fragments as stuffer DNA into an HD vector had a positive effect on the replication of HD vector versus helper virus. Moreover, an HD vector with HSU or AFO fragments showed a higher transgene expression in mice, than an HD vector with HPRT-derived stuffer DNA [128].

Until now, the most extensively studied HD vector contains the complete human α1-

antitrypsin (hAAT) locus and a 9 kb fragment from the HPRT gene, which brings the HD genome within the preferred size limits [124]. Intravenous administration of this HD vector in C57BL/6J mice yielded a high level of hAAT expression (50 μg hAAT/ml serum), which remained for at least 10 months. A first-generation Ad vector with a cDNA-based expression cassette induced a maximum expression level of 2 μg hAAT/ml serum, which declined to less than 0.1 μg/ml over 10 months. Since the latter vector contained hAAT cDNA in contrast to the HD vector, which harbours a genomic hAAT gene, some of the difference in serum levels may be explained by the different expression cassettes. The elevated and prolonged transgene expression from the HD vector was accompanied by a decreased acute and chronic toxicity. This was probably due to the complete elimination of viral gene expression and was even observed at the highest applied dose of 3.2×10^{11} virus particles [124,129]. Subsequent application of the HD-hAAT vector in non-human primates instead of in mice confirmed its performance: intravenous administration of this HD vector into baboons resulted in hAAT expression for more than 12 months in two out of three animals [126].

Similar results were obtained with HD vectors encoding the mouse erythropoietin (mEPO), mouse leptin, and human blood-clotting factors VIII and IX [50,125,130,131]. In mice, these vectors showed a significant attenuation of the host immune response and a prolonged transgene expression. Moreover, in comparison to its first-generation equivalent, the HD-mEPO vector demonstrated a 100-fold increase in transgene expression per infectious particle. This allowed considerable reduction of the vector dose, which prevented formation of anti-Ad neutralizing Abs and permitted, consequently, readministration of the HD vector.

The performance of the HD-mLeptin vector was, however, less successful when it was applied to a relevant animal model, the leptin-deficient, *ob*/*ob* mouse [131]. This could be attributed to the immunogenicity of leptin in this animal model, which resulted in the development of leptin-specific Abs and a gradual loss of vector DNA. The impact of the immunogenicity of the transgene product was confirmed by an HD vector encoding full-length dystrophin in combination with β-galactosidase [117,119]. Whereas LacZ transgenic mice revealed a prolonged cytoplasmic β -gal expression after intramuscular administration of the HD vector, non-transgenic mice showed a reduced expression period, which was combined with $CD4^+$ and $CD8^+$ T cell infiltration [132].

In general, the deletion of all Ad genes reduces the toxicity and inflammatory immune response and concomitantly results in a prolonged transgene expression, if the HD genome is stabilized by the insertion of stuffer DNA. These benefits are even more pronounced if the stuffer DNA is optimized for human application. Nevertheless, even after gene transfer with HD vectors host immune response may be mounted that are directed against the transgene product and viral capsid proteins [13].

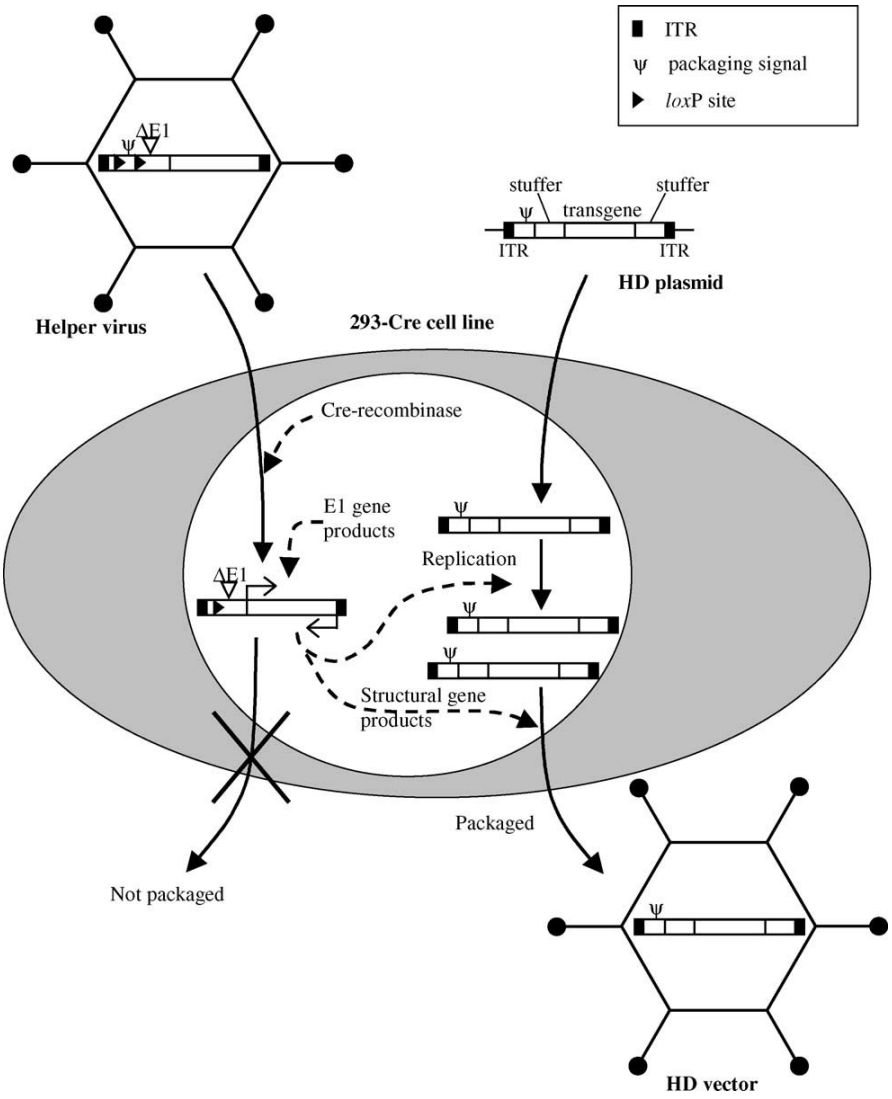

Fig. 3. Generation and propagation of the helper virus-dependent adenoviral (HD) vector. The adenoviral genes that are deleted from the HD vector are provided in *trans* by an E1-deleted helper virus and an E1-complementing cell line. To limit the simultaneous propagation of helper virus, the packaging signal of this virus is flanked by loxP-sites. The stable expression of Cre-recombinase in the helper cell line allows the excision of the packaging signal and renders the helper virus genome unpackagable. Besides the necessity of the *cis*-acting elements, the inverted terminal repeats (ITR) and the packaging signal (ψ), the HD vector backbone also contains a transgene. In order to allow stable propagation and efficient packaging, non-viral "stuffer" DNA is used to supplement the HD vector backbone up to 75% (27 kb) of the size of the *wt* adenovirus genome. 293-Cre: a 293-cell line derivative that stably expresses the Cre-recombinase.

1.4.3. The positive effects of adenovirus early region 3 (E3)

The E3 region is deleted from most adenoviral vectors, because it is not essential for viral replication and provides extra space for transgenes. It is, however, unlikely that a non-essential region, which covers about 10% of the viral genome, would have survived evolutionary selection. Indeed, several E3-encoded proteins are involved in the modulation of the host immune system [55–58]. Although the E3-encoded proteins E3-10.4K, E3-14.5K and

E3-14.7K are predominantly involved in the modulation of the innate immune response, the impact of their effect extends towards the adaptive immune system [57]. This was demonstrated in E3-14.7K transgenic mice, which revealed a significant reduction of the cellular immune response upon intratracheal administration of an Ad vector [133]. The humoral immune response against the Ad vector was, however, not markedly affected.

Another E3-encoded protein, i.e. E3-19K, might reduce the adaptive immune response more directly. This transmembrane 19 kDa glycoprotein is located in the endoplasmic reticulum (ER) and inhibits the transport of MHC-I molecules to the cell surface. To that end, it contains an MHC-I-binding domain and a microtubule-binding carboxyl terminus, which retains the resulting complex in the ER [134–136]. In vitro, E3-19K expression clearly reduced the level of MHC-I molecules on the cell surface and abrogated their recognition and subsequent lysis by specific CTLs [137]. In addition, in vivo administration of an Ad β-gal vector, which contained an E3-19K expression cassette, failed to stimulate the proliferation of Ad vector-specific, as well as, β-gal-specific CTLs [138]. This implies that the retention of MHC-I by E3-19K is not restricted to the adenoviral antigens, but extends to antigens of other origin as well [138]. Moreover, the application of an E3-19K-expressing Ad vector showed enhanced transgene persistence in the lung and the liver of B10.HTG mice [139]. However, E3-19K did not establish a similar effect in BALB/c mice. This might be explained by the different affinity of E3-19K for the various murine MHC-I haplotypes. Whereas E3-19K interacts efficiently with H-2D^{b}, H-2K^{d} and H-2L^{d} alleles, weak or no association was seen with H-2D^{k}, H-2D^{d} and H-2K^{b} alleles [139,140]. Although this might indicate a limited applicability of E3-19K in human gene therapy, it has been reported that E3-19K interacts with all analysed human MHC-I molecules, albeit with different affinities [141,142]. Notably, the E3-19K from human adenoviruses has evolved to function optimally in humans and might perform less well in other recipients. A similar functionality profile is reported for ICP47. This protein from Herpes simplex virus prevents the peptide loading of MHC-I molecules efficiently in man, but lacks this functionality in mouse [143,144].

Obviously, the most pronounced immune-modulating effect would be obtained if the potentials of E3-14.7K, E3-10.4K/14.5K and E3-19K were combined. This was most simply accomplished by the re-introduction of the whole adenoviral E3 region in the Ad vector [60,61]. Application of this vector in Gunn rats showed a clear inhibition of CTLs towards Ad-infected cells [60]. Remarkably, the humoral immune response towards the Ad vector was inhibited as well. This facilitated re-administration of the vector and consequently resulted in prolonged transgene expression. The unexpected inhibition of Ad-specific neutralizing Abs might be explained by a reduction of antigen release as a consequence of the diminished CTL- and TNF-α-induced cytolysis. In addition, the TNF- α-induced stimulation of dendritic-cell function can be hampered by the expression of E3-14.7K and E3-10.4K/14.5K and concomitantly limit the antigen presentation by these cells [60]. So, these preliminary in vivo

data reveal great potential for E3-encoded proteins. However, their performance in man and, subsequently, their impact for gene therapy with adenoviral vectors are to be addressed in future research.

1.4.4. The potential of gene and vector targeting

Although the re-insertion of the E3-region is presented as an adaptation of the adenoviral vector, E3-encoded proteins exert their effect via immunomodulation of the transduced cells. Consequently, this adaptation can be seen as a local adaptation of the recipient, as well. At first glance, the E3-induced ignorance of the immune system does not seem to cause any risk for the patient, since it did not prevent a normal immune response upon subsequent exposure to an E1-and E3-deleted vector [60]. However, one should note that the immune ignorance might not be limited to the antigens obtained from vector and transgene expression, but may hold for all antigens from the transduced cell. This implies that its transformation into a malignant cell might escape immune surveillance, as well, with all its consequences.

Preferably, adaptations of the adenoviral vector should reduce the host immune response to the vector and trans-gene product more specifically. Such specific attenuation of the immune response directed against the transgene product can be established by means of cell-specific promoters, which limit the transgene expression to the preferred cell type or tissue. Consequently, this would prevent unintended transgene expression in professional APCs, which initiate, at least partially, the observed cellular immune response [8–11,37,132]. Remarkably, the impact of the chosen promoter extends to the humoral immune response, as well. This is most clearly illustrated by the Ad-mediated expression of human α1-antitrypsin, which varies among different mouse strains [1,33]. Whereas hAAT expression, driven by a ubiquitous promoter (RSV or PGK) is prolonged in C57BL/6J mice (>8 weeks), hAAT expression in C3H/HeJ and CBA/J mice is limited (2–4 weeks). This strain-dependent variation in persistence of the hAAT protein is correlated to the development of hAAT-specific Abs [33]. In contrast, prolonged hAAT expression and absence of Ab development is shown when hAAT is expressed from its endogenous promoter in C3H/HeJ mice [124,145]. So, unless the recipient is somehow tolerant to the transgene product, the expression from ubiquitous promoters will be limited by a host immune response to the transgene product. The effect of an endogenous tissue-specific promoter was confirmed by a direct comparison of first-generation vectors, which expressed hAAT from a ubiquitous mouse PGK promoter or a liver-specific mouse-albumin promoter. The PGK promoter induced a hAAT-specific Ab response in C3H/HeJ mice, which limited the expression period. In contrast, expression from the albumin promoter was not hampered by any Ab response and persisted for more than 44 weeks [145]. Similar results were obtained after expression of human apo A-I [37]. When comparing vectors with the hepatocyte-specific *apo C-II* promoter and the ubiquitously

expressed murine *MHC II Eβ* promoter, the latter vector induced a major humoral immune response to the transgene product (apo A-I) whereas the hepatocyte-specific promoter did not induce a humoral response. This supports the hypothesis that promoters that are not expressed in the antigen-presenting cells may be applicable to prevent the humoral response. However, other reports suggest that the use of liver-specific promoters (viz. the human α1-antitrypsin gene promoter, a chimeric *apoE/apoCII* promoter, and the albumin gene promoter, respectively) was not sufficient to prevent the humoral responses against the secreted antigens [50,146]. This shows that although liver-specific promoters may reduce the induction of trangene-product-directed immune responses, their use may not be sufficient to prevent them altogether.

The immune response to a foreign antigen can be affected by the nature of the antigen itself, the dose and route of delivery, and the presentation of the antigen to immune-surveying cells. Since the first two options were similar in the comparative study in C3H/HeJ mice, the lack of hAAT expression in professional APCs might cause the observed tolerance to hAAT. Indeed, in baboons, the application of a similar vector, which contained the complete human hAAT locus, induced a prolonged expression of hAAT (>10 months). It could, however, not prevent the development of hAAT-neutralizing Abs in one out of three baboons [126]. Nevertheless, a well-chosen promoter, that limits the expression of the trans-gene to the tissue or cell type of choice, might contribute significantly to the reduction of the host immune response directed to the transgene product.

A similar or even more pronounced effect might be established by the modification of adenoviral tropism in order to transduce only the cell type of interest [52,147]. Since the adenovirus receptors are broadly expressed [148–152], specific targeting will prevent the transduction of unintended cells, like APCs [153,154]. This might result in a reduction of the cellular, as well as, the humoral immune response to vector and transgene product. In addition, adenoviral infection activates NF-κB, which stimulates cytokine production and thereby triggers the host immune response. A targeted Ad vector circumvents the natural infection route and might consequently prevent the stimulation of NF-κB and subsequent events [47,147]. Moreover, vector targeting will induce the efficacy of the vector. This permits the reduction of the administered dose and might concomitantly limit the inflammatory response.

Although the advantages of targeted adenoviral vectors are generally accepted, in vivo application of a properly targeted vector has not been reported yet. So, the actual impact of vector targeting on the immune response still has to be established.

1.4.5. Use of viral inhibitors of antigen processing

Cellular immune response against transgene-encoded *neo*antigens the may predominate upon use of the HD Ad vectors. Transgene-product immunity is prominent following

adenovirus-mediated transfer of the *Escherichia coli*-derived LacZ gene, encoding β–galactosidase. In rodents, strong immune responses against this *neo*antigen were observed following adenovirus-mediated gene transfer into liver, muscle, lung and brain, leading to local inflammation, destruction of the transduced cells, and loss of transgene expression [74,155–157]. Antigen-specific major histocompatibility complex class-I restricted cytotoxic T lymphocytes are the prime suspects responsible for target cell destruction [8,18,74,158]. This requires prior activation of $CD4^+$ T cells [6,7,11,18,51,159–162]. Also in primates a CTL response directed against the transgene product has been shown to occur after retrovirus-mediated gene transfer [163,164]. In a clinical trial aiming at inducing a graft-versus leukaemia response, 8 of 24 treated patients developed a specific cytotoxic $CD8^+$ T cell-mediated immune response against the cells genetically engineered to express the herpes simplex virus 1 (HSV1) thymidine-kinase (TK) gene. This led to the selective elimination of the modified cells [164].

Previous attempts to reduce the T cell responses against the *neo*antigens during gene therapy focused on blocking the MHC classes-I and -II restricted T cell responses, or the prevention of co-stimulation of T cells [12,75,80,158,160–162]. However, these approaches were either not fully effective or resulted in a general immunosuppression. In the ideal strategy the presentation of the transgene-derived peptides by MHC class-I is selectively prevented, which eliminates specifically the cellular immune response against the neoantigens encoded by the transgene.

Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class-I molecules. Given the important role of CTLs in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen-presentation [165–167]. In general, the viruses interfere with antigen presentation by frustrating the cell's capacity to generate or present antigenic peptides. In few cases, the inhibition blocks presentation of specific polypeptides. One such example comes from the Epstein–Barr Virus (EBV) nuclear-antigen 1 (EBNA-1). EBNA-1 is expressed in latent EBV-infected cells. Here it is indispensable for the virus as it is required for the maintenance of the viral episomes. Although EBNA-1-specific CTLs have been described in infectious mononucleosis patients and healthy carriers, they cannot recognize EBV-infected cells [168–173]. The failure to recognize endogenously expressed EBNA-1 has been attributed to the glycine-alanine repeat (GAr) domain in the EBNA-1 sequence that protects EBNA-1 from proteasomal degradation and subsequent presentation in the context of MHC class-I [174,175]. This successful immune-evasion strategy points to the unique opportunity to hide cells expressing transgenes from CTL-mediated target-cell destruction by incorporation of the GAr sequence into the transgene. We have demonstrated recently that fusing the β-galactosidase with the GAr sequence is compatible with enzyme function, and

prevents presentation of β-gal-derived peptides by MHC class-I. Hence, the cells were insensitive to antigen-specific CTL. Preliminary evidence suggests that the persistence of transgene expression was increased in vivo, as a result of the evasion of the anti transgene-product immunity [176].

1.4.6. Repetitive administration for prolonged transgene expression

In spite of all efforts to reduce the cell-mediated immune response against transduced cells, complete tolerance to transduced cells will not be a warrant for long-term trans-gene expression. This is mainly due to the episomal nature of the adenoviral DNA, which leaves the transgene mainly unintegrated in the host. Although the lack of efficient integration is beneficial in terms of the risks related to insertional mutagenesis, it makes vector persistence dependent on the absence of proliferation of the host cells. Obviously, re-administration of the adenoviral vector could obviate this lack in integrating capacity, if the efficiency of vector delivery at repeated administration was not limited by the development of Ad-specific Abs [7,25,177].

The classification of human adenoviruses into six subgroups is based on criteria like hemagglutination properties, oncogenicity and DNA homology. Within these subgroups a further division into different serotypes is based on their antigenic cross-reactivity [100,178]. On the basis of this classification, it was hypothesized that the Ad neutralizing Abs could be circumvented by the alternating use of different Ad serotypes. This was initially confirmed by a repetitive administration of Ad serotypes from different subgroups. The immunization of mice by an intraperitoneal administration of *wt* Ad7 (subgroup B) or *wt* Ad4 (subgroup E) virions did not affect subsequent gene transfer by an Ad5 (subgroup C) vector [179]. Similar results were obtained if mice were immunized by an intratracheal administration of *wt* Ad4 or *wt* Ad30 (subgroup D) virus [28]. Moreover, subsequent immunization with Ad4 and Ad30 virus could not prevent efficient gene transfer by the Ad5 vector either.

The benefit of using different Ad serotypes for repetitive administration was even extended to serotypes from the same subgroup by Mack et al. [27]. Two weeks after the first administration with an Ad2 or Ad5 vector, either the same or the other serotype was administered intratracheally. The administration of the alternating serotype established transgene expression at a level, which equalled that obtained in naive animals. In contrast, transgene expression after re-administration of the same serotype Ad vector was at least 70% less than the expression in naive animals.

This implies that the sequential use of multiple adenoviral vectors will help to evade anti-Ad neutralizing Abs generated by previously administered serotypes. However, this strategy requires the verification of each serotype for its safety and efficacy. In addition, each new Ad vector might need its own helper cell line, although the helper cell line might be interchangeable for serotypes within the same subgroup. From this point of view, the single

alteration of the immunodominant capsid components or, more precisely, of the immunodominant capsid epitopes would create more readily applicable vectors.

The adenovirus capsid mainly consists of three components: fibre, penton base and hexon. These components are therefore seen as the major targets for neutralizing antibodies. As can be deduced from In vitro analyses, the contribution of anti-penton-base antibodies to the neutralization of the Ad vector is limited [180,181]. This is partially explained by the observation that the RGD-epitope from the penton base, which interacts with αvβ3/β5 integrins and is involved in the internalization of the virus, escapes from antibody neutralization [182]. Although the anti-fibre antibodies neutralized the Ad vector rather efficiently in in vitro assays, they seem to lack neutralizing activity in vivo [181]. This was demonstrated by means of an Ad5 vector with Ad7-derived fibres (Ad5/F7) [183]. Whereas the chimerical Ad5/F7 vector was neutralized in Sprague–Dawley rats, which had been immunized intraperitoneally by *wt* Ad5 virus, it was unaffected in rats immunized by *wt* Ad7 virus. It has, however, not been demonstrated unequivocally that anti-fibre antibodies do not contribute to the neutralization of infection in vivo at all. The nature of the humoral immune response depends on the route of administration and the used intraperitoneal administration induces predominantly an anti-hexon Ab response [184,185]. Moreover, in vitro a synergistic effect of the anti-fibre and -penton base Abs is observed, which suggests an underestimation of their neutralizing effect if each of their contributions is considered separately [180].

Nevertheless, the anti-hexon Abs appear to be the most important effectors of Ad neutralization. The anti-hexon Abs exert their neutralizing effect via a very efficient single-hit mechanism, which requires just one Ab per virion [181]. Possibly, the attachment of one Ab is sufficient to prevent the conformational changes in the hexon that are necessary to rupture the endosome [181,186]. In addition, anti-hexon Abs occur both after intraperitoneal and intravenous administration of the Ad vector. Subsequent analysis of the hexon for type-specific antigenic determinants identified loop1 and 2 (L1 and L2, respectively) as the putative neutralizing epitopes [187]. This paved the way for strategies aiming at the evasion of anti-Ad neutralizing Abs by alteration of immunodominant capsid epitopes. To test the feasibility of this approach, L1 and L2 from the Ad5 vector were replaced by their counterparts from Ad2 [188]. Unfortunately, these modifications did not permit efficient transduction of Sprague–Dawley rats, which had been pre-immunized with an Ad5 vector, although neutralization was more pronounced after the re-administration of a native Ad5 vector or an Ad5 vector containing solely the Ad2-derived L2 loop. However, if the replaced hexon region was extended from L1 to L4 and derived from Ad12 instead of Ad2, the anti-Ad5 neutralizing Abs in immunized C57BL/6 mice were unable to neutralize the chimerical Ad5/H12 vector. The chimerical vector could efficiently transduce the liver of immunized mice, while transduction by the Ad5 vector was absent [189]. This implies that the inclusion of the L4 domain into the region of replacement is of more importance than would be expected on the basis of the relative conservation of the sequences. Additionally, the effect

might be augmented by shifting from the rather homologous serotype Ad2 to the most divergent serotype Ad12 (subgroup A). Of course, the use of a different animal model might affect the outcome as well.

A completely different approach for circumventing Ad-specific Abs consists of coating of the adenovirus capsid with an agent that shields the particle from the neutralizing antibodies. An initial attempt was performed with a combination of the cationic lipid GL-67 and dioleoylphosphatidylethanolamine-polyethylene glycol (DOPE-PEG) [190]. In vitro analysis of the GL-67/ DOPE-PEG-coated adenovirus showed that the particle was partially shielded from immune plasma. Furthermore, intranasal administration of a coated vector to immunized mice resulted in substantially higher transduction efficiencies than that of the unshielded Ad vector. However, intravenous administration revealed no significant protection against Ad-directed Abs in the circulation. Similar results were obtained with an alternative coating procedure, which included the covalent attachment of the activated PEG, tresylmonomethoxypolyethylene glycol (TMPEG) to the surface of the adenovirus particle [191]. In vitro, the TMPEG-coated adenovirus was almost completely shielded from the neutralizing Abs. Moreover, high titres of Ad-specific Abs in pre-immunized mice could not attenuate the infection efficiency of TMPEG-coated adenovirus upon intranasal administration. In a separate study PEGylation allowed significant gene transfer to the liver on readministration, suggesting protection from neutralizing immunity [192].

Since the HD vector and first-generation Ad vectors contain similar antigens, the same approaches to circumvent anti-Ad neutralizing Abs can be applied to the HD vector. However, the HD vector is more easily adapted to a different serotype, because it requires only a simple change of the helper virus serotype. In accordance with the humoral immune response to the first-generation Ad vector, the neutralizing Abs against an HD-Ad2 vector do not cross-react with an HD-Ad5 vector [193]. Whereas intravenous administration of an HD-Ad2 vector was a 100-times less efficient in HD-Ad2-immunized mice than in naive mice, the administration of an HD-Ad5 vector in the Ad2-immunized mice revealed no loss in transduction efficiency [193].

Remarkably, efficient re-administration is not necessarily limited to vectors from an alternative serotype, but can be established by the same vector as well. Initially, this was only accomplished by means of an intranasal administration, although this could not prevent the development of neutralizing Abs in the circulation [194]. However, Maione et al. [125] demonstrated that intravenous re-administration of an HD vector can be as effective as the first administration. This was due to the high vector efficiency, which provided a therapeutic transgene expression at a relative low virus dose (3×10^5 IU). Since the development of neutralizing Abs was not triggered up to 1.2×10^6 IU, re-administration of the HD vector established gene transfer at virtually 100% efficiency.

Although still preliminary, the latter data bring a repetitive use of the same Ad vector without tedious tricks or invasive treatment of the patient within reach. The potential of this

approach; however, is completely dependent on a low vector dose and requires a profound quest for efficient expression cassettes.

1.4.7. Prospects for an immune-tolerated adenoviral vector

Immunomodulation of the patient in order to prolong the effect of gene transfer seems inextricably associated with side effects. Consequently, modification of the Ad vector is the most desirable way of minimizing the induction of host immune responses and thereby increasing the feasibility of the Ad vectors for gene therapy of hereditary diseases. The most obvious approach for reducing the immune response to the Ad vector and Ad-transduced cells is the deletion of all adenoviral genes in order to eliminate the residual expression of viral antigens, which is responsible, at least partially, for the observed immunogenicity of the first-generation Ad vectors. Indeed, the HD vectors could bring about a stable expression in immunocompetent mice for at least 10 months, which resembled the performance of the first-generation Ad vector in immunodeficient mice [25,124]. Hence, HD vectors seem to outclass the second-generation Ad vectors in gene-therapeutic performance, although the HD vectors and second-generation Ad vectors were never compared directly.

Obviously, this will limit the application of the second-generation Ad vectors in favour of the HD vectors, but should not imply that their role in gene therapy is finished. As helper virus, needed for the construction and production of HD vectors, the second-generation Ad vector offers several benefits over the currently used first-generation Ad vector. Firstly, the use of a second-generation adenovirus will reduce the possibility of RCA formation. In addition, contamination of an HD vector batch with a second-generation Ad helper virus will be less immunogenic, due to the diminished expression of residual virus genes. However, double deletion-complementing cell lines are generally less efficient helpers, which result in a 1–2-log reduction of the virus yield [97,105]. Recently, this drawback was overcome by the generation of an efficient E1-and E2A-complementing cell line, the so-called E2T cell line, which allows the propagation of E1/E2A vectors to titres similar to those of E1 vectors propagated on 293 cells [98]. A derivative of this helper cell line, the E2T-Cre6 cell line stably expresses Cre-recombinase and might enable efficient propagation of an HD vector with the help of a doubly deleted helper virus [195]. Despite this progress, one should realize that the production and purification of HD vectors is still a multi-step process, which may be difficult to apply at an industrial scale.

Although the development of the HD vector holds considerable promise for in vivo gene therapy, it is unlikely that it will be sufficient to eliminate the host immune response completely. Specifically, it does not affect the immunogenicity of the transgene product. A carefully selected promoter that prevents transgene expression in professional APCs might diminish the host immune response to the transgene product. A similar effect might be accomplished by vector targeting. Moreover, specific transduction of target cells might

increase the vector's efficacy, as well, and concomitantly permit reduction of administered doses. Possibly, such adaptations might make the reinsertion of E3-19k, E3-14.7k and E3-10.4/14.7k superfluous. However, their use should be considered when application of a targeted HD vector appears to be insufficient to evade the host immune response. Especially, if it turns out that E3-14.7k and E3-10.4/14.7k attenuate the non-adaptive immune response, the addition of these regions might considerably affect the efficiency of the Ad vector.

It should be realized that the performance of most Ad vector modifications has been tested in animal models, which might be suggestive, but not necessarily representative for the performance in humans. Moreover, as demonstrated in mice, one specific vector can induce different immune responses in several strains of one species [1,33]. This makes it even harder to predict the effect of a vector modification in the genetically diverse human population.

In view of the Ad vector performance in humans, another point of concern is raised by pre-existing immunity to the adenovirus. Since most people have suffered from an adenovirus-mediated common cold, the efficiency of gene transfer by the Ad vector might be reduced by residual Ad-specific Abs. This concern was confirmed by the analysis of human sera, which revealed Ad-specific Abs in virtually all tested individuals [24,180]. However, in vitro analysis showed that only 40–55% of these Abs had neutralizing activity [24,189]. Moreover, pre-existing immunity did not prevent transduction by Ad vectors in vivo [196]. Nevertheless, the efficiency of gene transfer may be affected considerably, if the recipient has recently suffered from an adenoviral infection and carries large amounts of Ad-specific Abs. As a work around, vectors are being developed from serotypes with a very low prevalence of virus-neutralizing immunity in the humans, such as Ad35 [197,198].

2. Conclusion

Our extensive knowledge on adenovirus biology and the functions of its genes, in combination with our insight in the precise mechanisms that lead to the induction of cellular and humoral immune responses against Ad vectors, the Ad vector-transduced cells and the transgene products have facilitated the development of vectors that evade the immune system. The data obtained in murine models and in some cases non-human primates have underscored the considerable gain in vector performance that has been achieved. This suggests that the host's immune system may not be the insuperable hurdle for Ad-based gene therapy that it sometimes may seem to be. However, care should be taken when extrapolating results from animal experiments to humans. There is ample evidence for a marked interspecies heterogeneity in the responses of the innate and adaptive immune systems, and within a species considerable strain differences may occur [1,9,43,45,199]. In humans too, we should anticipate a significant heterogeneity in the immune responses to Ad vectors. In addition, vector-neutralizing immunity is known to occur in a significant fraction of the human population. These factors, in combination with the non-linearity of the dose response after intravenous administration of Ad vectors, make it difficult to predict the effects of the vector administration of the currently available Ad vectors. Several of the new vector modifications reviewed in this paper may lessen the impact of these factors. Yet extensive clinical testing will be needed to establish whether the suggested modifications suffice to allow the Ad vector to evade the host immune system to such an extent that Ad-based gene therapy for treatment of hereditary diseases comes within reach. Meanwhile, the Ad's capacity to provoke an immune response may be exploited for alternative purposes by using the adenovirus particles as adjuvant [200] or as safe vaccine against lethal pathogens [201,202].

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2.3 Transgene product directed immune responses

Whether for the treatment of genetic or acquired diseases, such as cancer and AIDS, the efficacy of most gene therapy protocols will depend on persistent, high-level expression of transgene-encoded proteins. In many instances, these proteins will constitute new antigens, and thus the induction of immune responses against transgene products is of concern for long-term efficacy of these therapies.

Transgene-product immunity is prominent upon adenovirus-mediated gene transfer, e.g. against the *Escherichia coli* β-galactosidase, and the Tet-transactivater protein in animal models. Intraperitoneal injection of a replication-defective (E1-deleted) adenovirus harboring the E.coli β-galactosidase LacZ gene induced a long-lasting β-galactosidase-specific cytotoxic T-cell response. This response was even more specific then when mice were vaccinated with the known LacZ immunogenic peptide. This indicates that immunization with rAd vectors promotes greater reactivity against naturally processed β-galactosidase (Juillard et al., 1995).

Instillation of a similar LacZ containing, E1-deleted adenovirus (Ad) in the lungs of mice elicited CTL responses both to the transgene and to the virus itself. The high expression of β-galactosidase (>80%) in the airways declined to undetectable levels by day 28 and was associated with peribronchial and perivascular lymphocytic infiltrates. Lymphocyte harvested from these animals showed specific lysis of cells infected with AdLacZ and a reduced level of specific lysis of adenovirus-infected cells expressing alkaline phosphatase. Cells infected with retroviruses expressing LacZ were also readily lysed and cells infected with retroviruses harboring alkaline phosphatase were spared from lysis, indicating that both transgene and vector virus elicited CTL. However, in mice tolerant to β-galactosidase there was also a transient expression of the β-galactosidase transgene observed. This indicates that although CTLs are readily induced by the transgene, the immune response against the virus is still capable of eradicating transduced cells (Yang et al., 1996b). Similar data were obtained when the virus was administered via the tail vein (Yang et al., 1996a).

A strong LacZ-specific response was observed after subcutaneous injection of tumor cells in syngeneic mice. Normally injection of these cells give rise to tumors, but when the LacZ transgene was inserted this was abrogated. Not only LacZ specific CLTs were found, but also CTLs against the cell line. Since the cells harboring LacZ were able to produce tumors in nude mice, the involvement of T cells is implicated (Abina et al., 1996).

Not only LacZ is capable of inducing an immune response. Immunocompetent mice receiving an intramuscular injection with identical replication-defective adenovirus, encoding either self (murine) or foreign (human) erythropoietin, showed markedly distinct reactions. Animals injected with the human erythropoietin displayed only transient elevations in hematocrits, followed by anemia, whereas animals injected with murine erythropoietin

displayed elevated hematocrits of approximately 80%, which were stable for at least 112 days. The main determinant of transgene loss was the appearance of CTLs against the transgene, but one has to keep in mind that in these vectors not only E1 was deleted, but also E3 (Tripathy et al., 1996).

There are also two studies on the immunogenecity of (HSV) thymidine kinase (TK). A fusion between hygromycin (hyg) and TK (HygTK) was expressed in renal cell carcinoma (RCC) cell lines. The cytotoxic T-cell responses obtained were not specific for RCC antigen, but for both the Hyg and TK genes. However, the CTL reactivity was predominantly directed against the TK epitope, whereas only a modest reactivity was observed against the Hyg epitope (Jung et al., 1998). Not only in vitro as in the last study was observed, but also in vivo there are CTLs against TK. This is even the first study to show that these immune responses are not restricted to rodents, but also occur in patients. In a clinical trial aiming at inducing a graft-versus-leukemia response, 8 of 24 treated patients developed a specific cytotoxic $CD8^+$ T cell-mediated immune response against donor T cells genetically engineered to express the TK gene. This led to the selective elimination of the modified cells (Thomis et al., 2001).

That non-human primates do also elicit an immune response against transgenes is evident from the study of Latta-Mahieu et al.. They used the tetracylcine-activated transcription factor commonly used in preclinical gene therapy, rtTA2-M2. In primates they injected intramuscular plasmid or adenoviral vectors encoding rtTA-M2 and looked at the cellular and humoral immune responses. They could show the presence of rtTA2-M2-sepcific interferon-γ (IFN-γ)-secreting cells, CTLs specific for rtTA2-M2 and specific anti-rtTA2-M2 antibodies. This all corresponded to a reduced expression and duration of the transgene that was transcribed under the control of rtTA2-M2 (Latta-Mahieu et al., 2002).

The last examples of proteins with known immunogenecity are the much-used visual reporter proteins, enhanced green and yellow fluorescent protein (eGFP and eYFP). These proteins are readily detectable using techniques of fluorescence microscopy, flow cytometry, or macroscopic imaging. In a first study it was shown that eGFP is readily detected by eGFP-specific CTLs after injection of adenovirus containing the eGFP gene into mice (Gambotto et al., 2000). A study by Morris et al. used lentiviral vectors encoding eGFP or eYFP. They transduced hematopoietic stem cells (HSCs) with these lentiviruses and observed complete disappearance of genetically modified eGFP/eYFP-expressing cells in 5 baboons that received the transplants. In 4 out of the 5 animals cytotoxic T cells specific for the transgenes were detected, demonstrating that immune reactions were responsible for the loss of transgene expression (Morris et al., 2004).

Since almost all of the studies above blame the specific cytotoxic T cells for the loss of the diverse transgenes it might be an option to circumvent CTL priming. Indeed, studies performed by Cordier et al. and Joos et al. highlight the effect of the vector on induction of CTLs and therefore expression of a transgene product. In mice with muscular dystrophy they had good results using a recombinant AAV (rAAV) vector expressing γ-sarcoglycan, a

subunit of the dystrophin-glycoprotein complex, which was missing in these mice. The expression of γ-sarcoglycan was driven by a muscle-specific promoter, a truncated version of muscle creatine kinase gene promoter. If the promoter was changed to ubiquitous cytomegalovirus (CMV) promoter lower levels of the trangene expression were observed and an immune respone to γ-sarcoglycan was apparent. This effect was also obtained when they changed γ-sarcoglycan in β-galactosidase (Cordier et al., 2001). Jooss et al. pinpointed this effect on dendritic cells (DCs) professional antigen presenting cells (APCs). Muscle fibers transduced with rAAV expressing LacZ elicited no CTL response or humoral immunity. When adenoviral vectors with LacZ were used there was a vibrant T-cell response to the transgene product that destroyed the targeted muscle fibers. Indeed these CTLs were also capable of eradicating rAAV-transduced muscle fibers. Moreover, adoptive transfer of DCs infected with AdLacZ lead to immune-mediated elimination of rAAV-LacZ transduced muscle fibers. AAV-LacZ transduced DCs failed to demonstrate β-galacosidase activity and were therefore also unable to elicit transgene immunity in adoptive transfer experiments (Jooss et al., 1998).

All in all there is ample evidence for transgene-induced immune responses in both rodents and (non-human) primates. The prime candidates for this target cell destruction are the MHC class-I restricted CTLs. Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class-I molecules. Given the important role of CTLs in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen-presentation. More on antigen presentation, viral immune evasion and our strategy to circumvent unwanted trangene-specific CTLs is given in the next part of the introduction (part 2.4).

Part 2.4 Antigen processing, DRiPs and viral immune evasion

2.4.1 Antigen processing

The adaptive immune system has evolved a remarkable array of cell surface molecules that are crucial mediators of cell-to-cell communication and thereby contribute to the organization of effective responses against invading pathogens and developing tumors. Antigen-presenting molecules, like MHC I and II, have a central role in the process of binding and presenting self-derived, pathogen-derived or tumor-associated antigen directly to T lymphocytes. The extracellular domains of these antigen-presenting proteins possess a high

degree of polymorphism that contributes to the diversity of peptides antigens that can be presented and subsequently recognized by T-cell receptors (TCR).

There are two distinct pathways operating to present peptides. The first involves the generation of T-cell epitopes through the processing and loading of exogenous derived antigens onto antigen-presenting molecules within the endocytic compartment of professional antigen presenting cells (APCs). This process has been recognized to constitute of the MHC II restricted antigen presentation to CD4+ T cells. The second constitutes of endogenous peptide presentation of self- and pathogenic proteins via the proteasomal cleavage and subsequently MHC I restricted antigen presentation to CD8+ T cells. More recently an alternative pathway, the so-called "cross-presentation" i.e. presentation of exogenous antigens by classical MHC I molecules, has been recognized for its importance especially in CD8+ T cell-mediated immunity against tumor, bacterial and viral antigens and tolerance to self-antigens. All these pathways will be discussed here in more detail.

Although MHC II molecules are nowadays also recognized to be capable of presented some endogenously synthesized antigen, they are primarily characterized as molecules that acquire and present exogenous antigens (Watts, 1997). There are specialized endolysosomal compartments in APCs, so-called MHC II compartments (MIICs), where the MHC II molecules acquire these antigens (Watts, 1997; Guagliardi et al., 1990; Peters et al., 1991). It is supposed that endocytic sorting motifs in the cytoplasmic tails of MHC II molecules have crucial roles to transport them to the MIICs (Bakke and Nordeng, 1999; Lizee et al., 2005). MIICs are thought to intersect with the endocytic pathway in APCs and contain internalized antigens derived from the extracellular environment (Amigorena et al., 1994; Hiltbold and Roche, 2002; Guermonprez et al., 2002). MHC II molecules are heterodimers, which consists of an α and a β chain. Each newly synthesized molecule binds to trimerized invariants chains (Ii) in the endoplasmatic reticulum (ER) lumen. Ii serves to prevent the binding of peptides derived from the secretory pathway and also mediates sorting from the trans-Golgi into MIICs of the endocytic pathway (Lotteau et al., 1990; Cresswell, 1996; Roche and Cresswell, 1990; Odorizzi et al., 1994). Once in this acidic environment of the MIIC, the Ii is degraded by proteolytic enzymes of the cathepsin family (Villadangos et al., 1999) to leave the class II-associated Ii peptide (CLIP) in the peptide-binding groove. The CLIP fragment is removed by the MHC-encoded HLA-DM molecule (Denzin and Cresswell, 1995). This is a non-classical MHC II molecule and promotes MHC II antigenic peptide binding by proteolytic removal of CLIP (Sherman et al., 1995; Alfonso and Karlsson, 2000). After antigenic binding the MHC II heterodimers are transported to the cell surface for presentation to CD4+ T cells. The heterodimers can recycle from the membrane through early endocytic compartments that are different from the MIICs, which are late endosomal compartments (Hiltbold and Roche, 2002; Harding et al., 1989). The pH is however low enough to facilitate peptide exchange, which can be a second, distinctly different, source of antigenic peptides to be presented on the cell membrane (Lindner and Unanue, 1996; Griffin et al., 1997).

MHC I molecules are classically known to present antigens that are synthesized intracellularly (Pamer and Cresswell, 1998). They consist of a membrane-integrated glycoprotein, the MHC heavy chain, a small soluble protein, β2 microglobulin (β2m) and present short peptides usually of 8-10 amino acids. There is a constitutive process in the cell, which cleaves cytosolic and nuclear proteins into peptides for loading on the MHC I. All proteins that are expressed in a cell are eligible for this processing, including viral or bacterial proteins synthesized endogenously. The most general entrance to the cytosolic protein degradation pathway (i.e. proteasomal degradation) of substrates involves the conjugation of ubiquitin on internal lysines (Ciechanover, 1994; Hochstrasser, 1996). The ubiquitinating mechanism involves three enzymatic activities, proteins called E1, E2 and E3. They work in a serial order activating ubiquitin first and then covalent linking of it to specific lysine residues in the target protein. Subsequently polyubiquitination can occur via conjugation of additional ubiquitin molecules to lysine residues in the ubiquitin itself (Hochstrasser, 1996). This polyubiquitin chain may serve two main purposes: one, to unfold the target proteins, and two, as recognition elements for cytosolic proteasome complexes. Not only old proteins have their fate in the proteasomal degradation machinery, but also so-called defective ribosomal products (DRiPs). The defective translation products of ribosomes, which are supposed to be error prone, are a rapid and important source of peptides for MHC I (Yewdell et al., 1996). More details on DRiPs will be given further on in this part.

The proteasome is a multi catalytic complex, which resides in the cytosol. It has a barrel-shaped catalytic core, the 20S proteasome, which consists of 4 heptameric rings. The two outer rings consist of 7 different but homologues α subunits (α1-α7) that provides the structure, control the access to the catalytic core and interact with regulatory factors (Groll et al., 1997). The two inner rings are each composed of 7 β subunits (β1-β7) of which three display catalytic activity (β1-d, β2-Z and β5-MB1). The central gate formed by the α subunits is normally closed by their N-termini and this keeps the proteasome in an inactive state (Groll et al., 1997; Groll et al., 2000). The 26S proteasome is formed through the ATP-dependent association of two 19S regulator complexes (Ferrell et al., 2000) with the two outer α-rings of the 20S core (Peters et al., 1994; Voges et al., 1999). The 19S base, which binds to the 20S core, is responsible for the ATP-dependent opening of the central gate and therefore the activation of the 20S core (Glickman and Ciechanover, 2002) as well as the unfolding of the protein substrates (Braun et al., 1999; Strickland et al., 2000). The 19S lid (the upper part) is thought to play a role in the recognition of poly-ubiquitinated proteins and is essentially required for their degradation.

The catalytic machinery of proteasomes is replaced under conditions of IFN-γ induction. There are three IFN-γ inducible proteasome subunits, βi1, βi2 and βi5, and these harbor active sites and replace the corresponding constitutive b-subunits upon de novo proteasome synthesis. In consequence, new 20S core complexes with altered proteolytic properties are generated. This altered proteasome system forms the immunoproteasome,

which has a connection with the cellular immune response (Aki et al., 1994; Hendil et al., 1998). This immunoproteasomes are constitutively expressed in cells with antigen presenting functions like cells in the thymus, spleen and lymphnodes (Macagno et al., 1999; Stohwasser et al., 1997; Eleuteri et al., 1997). The presence of the immuno-subunits enhances the presentation of a major subset of virus-derived antigen peptides (Schwarz et al., 2000; Sijts et al., 2000a; Sijts et al., 2000b; van Hall et al., 2000).

After peptide generation by the proteasome, the peptides are transported into the ER via the transporter associated with antigen processing (TAP). TAP is a heterodimer with two subunits, TAP.1 and TAP.2 (Monaco, 1992). Both TAP subunits have a N-terminal hydrophobic region with multiple transmembrane domains and a cytosolic C-terminal ATP-binding domain. In vitro studies have demonstrated the ability of TAP to translocate peptides across the ER membrane (Shepherd et al., 1993; Androlewicz et al., 1993; Neefjes et al., 1993). The binding site for peptides is comprised of regions of both TAP.1 and TAP.2 at the C-terminal end of the hydrophobic segment, adjacent to the cytosolic hydrophilic domain. Peptide binding to TAP is ATP-independent, while translocation is ATP-dependent (Neefjes et al., 1993; Androlewicz and Cresswell, 1994). There is a large range in peptide binding affinity depending on the peptide sequence and this may vary three orders of magnitude (Uebel et al., 1997). The current model of (nonameric) peptide binding to TAP suggests that the peptide backbone as well as side chain interactions at positions 1 to 3 and 9 are predominantly involved in this process (Schumacher et al., 1994; Uebel et al., 1997).

In the ER the transported peptides are mounted on the MHC I molecule. Proper folding of the MHC I molecules is a prerequisite for leaving the ER and shuttle to the membrane. The β2m subunit is obligate in this perspective and its absence leads to misfolding and degradation of the heavy chain and therefore no expression of MHC I molecules on the cell surface (Williams et al., 1989; Zijlstra et al., 1990). Also, absence of expression of either or both of the TAP genes leads to poor MHC I expression on the cell surface (Cerundolo et al., 1990; Salter and Cresswell, 1986). Since heavy chain-β2m dimers are formed, this suggests that bound peptide is an essential component of a class I molecule as well as β2m expression is. The native conformation of an MHC class I molecules requires therefore peptide association and β2m to satisfy ER quality control processes before shuttling to the membrane via the trans-Golgi network. Here they present the peptides to the CD8+ cells. After several hours they are transported to the lysosomes for degradation.

In most somatic cells this is the only pathway of MHC I presentation. In contrast, in APCs there is an alternative pathway (Yewdell et al., 1999; Heath and Carbone, 2001). APCs can present exogenously derived antigens in the context of MHC I molecules in a process called cross-presentation. Upon phagocytosis of exogenous proteins there is limited proteolysis and these degradation products are exported to the cytosol. Loading of exogenous antigens onto MHC I molecules occurs within a specialized endocytic (lysosomal-associated membrane protein-1 (LAMP-1) positive) compartments, rather than in the ER (Lizee et al.,

2003; Guermonprez et al., 2003; Houde et al., 2003; Ackerman et al., 2003). These compartments contain all components necessary for cross-presentation, like MHC I, β2m and TAP and even proteasomal complexes, which are associated with the cytoplasmic face of the compartments (Guermonprez et al., 2003; Houde et al., 2003; Ackerman et al., 2003). It is now known that this cross-presentation of exogenous antigens to CD8+ T cells leads to cross-priming, a process which is essential for establishing tolerance to self-antigens, as well as for generating optimal cell-mediated immune responses against numeral viral, bacterial and tumor antigens *in vivo* (Kurts et al., 1996; Sigal et al., 1999; Svensson and Wick, 1999; Huang et al., 1994; Lizee et al., 2003).

2.4.2 Defective Ribosomal Products (DRiPs)

The CD8+ T cells play an important role in the immune responses to many intracellular pathogens. Peptides of 8 to 11 residues are presented to the CD8+ T cells via the MHC class I. These peptides are derived from a diverse set of cellular and foreign proteins and no proteins are known to escape peptides generation by this mechanism (Yewdell and Bennink, 1992; Townsend and Bodmer, 1989).The vast majority of class I bound peptides derive from endogenously synthesized proteins, but also exogenous proteins, introduced either artificially or during bacterial or viral infection, can be processed for presentation by the MHC class I pathway (Yewdell and Bennink, 1992). Most of these small peptides are generated by the proteasome (Voges et al., 1999). The proteasome is the major protease used in eukaryotic cells to degrade damaged or misfolded proteins. After proteasomal cleavage the peptides are transported to the ER via the TAP transporter. Here they are loaded onto the newly synthesized MHC class I molecules and transported via vesicles to the cell membrane where they are presented to the CD8+ T cells.

The sources of proteasomal substrates are not clearly defined. In the initial model old proteins were degraded by the proteasome. A major problem to this theory is that virally infected cells can be recognized by CD8+ T cells in less then 60 minutes after viral penetration (Esquivel et al., 1992). Since it takes MHC class I molecules 10 to 15 minutes to reach the cell surface once they are loaded with peptides, it is hard to believe that there are already virus proteins that served there goal and are degraded “of old age”. The current model is that most of the peptides produced for MHC class I loading are derived from the so-called Defective Ribosomal Products (DRiPs). There is a wide variety of DRiPs identified. Not only truncated or misfolded proteins, but also the products that are translated from alternative open reading frames are considered as DRiPs (Yewdell et al., 2003; Yewdell et al., 1996). Indeed several proteins are produced by translation of alternative ORFs (Fetten et al., 1991; Malarkannan et al., 1995; Shastri et al., 1995; Wang et al., 1996; Mayrand and Green, 1998; Mayrand et al., 1998; Schwab et al., 2003) and from non-AUG start codons (Malarkannan et al., 1995; Shastri et al., 1995; Schwab et al., 2003). These DRiPs are shown

to play an important role in peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001).

DRiPs are also identified as a source for new tumor antigens. Here an increasing number of unexpected transcriptional or translational products have been found. Tumor-specific T cells were shown to recognize epitopes that were derived from intron sequences (Coulie et al., 1995; Guilloux et al., 1996; Lupetti et al., 1998; Robbins et al., 1997), from alternative ORF products (Ronsin et al., 1999; Probst-Kepper et al., 2001; Wang et al., 1996; Aarnoudse et al., 1999; Wang et al., 1998) or even from reverse strand transcription (Van Den Eynde et al., 1999).

DRiPs are nowadays more and more understood and there is ample evidence that especially in peptide generation and therefore CTL destruction of infected cells, they play a large role and are necessary.

2.4.3 Viral immune evasion

It should come to no surprise that many viruses have evolved strategies to counter proteasomal degradation, peptide generation, and MHC class I transport. The human cytomegalovirus (HCMV) is the most notorious virus in this perspective and encodes in its unique short region of the viral genome for at least five proteins (US2, US3, US6, US10 and US 11) that inhibit MHC class I pathway (Tortorella et al., 2000; Furman et al., 2002). There are numerous sites in the pathway where viruses can act and HCMV targets them all.

TAP represents an obvious target for viral inhibition, because the vast majority of peptides presented by MHC I molecules are generated in the cytosol and require translocation across the ER membrane. Both the herpes simplex virus (HSV) ICP47 and the HCMV US6 gene products exploit this bottleneck. ICP47 inhibits peptide binding to TAP, but does not affect ATP binding (Tomazin et al., 1996; Ahn et al., 1996b). ICP47 acts as a competitive inhibitor of peptide binding, because its affinity for TAP is 10-1000 fold greater than most peptides. It is not translocated across the ER membrane and it remains TAP associated, thereby blocking the TAP for other peptides. US6 is ER localized and acts totally opposite, in that it inhibits peptide translocation, but not peptide binding (Lehner et al., 1997; Hengel et al., 1997; Ahn et al., 1997). It prevents conformational rearrangements of TAP and ATP binding to TAP (Hewitt et al., 2001; Kyritsis et al., 2001). Since ATP binding is crucial, it starves the TAP from its energy and no peptides will enter the ER.

Another option is destruction or retention of MCH I molecules in the ER. HCMV US2 and US11 are expressed in the ER and expression of either of these proteins causes a rapid degradation of newly synthesized MHC I heavy chains (Wiertz et al., 1996b; Wiertz et al., 1996a). Both proteins can redirect the MHC I to the cytosol where it is degraded by the proteasome. Moreover, HCMV US3 retains MHC I in the ER by binding to it and therefore

sequesters them in the ER (Ahn et al., 1996a). The adenovirus E3-19K protein forces retention of the MHC I in the ER via an ER retrieval signal in its cytoplasmic tail (Paabo et al., 1989). Murine CMV (MCMV) gp40 also retains MHC I in the cell, but does so in the cis-Golgi compartment (Ziegler et al., 1997). A different way of destructing MHC I has the human herpesvirus 7 (HHV 7). Its U21 gene product binds to the MHC I in the ER and targets them subsequently to the lysosome, where they are both degraded (Hudson et al., 2001).

Arrival at the cell membrane does not protect MHC I from viral interference. The Kaposi's sarcoma-associated herpes virus (KSHV or HHV 8) encodes two proteins K3 and K5 and expression of either of them causes rapid down-regulation of MHC I from the plasma membrane by clathrin-dependent endocytosis (Ishido et al., 2000; Coscoy and Ganem, 2000). Once inside the cell the MHC I molecules are sorted into an acidic endocytic compartment where they are degraded by acidic proteases (Coscoy and Ganem, 2000; Lorenzo et al., 2002). Also human immunodeficiency virus 1 (HIV-1) Nef down-regulates MHC I on the cell surface. This Nef mediated internalization of MHC I is clathrin-independent and the class I molecules are sequestered in the trans-Golgi network (Schwartz et al., 1996; Le Gall et al., 2000; Greenberg et al., 1998). But already in the first step of antigen generation, proteolysis of proteins, two examples exist that display interference with this proteolysis. HCMV expresses a viral phosphoprotein, pp65, which inhibits the generation of HCMV specific T-cell epitopes (Gilbert et al., 1996).

More important for this thesis is the Epstein-Barr virus (EBV) encoded nuclear antigen I (EBNA 1) proteins which interferes with its proteasomal degradation. Epstein Barr Virus (EBV) is a gamma herpes virus and infects over 90 % of the human adult population. It is considered to be the classic example for immune surveillance of persistent viral infections in humans (Klein, 1994). It has three latency programs, which differ in protein expression profiles. However, the Epstein Barr virus nuclear antigen 1 (EBNA-1) is expressed in all latency programs and is therefore associated with all EBV-related malignancies.

EBNA-1 is essential for the maintenance of the viral genome as stable episomes during latency. It binds as a dimer to the viral origin of replication and ensures B-cell growth (Bochkarev et al., 1996; Shah et al., 1992; Yates et al., 1985; Rowe et al., 1992). Although EBNA-1-specific cytotoxic-T lymphocytes (CTLs) circulate in patients (Blake et al., 1997; Blake et al., 2000; Rickinson and Moss, 1997), the EBNA-1-positive cells are not recognized and killed. This has been attributed to the presence of the large glycine and alanine-rich repeat (GAr), which is not required for genome maintenance or cellular transformation (Lee et al., 1999), but has an immune evasion function. The GAr domain of EBNA-1 prevents cytotoxic T-lymphocyte (CTL)- epitope generation (Ossevoort et al., 2003) by inhibiting the proteasomal degradation (Levitskaya et al., 1997; Levitskaya et al., 1995). The inhibition requires the interaction of at least three alanine residues of the GAr with adjacent hydrophobic binding pockets of a putative receptor at the proteasome (Sharipo et al., 2001). Although efficient, the GAr may not be sufficient to prevent the generation and presentation of

antigenic peptides altogether (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). Especially, the GAr may not inhibit the synthesis of defective ribosomal products (DRiPs) that may generate antigen-specific antigenic peptides.

We used this GAr in our studies to create 'stealthed' transgenes for use in gene-therapy applications. The choice for the GAr domain of EBNA-1 was based on the small impact of the strategy to other endogenous expressed proteins. The strategies described in this part that decrease the MHC I content on the cell membrane have a major limitation. Cells deficient in self-MHC I products are recognized by natural killer (NK) cells and killed by these. Since the GAr acts purely *in-cis*, this should be no problem for our approach.

Chapter 1

Introduction

Part 3

References

Part 3

References

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Chapter 2

Creation of immune ‘stealth’ genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1

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RESEARCH ARTICLE

Creation of immune ‘stealth’ genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1

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A major obstacle in gene-therapy protocols is T-cell-mediated destruction of transgene-expressing cells. Therefore new approaches are needed to prevent rapid clearance of transduced cells. We exploited the Gly-Ala repeat (GAr) domain of the Epstein–Barr virus nuclear antigen-1, since the GAr prevents cytotoxic T-lymphocyte-epitope generation. Here we show that three different enzymes (viz. the E. coli LacZ gene encoded b-galactosidase, firefly luciferase, and HSV1 thymidine kinase) fused with the GAr retained their function. Moreover, linking GAr with b-galactosidase successfully prevented recognition of GAr-LacZ-expressing cells by b-galactosidase-specific CTL. Nonetheless, vaccination with a GAr-LacZ adenovirus or with an allogeneic cell line expressing GAr-LacZ resulted in the induction of b-gal-specific CTL. This demonstrates that the GAr domain does not inhibit crosspresentation of antigens, but only affects breakdown of endogenously synthesized proteins. These data demonstrate how the GAr domain can be exploited to create immuno‘stealth’ genes by hiding transgene products from CTL-mediated immune attack.

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Introduction

The transfer of genes holds promise as a therapeutic approach for the treatment of a wide variety of diseases. One of the limitations imposed onto gene therapy is the immune response directed against vector and/or trans-gene product.[1–4] While beneficial for the development of recombinant vaccines against infectious agents[5,6] and tumor cells,[7,8] it significantly impedes the development of those gene-therapy approaches where persistent expression of the transgenes encoding neoantigens is required. Long-term humoral and cellular immunity against several viral-vector systems prevails in a large part of the population, or may be induced upon the first vector administration. This may frustrate (re)administration of the vector and lead to elimination of the transduced cells. Developments in the viral and nonviral technology greatly improved the efficiency of the gene-transfer vectors. Retroviral, lentiviral, adenoviral, and adeno-associated viral vectors are available from which all virus-derived protein-coding genes have been re-moved.[9] This eliminated, albeit not completely,[10] the problem associated with the cellular-immune response against the vector-derived antigens.

However, a cellular immune response against neoantigens encoded by the transgene may still be induced. This problem has been most prominently described following adenovirus-mediated transfer of the E.coli-derived LacZ gene, encoding b-galactosidase. Strong immune responses against β-galactosidase have been observed in rodents following adenovirus-mediated gene transfer into liver, muscle, lung, and brain, leading to local inflammation, destruction of the transduced cells, and loss of transgene expression.[4,11–13] Antigen-specific major histocompatibility complex (MHC) class-I restricted cytotoxic T lymphocytes (CTL) are the prime suspects responsible for target cell destruction.[13–16] This requires prior activation of $CD4^+$ T cells.[1,16–23] Also in primates a CTL response directed against the transgene product has been shown to occur.[24,25] In a clinical trial aiming at inducing a graft versus leukemia response, eight of 24 treated patients developed a specific cytotoxic $CD8^+$ T-cell-mediated immune response against the cells genetically engineered to express the Herpes Simplex Virus 1 (HSV1) thymidine-kinase (TK) gene. This led to the selective elimination of the modified cells.[25]

Previous attempts to reduce the T-cell responses against the neoantigens during gene therapy focused on blocking the MHC class I-and class II-restricted T-cell responses, or the prevention of costimulation of T cells.[14,18–20,26–28] However, these approaches were either not fully effective or resulted in a general immunosuppression. The ideal strategy would selectively prevent the presentation by MHC class I of the transgene-derived peptides.

Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class I molecules. Given the important role of CTL in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen presentation.[29,30] One such example comes from the Epstein–Barr Virus (EBV) nuclear-antigen 1 (EBNA-1). EBNA-1 is expressed in latent EBV-infected cells. Here it is indispensable for the virus as it is required for the maintenance of the viral episomes. Although EBNA-1-specific CTL have been described in infectious mononucleosis patients and healthy carriers, they cannot recognize EBV-infected cells.[31–36] The failure to recognize endogenously expressed EBNA-1 has been attributed to the glycine-alanine repeat (GAr) domain in the EBNA-1 sequence that protects EBNA-1 from proteasomal degradation and subsequent presentation in the context of MHC class I.[37,38] This successful immune-evasion strategy points to the unique opportunity to hide cells expressing transgenes from CTL-mediated target-cell destruction by incorporation of the GAr sequence into the transgene.

Here, we show that inclusion of the full EBNA-1-derived GAr domain does not inhibit enzyme function. A recombinant adenovirus expressing the GAr-containing LacZ gene as its transgene was able to deliver a functional enzyme after infection in mice invivo, resulting in the induction of a strong β-galactosidase-specific CTL response in the recipient mice. However, these murine antigen-specific CTL did not recognize cells expressing the GAr-transgene fusion, demonstrating that the EBNA-1-derived GAr can be exploited to create 'stealth' transgenes by hiding the transgene-expressing cells from CTL-mediated immune-attack.

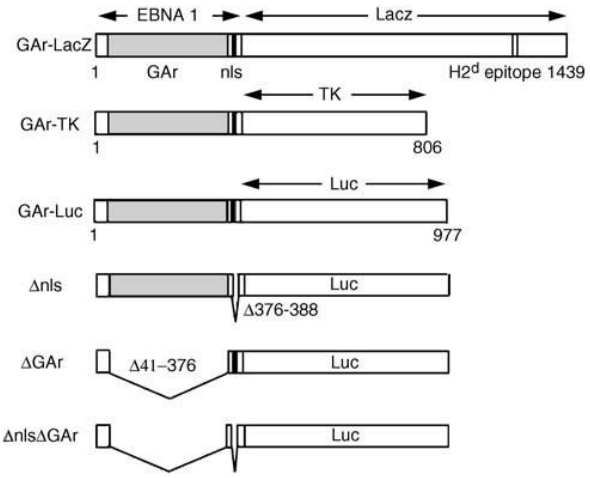

Figure1
Schematic outline of the chimaeric Gly-Ala repeat constructs. Indicated are the Gly-Ala repeat unit (GAr; aminoacids 90–328) and the nuclear localization signal (nls, aa 378–386). The open reading frames of

E.coli β-galactosidase, HSV1-TK, and firefly luciferase start at aa 427. In the C-terminal part of LacZ, the H-2^{d} CTL epitope is indicated (aa 1303–1311). Also indicated are the deletions of the GAr region and the nls that were made in luciferase.

Results

GAr does not inhibit protein function

To study whether the GAr domain can be exploited to enhance the persistence of gene expression by minimizing CTL-mediated recognition of transgene-expressing cells, we first tested whether enzymes retained their function if fused with the full-length GAr domain. To this end, we constructed plasmids encoding GAr-β-galactosidase (pGAr-LacZ), GAr-HSV-thymidine kinase (pGAr-TK), and GAr-luciferase (pLXRN-GAr-Luc; Figure 1). Transfection experiments revealed that the functional properties of the enzymes were not affected as evidenced from β-galactosidase activity in Hep2 cells (Figure 2a), [^{3}H]thymidine incorporation and ganciclovir sensitivity in Rat2 cells (Figure 2b), and luciferase activity in 911 cells (Figure 2c), respectively. Thus, these data indicate that the GAr does not inhibit the function of proteins harboring the full-length GAr. A slight (three-fold) reduction in luciferase activity can be attributed to the presence of the GAr in the transcription unit, and a mere two-fold reduction to the nls. However, functionally the vectors are equivalent to the unmodified predecessors.

Delivery of GAr-LacZ by a recombinant adenovirus

As recombinant viruses most efficiently facilitate gene transfer, we generated a recombinant adenovirus (rAd5) with the GAr-LacZ gene (rAd5-GAr-LacZ) to test the stability of the repeats in the adenovirus backbone. Whereas manipulation of plasmids with

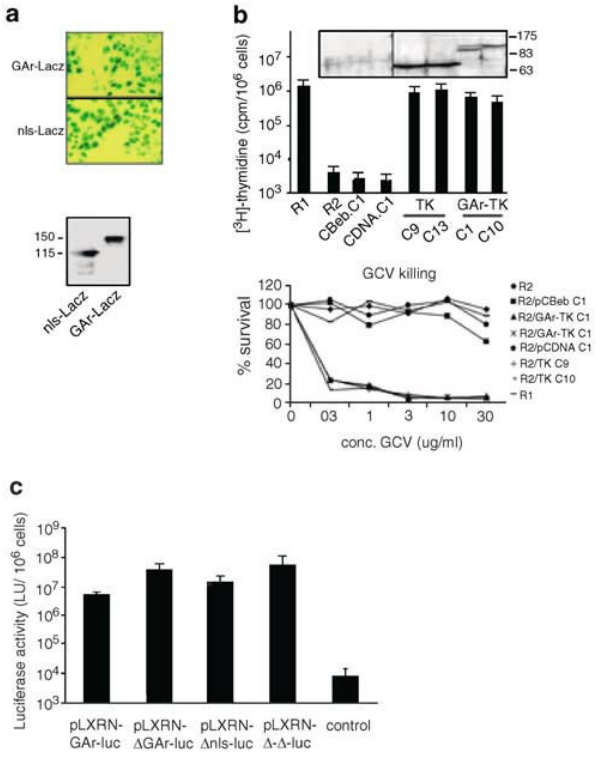

Figure 2

GAr fusion enzymes retain their activity. (a) Activity of GAr-β-galactosidase. Hep2 cells were infected with rAd5-nls-LacZ or rAd5-GAr-LacZ (4 PFU/cell). At 48h postinfection, the cells were fixed and stained for β-galactosidase activity. In addition, H_{1299} cells grown in 10-cm dishes were infected with rAd5-nlsLacZ or rAd5-GArLacZ with 10 PFU/cell. After 48h, protein extracts were made and size-fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed with an anti-LacZ mouse monoclonal antibody (Roche, Basel, Switzerland). (b) Activity of GAr-TK. The TK-deficient cell line Rat2 (R2, TK^-) was used to generate stable cell lines containing the various plasmids. CBeb.C1 and CDNA.C1 are G418-resistant Rat2 cells obtained after transfection of the empty pCBeb and pCDNA3.1 plasmids, respectively. GArTK.C1 and C10 are independent G418-resistant clones of Rat2 cells stably expressing the pGAr-TK plasmid. TK.C9 and C13 are two independent G418-resistant Rat2 clones derived by transfection of the plasmid pCDNA-TK. Rat1 (R1) is the TK^+ parental cell line from which Rat2 had been derived. [^{3}H] thymidine incorporation (± s.d.) is represented per 10^6 cells. The inset depicts a Western analysis of the Rat2 clones with a polyclonal goat anti-HSV-TK antibody. The faster-migrating band in clone GAr-TK.C1 may result from splicing within the TK coding region.[59] The same cell lines were analysed for their ganciclovir sensitivity by growing these cells for 48h in the presence of varying concentrations of ganciclovir. Cell viability was determined with the WST-I colorimetric assay. (c) Activity of GAr-luciferase. Cultures of 911 cells were transfected with pCBeb (as a negative control), pLXRN-GAr-luc and the GAr- and nls-deletion derivatives. Cells were lysed 18h post-transfection and the luciferase activity was measured in the lysates. The mean of three experiments is shown, expressed as light units/10^6 cells ± 1 s.d.

the full-length GAr proved difficult as the repeats lead to frequent internal deletions in the GAr domain (DJMvdW and MO, unpublished observations), rAd5 vectors carrying the GAr-LacZ fusion gene could be generated and be propagated with titers similar to those of vectors lacking the GAr domain. Western analysis of infected cells detected β-galactosidase at the expected molecular weights of 150 kDa for GAr-LacZ and 115 kDa for nls-LacZ (Figure 2a). This confirms that the entire GAr-β-galactosidase fusion protein was synthesized. Infection of

Hep2 cells with rAd5-nls-LacZ as well as rAd5-GAr-LacZ with 4 plaque-forming units (PFU)/cell yielded β-galactosidase activity in approximately 50% of the cells 2 days after infection (Figure 2a). Similarly, intracellular staining revealed that the amount of β-galactosidase present after infection of Hep2 cells with rAd5-GAr-LacZ with 10 PFU/cell was similar to the amount observed after infection with rAd5-nls-LacZ (data not shown). Thus, rAds harboring the GAr repeats can be stably propagated and used to deliver efficiently functional GAr fusion genes to cells.

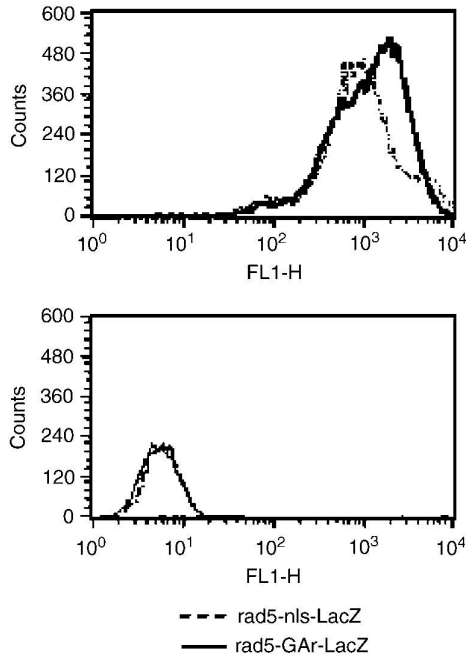

Figure 3

Delivery by rAd leads to the expression of the entire GAr-LacZ with the same efficiency as delivery of nls-LacZ. To determine the efficiency of gene delivery, MEC were infected with 10 PFU/cell of rAd5-GAr-LacZ and rAd5-nls-LacZ, respectively. At 2 days postinfection, the β-galactosidase activities were determined by intracellular FACS staining with the fluoreporter LacZ flowcytometry kit (Molecular Probes Inc., Eugene, OR, USA). The dotted line represents the signal in rAd5-nls-LacZ-infected cells, the solid line depicts the signal in rAd5-GAr-LacZ-infected cells. In the lower panel, the negative controls are shown for both cell populations.

GAr inhibits recognition of LacZ-expressing cells by CTL

As recognition of transgene expressing cells by CTL is an important limitation for prolonged transgene expression invivo, we investigated whether target cells infected with rAd5-GAr-LacZ could present the H-2L^d-restricted CTLepitope Lac876–884 (TPHPARIGL)[39] to LacZ-specific CTL. Therefore, BALB/c mouse embryo cells (MEC) were infected with either rAd5-nls-LacZ or rAd5-GAr-LacZ. After 2 days, when both infected MEC populations expressed similar transgene levels as determined by intracellular FACS staining (Figure 3), as well as by determination of β-galactosidase activity (data not shown),

the cells were used as stimulators for LacZspecific CTL. LacZ-expressing MEC but not GAr-LacZ-expressing MEC were efficiently recognized by LacZ-specific CTL as determined by an interferon-γ production assay (Figure 4). The GAr-LacZ-expressing and LacZ-expressing MEC did not differ in MHC expression and the GAr-LacZ-expressing MEC were efficiently recognized by LacZ-specific CTL after loading with $Lac_{876–884}$ peptides (data not shown). These findings indicate that, despite similar transgene expression, the GAr inhibits the generation of CTL epitopes derived from LacZ.

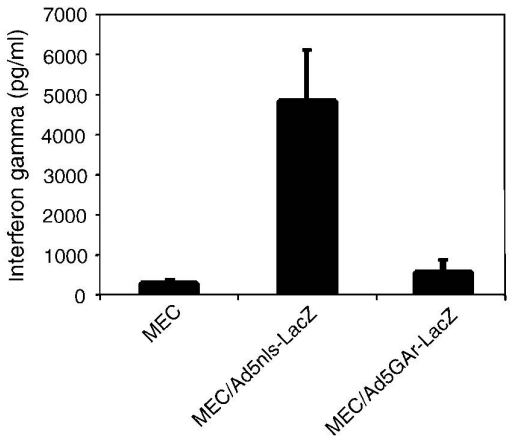

Figure 4
GAr prevents the presentation of the LacZ-encoded CTL epitope TPHPARIGL to LacZ-specific CTL. Xc9 MEC were mock-infected, infected with 5 PFU/cell rAd5-nls-LacZ or rAd5-GAr-LacZ. After 2 days, at the time when 10 000 LacZ-specific CTL were added to 15 000 infected cells (E:T ratio 1:1.5), the LacZ expression of MEC infected with rAd5-nls-LacZ or rAd5-GAr-LacZ was similar to LacZ expression as determined by intracellular FACS with a fluoreporter LacZ flowcytometry kit (Figure3). After 24h CTL activation was determined by measuring the IFN-γ produced by LacZ-specific CTL in the supernatant. Xc9 MEC infected with rAd5-nls-LacZ but not with rAd5-GAr-LacZ are efficiently recognized by β-galactosidase-specific CTL.

Induction of β-galactosidase specific CTL

The above studies show that GAr-LacZ-expressing target cells are poorly recognized by β-galactosidase-specific CTL. To study whether the GAr fused with β-galactosidase would also inhibit CTL induction *in vivo*, we immunized mice with rAd5-GAr-LacZ as well as with rAd5-nls-LacZ viruses (10^8 PFU/mouse), as controls. At 21 days postinjection, splenocytes were harvested and analyzed for the presence of β-galactosidase-specific CTL. Mice vaccinated with either rAd5-nls-LacZ and rAd5-GAr-LacZ viruses mounted a strong β-galactosidase-specific CTL response (Figure 5). Together, these data indicate that the GAr does inhibit CTL recognition but not CTL induction.

Since presentation of β-galactosidase-derived CTL epitopes by GAr-LacZ-expressing target cells is strongly inhibited by the action of the GAr, it is conceivable that CTL directed against GAr-containing proteins are primed via an indirect pathway, which is not hampered

by the activity of the GAr. To study the mechanism responsible for priming of CTL directed against GAr-containing antigens, we set out to vaccinate BALB/c mice (H-2^d) with GAr-LacZ-expressing C57BL/6 (H-2^b) cells unable to present the LacZ epitope to β-galactosidase-specific CTL directly. MEC from C57BL/6 mice were infected with 1 PFU/cell rAd5-GAr-LacZ. After 2 days, the cells were thoroughly washed, irradiated,[40] and used for vaccination of the completely allogeneic BALB/c mice (H-2^d). As the allogeneic H-2^b MHC expressed by the immunizing MEC cannot prime H-2^d-restricted host CTLs directly, generation of β-galactosidase-specific CTL requires crosspriming, that is, the uptake and H-2^d-restricted representation of antigen by host antigen-presenting cells (APC). Vaccination of BALB/c mice with the completely allogeneic vaccine resulted in the induction of a β-galactosidase-specific CTL response, indicating that GAr-LacZ crossprimes CTL (Figure 6). To exclude the possibility that BALB/c anti-C57BL/6 allospecific CTL induced by immunization of BALB/c mice with C57BL/6 cells crossreacted on the β-galactosidase epitope, we vaccinated BALB/c mice with uninfected C57BL/6 cells. These mice, however, did not generate β-galactosidase-specific immunity (Figure 6).

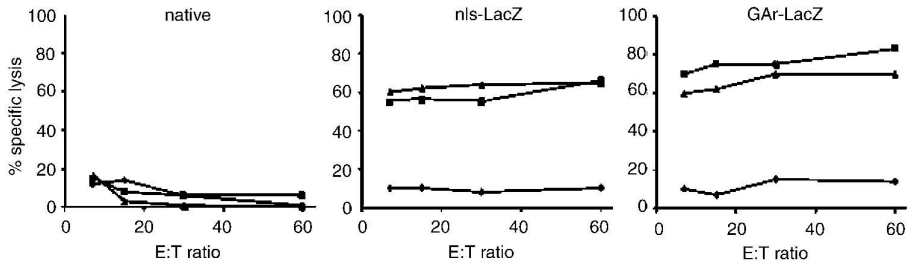

Figure 5

Vaccination with rAd5-GAr-LacZ leads to induction of a strong β-galactosidase-specific CTL response. BALB/c mice were naive, were immunized with 10^8 PFU rAd5-nls-LacZ or with 10^8 PFU rAd5-GAr-LacZ by intraperitoneal administration. After 3 weeks, β-galactosidase-specific CTL activity was analysed in a cytotoxicity assay on BALB/c MEC (H-2^d; control, ♦), LacZ-peptide-loaded BALB/c MEC (▲), and LacZ-transfected BALB/c MEC (■). The rAd5-GAr-LacZ-immunized animals have mounted a β-galactosidase-specific CTL response similar to rAd5-nls-LacZ vaccinated animals.

We were unable to isolate detectable amounts of rAd5-GAr-LacZ virus from the C57BL/6 MEC 2 days postinfection (<0.01 PFU/cell) by washing or freeze– thawing the vaccine cells. Hence the maximum amount of free viruses administered with the vaccine (<400 PFU) is well below the minimal amount of viruses required to induce an immune response (>10^6 PFU/ mouse; data not shown). Thus the induction of the immune response cannot be attributed to carry-over of free rAd5-GAr-LacZ vector viruses with the vaccine. Taken together, these data indicate that GAr-β-galactosidase crossprimes CTL and explain

why LacZ-specific CTL induction is not blocked, whereas recognition of GAr-LacZ-expressing cells is inhibited by the action of the GAr.

Prolonged LacZ expression in vivo by delivery of GAr-containing LacZ

We have demonstrated that while the GAr domain protects antigens from destruction by antigen-specific CTL *in vitro*, it does not inhibit the induction of antigen-specific CTL *in vivo*. To assess whether insertion of the GAr will result in prolonged transgene expression *in vivo*, we injected either rAd5-GAr-LacZ or rAd5-nls-LacZ into the gastrocnemius muscle of two groups of BALB/c mice (H-2^d). At days 8 and 19 postinjection, the muscles were analyzed for β-galactosidase activity as a read-out for transgene expression. At day 19, β-galactosidase-positive cells are present in the muscle transduced with rAd5-GAr-LacZ. In contrast, the β-galactosidase-positive cells were eradicated in the rAd5-nls-LacZ-injected muscle, demonstrating that the GAr prolongs transgene expression *in vivo*(Table 1). Similar results were obtained after injection of rAd5-GAr-LacZ in the gastrocnemius muscle of the right leg and rAd5-nls-LacZ left leg of the same mouse (Table 2), excluding a bias due to difference in immunity of the GAr-LacZ and nls-lacZ proteins. Thus, the GAr-β-galactosidase persists longer than the nls-LacZ-derived control protein.

Table 1
Intramuscular β-galactosidase expression at various times after vector administration

Day	rAd5-GAr-LacZ	rAd5-nls-LacZ	rAd5-nls-LacZ (nu/nu)
8	4(4)	4(4)	1(1)
19	4(4)	1(4)	1(1)

The vectors rAd5-GAr-LacZ or rAd5-nls-LacZ were injected (10^8 PFU/injection) into the left gastrocnemius muscle of two groups of BALB/c mice (H-2^d) and as controls into BALB/c *nu/nu* mice. At days 8 and 19, the muscles were taken out and sections were stained for β-galactosidase activity, as a read-out for transgene expression. No β-galactosidase expression was discernable in the contralateral muscles. The values represent the number of mice showing β-galactosidase activity at the site of injection and the total number of mice tested (in parenthesis).

Table 2
Intra-animal comparison of intramuscular β-galactosidase expression after adenovirus-mediated transfer of nls-LacZ and GAr-LacZ

Day	rAd5-GAr-LacZ	rAd5-nls-LacZ	rAd5-nls-LacZ (nu/nu)
8	3(3)	2(3)	1(1)
19	3(3)	0(3)	1(1)

The vectors rAd5-GAr-LacZ and rAd5-nls-LacZ were injected (10^8 PFU/injection) into the left and right gastrocnemius muscle of BALB/c mice (H-2^d), respectively. BALB/c *nu/nu* mice served as controls. At days 8 and 19, the muscles were taken out and sections were stained for β-galactosidase activity. The values represent the number of mice showing β-galactosidase activity at the site of injection and, the total number of mice tested (in parentheses).

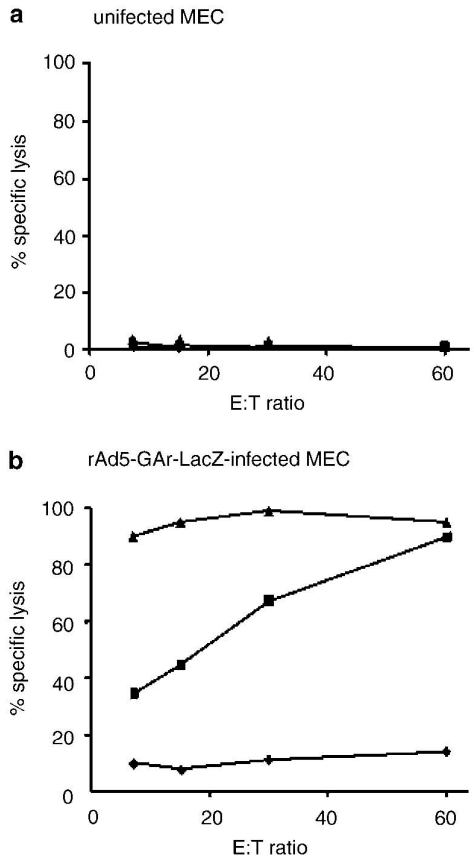

Figure 6
Vaccination of BALB/c mice with rAd5-GAr-LacZ-infected allogeneic cells induces LacZ-specific CTL. BALB/c mice were immunized with uninfected (a) or 4×10^4 rAd5-GAr-LacZ-infected MEC derived from a C57BL/6 mouse (b). After 3 weeks, LacZ-specific CTL activity was analyzed. The β-galactosidase-specific CTL activity was analysed in a cytotoxicity assay on BALB/c MEC ($H\text{-}2^d$; control, ♦), LacZ-peptide-loaded BALB/c MEC (▲), and LacZ-transfected BALB/c MEC cells (■). Mice vaccinated with infected C57BL/6 MEC, but not immunized with uninfected MEC, have mounted a strong β-galactosidase-specific CTL response, indicating that the GAr does not inhibit crosspriming of CTL.

Discussion

In this study, we evaluated the use the EBNA1-derived GAr element to prevent presentation of transgene-derived *neo*antigens. We provide three examples where fusion of the GAr domain did not impede the function of three popular reporter proteins. We also show that the GAr element does not affect induction of transgene-directed CTL activity upon adenovirus-mediated transfer and expression of a GAr-LacZ fusion gene. However, recognition of transgene-expressing cells by antigen-specific CTL is abolished sufficiently to prevent cytolysis by β-galactosidase-specific CTL.

The mode of action employed by the GAr domain to inhibit antigen processing is not fully understood. Studies by Levitskaya *et al*[37] showed that the GAr domain of EBNA-1 acts as a cis-inhibitory activity of MHC class I-restricted presentation. The GAr domain inhibited the ubiquitin/proteasome-dependent proteolysis pathway by interference with protein folding or otherwise inhibiting the capacity of the target protein to bind components of the ubiquitin-dependent proteasome pathway.[38] Recent studies demonstrate that small synthetic GAr-derived peptides do not inhibit polyubiquitination, but rather inhibit stable association of the protein with proteasomes.[41] However, the effect of these peptides on antigen presentation was not determined.[42,43] Subsequent studies have shown that cotranslational ubiquitination of EBNA-1 can override the GAr-mediated inhibition of proteasomal degradation and restore the endogenous processing and presentation of MHC class I-restricted CTL epitopes.[44] This suggests that the GAr domain not only prevents proteasomal degradation but also ubiquitination of GAr domain-containing proteins. Alternatively, experiments with p53 GAr fusion proteins suggest that the GAr domain acts on events between ubiquitination and proteasomal degradation.[45] The observation that the GAr does not inhibit priming of CTL is important and consistent with reports describing the presence of EBNA-1-specific CTL in EBV-seropositive individuals.[31–36] As antigen presentation by professional APC, most likely DC, is crucial to the initiation of virus-specific CTL responses, the presence of EBNA-1-specific CTL in EBV-positive donors suggests that antigen processing for MHC class I by specialized APC is not hampered by the GAr. In case EBV-specific CTL are induced through the direct route following EBV infection of DC, these observations would indicate that antigen presentation in DC differs intrinsically from presentation by 'nonprofessional' APC. Alternatively, EBV-specific CTL could have been primed in an indirect manner (crosspriming), as this is likely representing the dominant way by which CTL responses are induced *in vivo*.[40,46,47]

In case EBV-specific CTL are indeed primed in an indirect manner following uptake of viral antigens derived from EBV-infected cells, the presence of EBNA-1-specific CTL points to the possibility that the GAr does not affect the processing of exogenously acquired EBNA-1-antigens.[34–36] The GAr domain does not inhibit CTL priming via the indirect pathway, as vaccination with completely allogeneic tumor cells expressing GAr-LacZ resulted in a strong LacZ-specific CTL response. As these completely allogeneic tumor cells lack the proper MHC class I restriction element, generation of Lac-Z-specific CTL must involve uptake and H-2^d-restricted re-pre-sentation of antigen by host APC. Although not the subject of this study, it is tempting to speculate that the (GAr-containing) antigens taken up by DC are initially processed in endosomal/lysosomal compartments resulting in the liberation of the GAr from the antigenic CTL epitope. In this way, the processing of the CTL epitope is separated from the inhibitory influence of the GAr on proteasomal antigen degradation and

would explain the observation that CTL priming proceeds in an uninhibited fashion, while recognition of GAr-LacZ-expressing target cells is severely inhibited.

The immune response against *neo*antigens encoded by the vector or by the transgene represents a major limitation for the successful clinical application of gene therapy for the treatment of chronic diseases where long-term transgene expression is desired. Currently, vector systems are being developed from which all vector-derived protein-coding regions have been removed. Nonetheless, the immune response against the transgene product itself is not circumvented by these approaches, limiting the persistence of transduced cells. As CTL-mediated immune attack is the most important effector mechanism responsible for the destruction of transgene-expressing cells, we sought to determine whether transgene-expressing cells could be rescued by the insertion of the GAr in a transgene of interest. Indeed, prolonged transgene expression was detected in mice treated with rAd5-GAr-LacZ, consistent with the prominent role of the cellular immunity to transgene-encoded products on the persistence of transgene expression.[3] Nonetheless, we did not achieve persistent transgene expression *in vivo*, which is likely to be the result of the use of the first-generation E1-deleted adenoviral vectors. These vectors are notorious for their immunogenicity due to the leaky expression of viral genes residing in the vector.[3,48]

The GAr provides a powerful and specific tool to inhibit the presentation of transgene-derived antigens to $CD8^+$ CTL. Provided that the GAr is also functional in human cells, it may be exploited in gene-therapy applications involving expression of new antigenic proteins, for example, in hereditary protein deficiencies, in enzyme/prodrug or 'suicide' strategies that employ bacterial or viral enzymes, and in hiding bacterial transcription-regulating proteins, that is, those used in the tetracycline-regulated gene-expression systems. Since the GAr specifically targets the proteasome pathway and therefore the destruction of transgene-expressing cells via $CD8^+$ CTL, it is questionable if the system works as well for secreted and proteolytically activated proteins like blood-clotting factors FVIII and FIX. Although this study did not look into this, it is unlikely that the GAr system will be effective in such application. Whereas the longevity of the cells making the secreted proteins may increased, fragments of the secreted proteins may still be processed and presented despite the presence of the GAr.

The GAr approach may eliminate the risks that are associated with other immune suppressive approaches that have been described. Many of these are not antigen specific and aim at a more general immunosuppression, which enhances the risk of opportunistic infections that cannot be counteracted by the immune system. In contrast, the GAr provides an antigen-specific approach to temper CTL-mediated immune destruction, as is also evident from the effectiveness by which EBV exploits the GAr to prevent CTL-mediated destruction of EBNA1-expressing B cells in humans.

Materials and methods

Construction of the GAr fusion constructs

The LacZ gene carrying the EBNA-1 Gly-Ala repeats was constructed by inserting the LacZ gene into plasmid pCMV-EBNA (Invitrogen, Groningen, The Netherlands) (Figure 1). Firstly, plasmid pCBeb was created from plasmid pCMV-EBNA by converting the BstXI site to a *Bgl*II site by insertion of a *Bgl*II adapter that was created by annealing the synthetic oligonucleotides 5'-TACGAGATCTGAAG-3'and 5'-AGATCTCGTACTTC-3' (the *Bgl*II site is underlined). Secondly, a 3072 bp *Bam*HI fragment of the retroviral vector pBag,[49] which carries the LacZ-coding region except the first two codons, is inserted into the *Bgl*II site of pCBeb to generate pGAr-LacZ.

Similarly, the plasmids pGAr-TK and pGAr-Luc were created. For the first, a PCR was performed on the wtHSV1 (strain17) DNA to obtain the TK gene. Primers TK-for (5'-CAGGATCCTGACCATGGCTTCGTACCCCT GCCATC-3') and TK-rev (5'-GTGGATCCTGATCAGTTAGCCTCCCCCATCTCCCG-3') containing extensions with *Bam*HI restriction sites (underlined) were used and subsequently the TK gene was cloned into the pCBeb plasmid. For the latter, another PCR was performed on pCMVluc, a plasmid containing the luciferase gene. Primers Cl.luc-for (5'-GCAGATCTCCATGGAAGACGCCAAAAACAT-3') and Cl.luc-rev (5'-GCAGATCTC GAGCTAGCTCAATTTGGACTTTCCGCC-3') containing *Bgl*II sites (underlined) were used and the fragment cloned into pCBeb. The modified GAr-luc gene was inserted into vector pLXRN, in which the Mo-MuLV LTR promoter drives expression of the luc gene. The nls and GAr region were deleted by PCR mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer 's protocols. In all plasmids, the presence of the sequences coding for the GAr was verified by restriction analyses and DNA sequencing, as these repeats negatively affected the replication of the plasmid DNA and, as a result, had a tendency to be lost during propagation. The control plasmid pCDNA-TK contains the unmodified HSV-TK cDNA.

Adenoviral vector construction

The adenoviral vector pAd-GAr-LacZ was constructed following the procedures described.[50] The CMV-EBNA-LacZ cassette was excised from pGAr-LacZ as a 5125 bp *Bam*HI–*Hin*dIII fragment and inserted into plasmid pShuttle-CMV,[50] digested with *Bgl*II and *Hin*dIII, to create pSEbLacZ. The adenoviral vector plasmid pAd5-GAr-LacZ was made by homologous recombination between pSEbLacZ and pAdEasy1[50] in *E.coli* BJ5183. To generate recombinant viruses, *Pac*I-digested pAd5-GAr-LacZ was transfected into 911 cells.[51] In all experiments, the vector rAd5-nls-LacZ served as a control. This vector contains the LacZ codons fused with the SV40 large T-derived nuclear localization signal (nls). Amplification, propagation, and screening for replication-competent adenovirus were performed as described.[52,53]

Plaque assays were performed essentially as described.[51] Briefly, adenovirus stocks were serially diluted in 1 ml DMEM/2% HS and added to near-confluent 911 cells in six-well plates. After 2 h of incubation at 37°C/5% CO2, the medium was replaced by agar-containing culture medium. All batches were checked for integrity of the transgene by PCR and Southern analysis.

Western analyses

H_{1299} cells were infected with rAd5-nlsLacZ or rAd5GArLacZ with 10 PFU/cell. After 48 h, protein extracts were made and size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes, and probed with an anti-LacZ mouse monoclonal antibody (Roche, Basel, Switzerland). After incubation with a peroxidase-conjugated goat-anti-mouse secondary antibody, the protein was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Similarly, Rat2 clones were analyzed for the presence of HSV-TK. Protein extracts were made and 50 mg was size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes, and probed with a rabbit anti-HSV-TK antiserum (1/250 diluted), kindly provided by Dr M Janicot (Aventis-Gencell, Vitry-sur-Seine, France) and visualized with a peroxidase-conjugated goat-anti-rabbit secondary antibody.

Enzymatic assays

Luciferase and β-galactosidase activity assays were performed as described.[54,55] The [^{3}H]thymidine incorporation assays and the ganciclovir sensitivity assays to detect HSV-TK activity were performed in Rat2 cells as described.[56] Cell viability was assessed with the WST-1 colorimetric assay (Roche diagnostics, Almere, The Netherlands) according to the manufacturer 's description.

Generation and analysis of CTL bulk cultures

BALB/c mice were vaccinated by intraperitoneal injection as described in the legends to the figures. After 3 weeks, β-galactosidase-specific CTL were generated as follows: 5×10^{6} spleen cells per well were cocultured for 6 days with 10% irradiated BALB/c-derived stimulator cells expressing the LacZ gene (P13.1 cells)[57] in 24-wells plates. Next, effector cells were harvested and dead cells were removed by density centrifugation on Lympholyte M (Cedarlane, Hornby, Canada). These cells were used in a cell-mediated lymphocyte cytotoxicity assay as described previously.[58]

LacZ expression in vivo

At day 0, 12 Balb/c mice received an injection into the gastrocnemius muscle of one leg with 1×10^{8} PFU rAd5-nls-LacZ. Another group of 12 Balb/c mice received i.m. in one leg with 1×10^{8} PFU rAd5-GAr-LacZ. Additionally, three nude mice were injected i.m. with 1×10^{8} PFU rAd5-nls-LacZ in one leg and 1×10^{8} PFU rAd5-GAr-LacZ in the other. At days 8 and 19, four animals of each group and a nude mouse were killed and the gastrocnemius muscles were isolated from both legs. As negative control a naive mouse was killed, whereas the nude mouse served as positive control.

Alternatively, six Balb/c mice and two Balb/c$^{nu/nu}$ nude mice were injected i.m. with 1×10^{8} PFU rAd5-nls-LacZ in one leg and 1×10^{8} PFU rAd5-GAr-LacZ in the other. These muscles were collected at days 8 and 19 (three Balb/c and one nude). A naive mouse served as negative control. In both protocols, the muscles were flash frozen in TissueTek using liquid nitrogen. Sections (10 mm) that were cut were either directly stained or stored at −80°C until further use.

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Chapter 3

The Nested Open Reading Frame in the Epstein Barr Virus Nuclear Antigen 1 mRNA Encodes a Glycine, Glutamic-acid, and Glutamine-Rich Protein, Which Functions As an *In Cis*-Acting Inhibitor of Antigen Processing

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The Nested Open Reading Frame in the Epstein Barr Virus Nuclear Antigen 1 mRNA Encodes a Glycine, Glutamic-acid, and Glutamine-Rich Protein, Which Functions As an *In Cis*-Acting Inhibitor of Antigen Processing

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Running title: Immune Evasion by Nested Open Reading Frame protein in EBNA-1

Abstract

The Epstein Barr Virus Nuclear Antigen-1 (EBNA-1) has a 249 amino-acids long glycine and alanine-rich repeat, which can function as an *in cis*-acting inhibitor of antigen processing. This cannot fully prevent antigenic peptide presentation since such peptides may be derived from defective ribosomal products. These DRiPs are an important source of immunogenic peptides and may be derived from translation of alternative open reading frames of the messenger. The EBNA-1 messenger contains a large nested open reading frame. Translation starting at an alternative initiator AUG downstream of the canonical initiation codon would yield a strongly acidic protein with a calculated molecular weight of 40.7 kDa. This protein would contain a 238 amino-acids long glycine, glutamine, and glutamic acid-rich repeat. We fused the nested ORF with the *Escherichia coli*-derived LacZ gene and showed β-galactosidase activity upon transfer of the fusion gene into cultured cells. Cells producing the unmodified β-galactosidase readily present the H-2L^{d}-restricted CTL epitope TPHPARIGL. In contrast, the same CTL epitope is not presented upon expression of the fusion gene. Deletion of two-thirds of the repeat does not affect its capacity to inhibit antigenic peptide generation. From these data we conclude that the glycine, glutamine, and glutamic acid-rich repeat of the nested ORF protein of EBNA-1 can act as a cis-acting inhibitor of antigen processing.

Introduction

Epstein Barr Virus (EBV) is a gamma herpes virus and infects over 90% of the human adult population. It is considered to be the classic example for immune surveillance of persistent viral infections in humans (Klein, 1994). It has three latency programs, which differ in protein expression profiles. However, the Epstein Barr virus nuclear antigen 1 (EBNA-1) is expressed in all latency programs and is therefore associated with all EBV-related malignancies.

EBNA-1 is essential for the maintenance of the viral genome as stable episomes during latency. It binds as a dimer to the viral origin of replication and ensures B-cell growth (Bochkarev et al., 1996; Shah et al., 1992; Yates et al., 1985; Rowe et al., 1992). Although EBNA-1-specific cytotoxic-T lymphocytes (CTLs) circulate in patients (Blake et al., 1997), the EBNA-1-positive cells are not recognized and killed. This has been attributed to the presence of the large glycine and alanine-rich repeat (GAr), which is not required for genome maintenance or cellular transformation (Lee et al., 1999), but has an immune evasion function. The GAr domain of EBNA-1 prevents cytotoxic T-lymphocyte (CTL)- epitope generation (Ossevoort et al., 2003) by inhibiting the proteasomal degradation (Levitskaya, et al., 1997; Levitskaya et al., 1995). The inhibition requires the interaction of at least three alanine residues of the GAr with adjacent hydrophobic binding pockets of a putative receptor

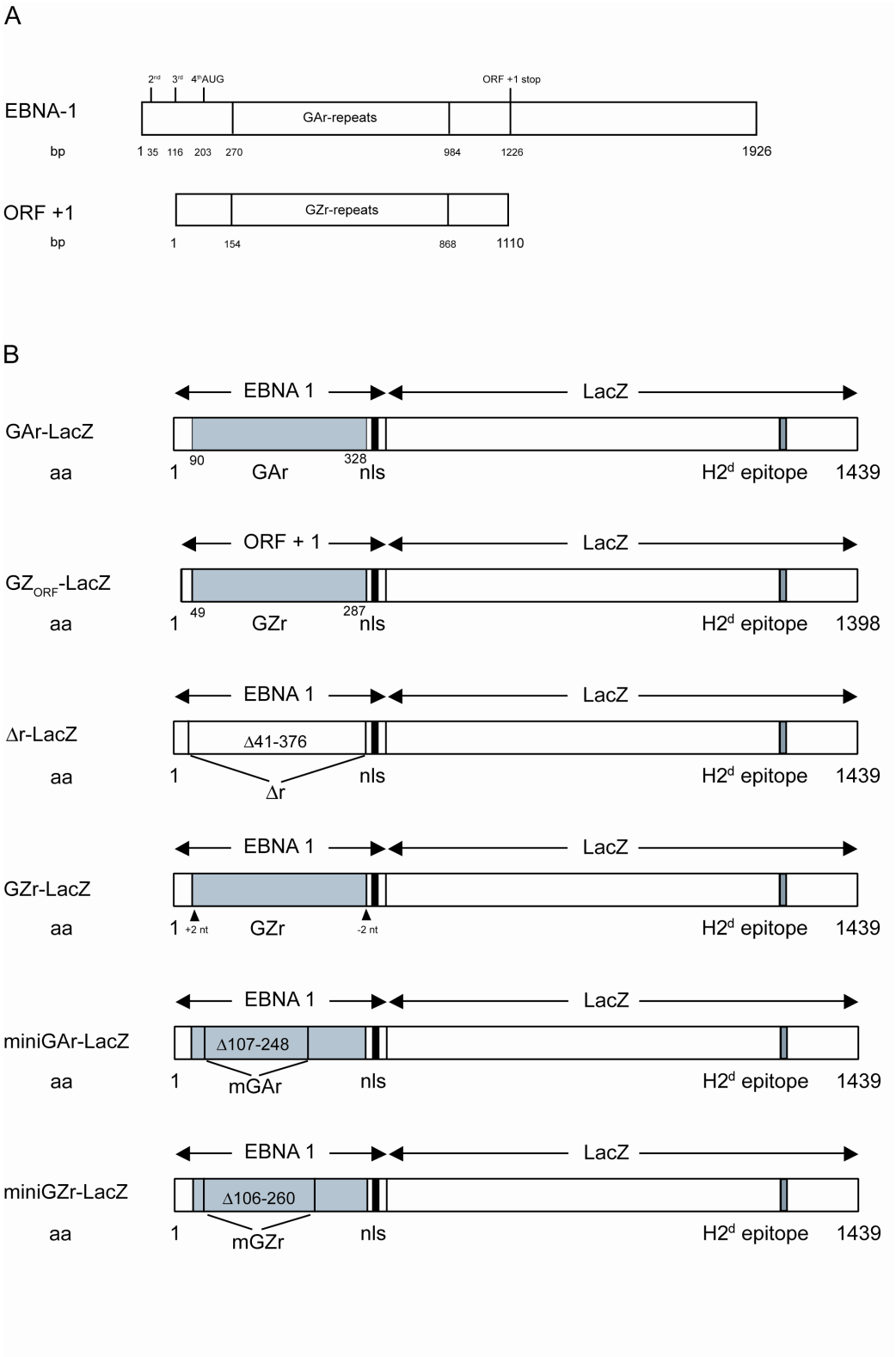
at the proteasome (Sharipo et al., 2001). Although efficient, the GAr may not be sufficient to prevent the generation and presentation of antigenic peptides altogether (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). Especially, the GAr may not inhibit the synthesis of defective ribosomal products (DRiPs) that may generate antigen-specific antigenic peptides. These DRiPs are shown to play an important role in peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001). Here we show that the EBNA-1 messenger RNA contains a nested open reading frame, preceded by a translation-initiation codon. The putative initiation codon adheres to the Kozak consensus sequence, in that it has an A at position -3 and a G in position +4 (Kozak, 1986; Kozak, 1997). Use of this ORF would yield a strongly acidic 40.7 kDa protein. Here we demonstrate that this protein can function as a cis-acting inhibitor of antigen presentation. These data indicate that also translation of the alternative ORF of the EBNA-1 mRNA will not result in abundant presentation of antigenic peptides and also this ORF can function as a mechanism to prevent the presentation of antigenic peptides.

Results

'In silico' analysis of the EBNA-1 mRNA sequences revealed a long open-reading frame that initiates at the third AUG codon. This start site is in open reading frame (ORF) +1 relative to the EBNA-1 ORF. Translation of the +1 ORF would yield a long and acidic protein of 370 amino acids and an estimated molecular weight of 40.7 kDa. The protein is strongly acidic and would contain 127 Glutamic-acid residues, 90 Glutamine residues and 69 Glycine residues. The polypeptide tract rich in Gly and Glx residues was named GZ- repeat (GZr) region (fig. 1). The AUG that could serve as translation initiation codon is part of the sequence 5'-ACCAUGG and is identical to the optimal Kozak consensus sequence.

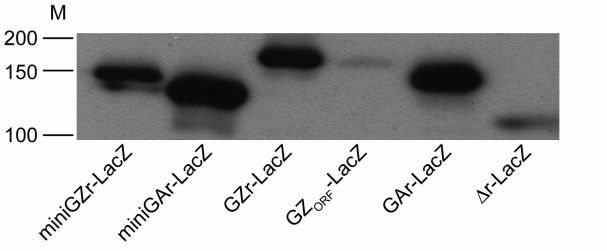
To study if the alternative open reading frame could be used, we generated a fusion of the GZr protein with the *E.coli*-derived LacZ gene. From the plasmid pGAr-LacZ, we deleted two nucleotides distal of the GAr region, merging the GZr protein ORF with the LacZ ORF. Transfection of the resulting plasmid pGZ_{ORF} into 293T cells resulted in readily detectable β-galactosidase activity. This demonstrates that under the transfection conditions translation

Figure 1 (page 95)
Schematic outline of EBNA-1 and the alternative ORF+1 proteins (A) and the fusion proteins with β-galactosidase (B). (A) The EBNA-1 coding region is depicted. The putative start codons of the alternative ORF (ORF +1) are indicated above the EBNA-1 sequence (bp = base pairs). Also indicated are the codons for the GAr in EBNA-1, and GZr in the ORF +1 protein, as well as the stop for the ORF +1 polypeptides. (B) Indicated are the Gly-Ala repeat region GAr, amino acids 90-328, the nuclear localization signal nls, aa 378-386, the entire repeat deletion aa 41-376, the deletions creating the miniGAr aa 107-248 and the miniGZr aa 106-260. The LacZ gene starts after aa 420 of the EBNA-1 ORF. In the LacZ gene the $H2^d$ epitope aa 1303-1311 is indicated. In GZ_{ORF}-LacZ the third AUG is depicted as start of the alternative ORF +1. The arrows flanking the GZr in GZr-LacZ indicate the site of the 2 bp the insertion and deletion, respectively.


A
2nd
3rd
4thAUG
ORF +1 stop
EBNA-1
GAr-repeats
bp
1 35 116 203 270 984 1226 1926
ORF +1
GZr-repeats
bp
1 154 868 1110
B
EBNA 1
LacZ
GAr-LacZ
aa
1 90 GAr 328 nls H2d epitope 1439
ORF + 1
LacZ
GZORF-LacZ
aa
1 49 GZr 287 nls H2d epitope 1398
EBNA 1
LacZ
Δr-LacZ
Δ41-376
aa
1 nls H2d epitope 1439
Δr
EBNA 1
LacZ
GZr-LacZ
aa
1 +2 nt GZr -2 nt H2d epitope 1439
EBNA 1
LacZ
miniGAr-LacZ
Δ107-248
aa
1 mGAr nls H2d epitope 1439
EBNA 1
LacZ
miniGZr-LacZ
Δ106-260
aa
1 mGZr nls H2d epitope 1439

initiation can initiate at the 3rd AUG of the EBNA1-containing mRNA's (fig. 2 and 3). This indicates that, under normal conditions, not only the GAr-containing EBNA-1 could be synthesized, but also GZr-containing polypeptides from the nested ORF. Although the GZ_{ORF}-LacZ protein is present in the transfected cells in relatively low amounts, the polypeptide may be degraded by proteasomes and yield antigenic peptides.

To facilitate studies on this protein, we enhanced its synthesis by constructing a plasmid in which the GZr region was linked to the *bona fide* EBNA-1 N terminus, allowing translation initiation to initiate at the 1st AUG. This construct allows a side-by-side comparison of GAr-LacZ and GZr-LacZ. In addition, we generated plasmids from which we deleted the GAr or GZr repeats from the LacZ fusion genes. The resulting plasmid encodes a LacZ with a small N-terminal extension consisting of 85 amino acids of the EBNA-1 ORF. In addition, we isolated some spontaneous deletion plasmids with repeat lengths considerably smaller than the 239 amino acids of the GAr and GZr. Two of these, with repeat lengths coding for 97 (in pMiniGAr-LacZ) and 82 amino acids (in pMiniGZr-LacZ), were used for further studies. All constructs are depicted in figure 1.



Protein	repeat size (aa)	Molecular weight (kD)
GAr-LacZ	239	149
miniGAr-LacZ	97	140
Δr-LacZ	none	124
GZr-LacZ	239	161
miniGZr-LacZ	82	144
GZ_{ORF}-LacZ	239	158

Figure 2

Western-blot analysis of the different LacZ fusion proteins. 293T cells and 293T cells expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ genes were lysed and protein extracts were size fractionated by SDS-PAGE. The western blot was analyzed with a monoclonal mouse anti-LacZ antibody. All proteins migrate according to their expected size (indicated in the table underneath) and no smaller products are visible.

The plasmids were transferred into 293T cells and the translated proteins were analyzed by Western-blot analysis. All LacZ fusion genes yielded equivalent amount of β-galactosidase protein (fig. 2), except the GZ_{ORF}-LacZ fusion. The apparent molecular weight, as deducted from the mobility of the proteins on SDS PAGE gels, was in good agreement with the calculated molecular weight. *In-situ* staining of the transfected 293T cells for β-galactosidase activity demonstrated that the fusion proteins were biologically active (fig. 3A). Furthermore, analysis of β-galactosidase activity in protein lysates by galactolight assay further confirmed the activity of the fusion proteins. When the ratio of the activity and protein contents is plotted, it shows a clear difference between the constructs starting at the original EBNA-1 ATG and the GZ_{ORF}-LacZ. All repeat- and mini repeat containing LacZ as well as the LacZ deleted for the repeats show equivalent levels of activity (fig. 3B). Only the GZ_{ORF}-LacZ plasmid shows a significantly reduced activity level. In contrast, the GZr-LacZ, in which the GZr-LacZ fusion protein is translated from the AUG normally used by EBNA-1, has a lacZ level similar to the GAr-LacZ fusions. Since both proteins contain a GZr region of the same length, we decided to use GZr-LacZ for all further research.

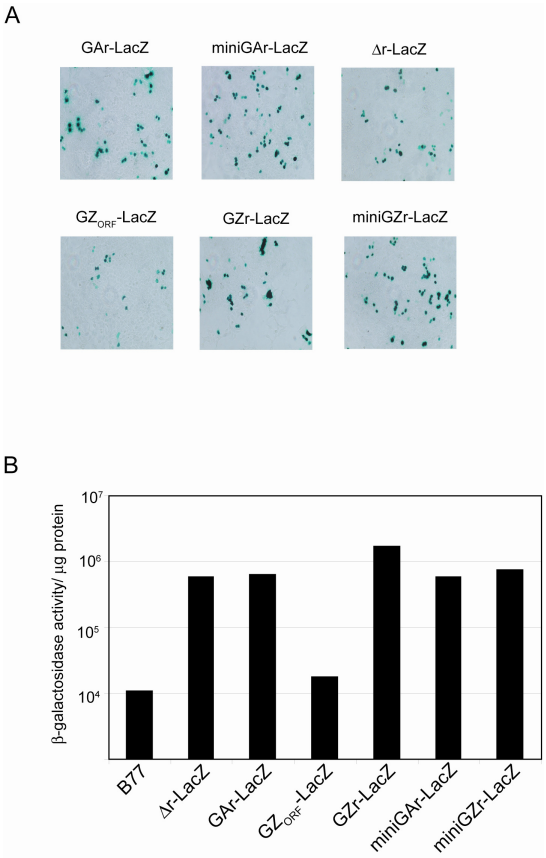


Figure 3

In-situ β-galactosidase activity assay (A) and galactolight assay (B) of the transfected 293T cells. A 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ-encoding plasmids. After 48 hours, the cells were fixed, and stained overnight. (B) 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ encoding plasmids. After 48 hours, the cells were lysed and galacton was added. The β-galactosidase activity is normalized for the protein concentration in the lysate.

For further studies we generated cell lines stably expressing the fusion genes. We tested the stable cell lines by a western-blot analysis and found the proteins being present in similar amounts (fig. 4A). All constructs yielded protein of the expected size and no degradation products were apparent. These cell lines were tested for β-galactosidase activity by *in-situ* staining (fig. 4B). As a positive control served the cell line BB16, a stable B77-derived cell line expressing an unmodified LacZ gene (Hoeben et al., 1991). In contrast to the fusion constructs, which contain the EBNA-1-derived nuclear localization signal, β-galactosidase is distributed in the cytoplasm of the BB16 cells. The cell lines expressing the fusion gene and the BB16 cells all had similar levels of activity, as is evident by the *in-situ* staining. The expression levels were further confirmed by galactolight assays (fig. 4C). Taken together, our data show that all cell lines have similar amounts of β-galactosidase antigen and activity.

To measure antigenic peptide presentation, these cell lines expressing the fusion genes were tested for the presentation of LacZ-derived peptides (H_2L^d, TPHPARIGL, indicated in fig. 1) with an interferon-gamma (IFNγ) production assay using the cells expressing the fusion genes as targets. The generation of LacZ-specific CTLs was effective and the CTL recognized the positive control BB16 cells, but not the parental LacZ-negative B77 cells (fig 5A). Fusion of the full-length GAr reduced the amount of IFNγ produced clearly. Also fusion of the GZr repeat reduced INFγ production. Remarkably, the MiniGAr and MiniGZr mini repeats are more efficiently inhibiting INFγ production. When we loaded the cell lines with β-galactosidase peptide, all cell lines stimulated the antigen-specific CTLs (data not shown), demonstrating that the presence of the repeats does not frustrate the cells' capacity to present the β-galactosidase peptides. Naïve CTLs did not induce an IFNγ response.

From these data we conclude that GZ repeat that is derived from nested open reading frame in the EBNA-1 mRNA is able to inhibit presentation of linked antigens. These findings demonstrate that not only the GAr-derived sequences, but also other simple repeat sequences can inhibit antigen presentation. Further research should reveal which step in the chain of processes that leads to the presentation of antigenic peptides is inhibited.

Discussion

The Gly-Ala repeat (GAr) domain of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) prevents cytotoxic T-lymphocyte (CTL)- epitope generation (Ossevoort et al., 2003) by inhibiting the proteasomal degradation (Levitskaya et al., 1997; Levitskaya et al., 1995). However, this may not be sufficient to prevent the generation and presentation of antigenic peptides, since the GAr might not prevent formation of defective ribosomal products (DRiPs) (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). There is a wide variety of DRiPs identified nowadays. Not only truncated or misfolded proteins, but also the products that are

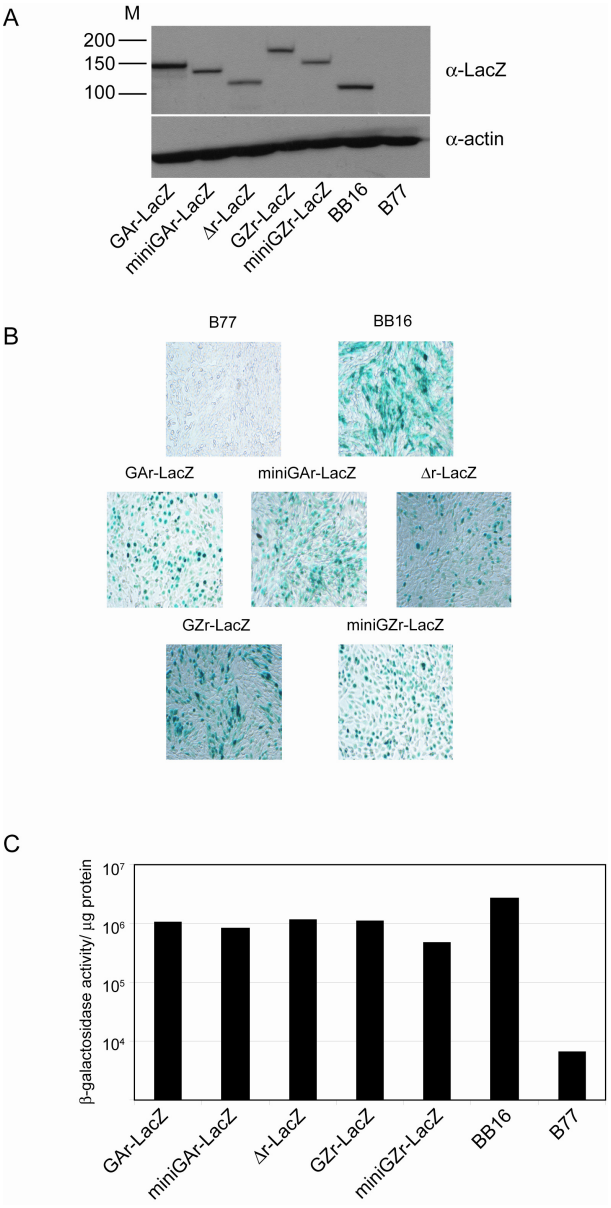


Figure 4

Characterization of the stable B77 cell lines. B77 cells were transfected with plasmids encoding miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ proteins respectively. After establishing stable cell lines, the cells expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ, as well as B77 and BB16 cells (B77 cells expressing LacZ) were lysed and protein extracts were size fractionated by SDS-PAGE (A). The western blot was analyzed with a monoclonal mouse anti-LacZ antibody. All proteins migrate at the expected size, and no smaller products are visible. (B) The same cell lines, as well as B77 and BB16 were fixed stained *in-situ*. (C) Stable B77 cell lines expressing the miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ constructs, and B77 and BB16 cells were lysed and galacton was added. Plotted is the normalized β-galactosidase activity.

translated from alternative open reading frames are considered as DRiPs (Yewdell et al., 2003; Yewdell et al., 1996). Indeed several proteins are produced by translation of alternative ORFs (Fetten et al., 1991; Malarkannan et al., 1995; Shastri et al., 1995; Wang et al., 1996; Mayrand & Green, 1998; Mayrand et al., 1998; Schwab et al., 2003) and from non AUG start codons (Malarkannan et al., 1995; Shastri et al., 1995; Schwab et al., 2003). These DRiPs are shown to play an important role in peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001).

Here we describe that polypeptides derived after translation of an alternative open reading frame of the EBNA-1 mRNA can inhibit presentation of linked antigens. This alternative ORF was observed after *in-silico* analysis of the EBNA-1 mRNA sequences and revealed a long open reading frame that initiates at ORF +1 from the original translation initiation site. It contains a repeat unit of the same length as the GAr and is rich in Gly (G) and Glx (Z) residues. Therefore, it was named GZ-rich repeat (GZr) region (fig. 1). We show that the alternative ORF is translated upon transfection of an EBNA-1 expression vector, although the steady state level of the GZr-fusion protein is lower than of the *bona fide* GAr fusion protein.

There are two possible alternative translation-initiation sites. The second AUG is followed by a single sense codon before a nonsense codon is encountered. The third and the fourth AUG codons in EBNA-1 are both in the +1 frame and would result in translation of the GZr containing polypeptide. The third AUG is has an optimal Kozak consensus sequence. It contains the A at -3 and a G at +4 and it has two Cs at positions -1 and -2. The fourth AUG contains a G residue at +4 and -3 (Kozak, 1986; Kozak, 1997). As can be seen in figures 2 and 3, the GZ_{ORF} fusion protein is present in low levels. This is not unusual for alternative ORFs (Fetten et al., 1991; Malarkannan et al., 1995; Shastri et al., 1995) and does not seem to interfere with the capacity to generate peptides and elicit a CTL response (Wang et al., 1996; Mayrand & Green, 1998; Mayrand et al., 1998; Schwab et al., 2003).

To enhance fusion protein synthesis and to make a more fair comparison between the GAr and the GZr fusion proteins, we made a GZr-LacZ fusion starting at the original AUG of EBNA-1 translation initiation. We also constructed a deleted version lacking the repeat regions and isolated comparable mini-repeats for both GAr and GZr. We could map which parts were deleted, and this mini-repeats were included in this study since it is known that short EBNA-1 derived glycine and alanine-rich sequence of only 8 amino acids are able to significantly inhibit proteasomal degradation of instable reporter protein (Sharipo, et al., 2001; Sharipo, et al., 1998; Dantuma, et al., 2000).

All these constructs were well expressed and retained their β-galactosidase activity. The stable cell lines all exhibited similar amounts of the fusion proteins and the biological activities of the proteins were equivalent (fig. 4). The cell lines were tested for peptide presentation (fig. 5).

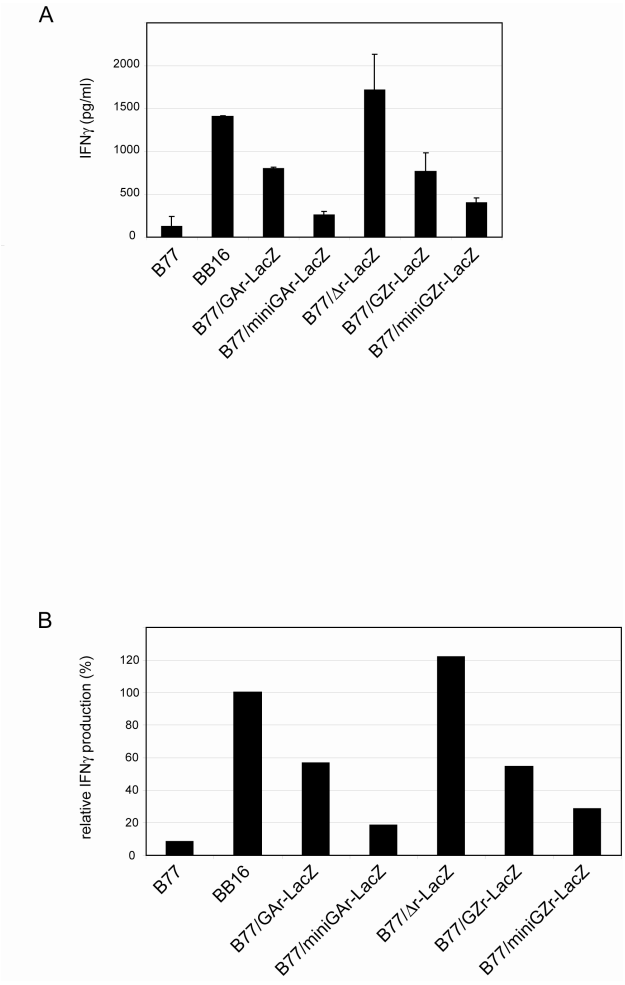


Figure 5

LacZ-specific CTLs were generated and co-cultured for 48 hours together with B77, B77 expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ genes. Thereafter, the supernatant was collected and the amount of IFNγ was determined. (A) Absolute IFNγ production of the different cell lines. (B) Relative IFNγ production. BB16, the B77-derived cell line expressing unmodified β-galactosidase, is set to 100 percent.

Introduction of a full length repeat, both GAr and GZr, significantly decreased the IFNγ production. However, the mini-repeats blocked presentation more efficiently, as is evident by reduced IFNγ production. The mechanism for the more potent inhibition is unclear. Maybe these shorter proteins form less DRiPs then longer proteins, on the basis of size and inherent difficulties in folding or assembly (Schubert et al., 2000). Furthermore, we know that over-expression of either GAr- or GZr-containing polypeptides in B77 and MEC cells does not decrease the amount of MHC I on the cell surface (data not shown). These findings demonstrate that not only the GAr-derived sequences, but also other simple repeat sequences can inhibit antigen presentation.

The observation that also the GZr repeat blocks presentation of linked antigens is intriguing. Sharipo et al. (2001) suggested that the inhibitory effect of the GAr requires at least three alanine residues in a β-strand conformation with adjacent hydrophobic binding pockets of a putative receptor (Sharipo, et al., 2001). In the case of the GZr there are no alanine residues present and since the whole repeat is strongly acidic, it is highly unlikely that there will be hydrophobic pockets involved in the receptor part. This suggests that the GZr repeat functions in a different way. It remains to be established whether the GZr repeat protein is produced EBV infected cells and whether this protein has any physiological function.

In conclusion, we demonstrate that the GZr protein that can be translated from the nested ORF of the EBNA-1 mRNA can function as an *in cis*-acting inhibitor of antigen presentation. This ORF may function as a mechanism to prevent the presentation of antigenic peptides that are generated by translation of alternative open reading frames of the EBNA-1 messenger RNA. Further research should reveal which step in the chain of processes that leads to the presentation of antigenic peptides is inhibited. Nonetheless, the new GZ repeat may be used to stealth antigenic proteins in gene-therapy applications in which eradication of the transduced cells would frustrate the therapy.

Materials and Methods

Construction of the GAr-fusion plasmids

To obtain a fusion gene of LacZ and the EBNA-1 Gly-Ala repeats, the LacZ gene was inserted into plasmid pCMV-EBNA (Invitrogen, Groningen, The Netherlands). Firstly, plasmid pCBeb was created from plasmid pCMV-EBNA by converting the *Bst*XI site to a *Bgl*II site by insertion of a *Bgl*II adapter that was created by annealing the synthetic oligonucleotides 5'-TACGAGATCTGAAG-3' and 5'-AGATCTCGTACTTC-3' (the *Bgl*II site is underlined). Secondly, a 3072 bp *Bam*HI fragment of the retroviral vector pBag (Price et al., 1987), which carries the LacZ-coding region except the first two codons, was inserted into the *Bgl*II site of pCBeb to generate pGAr-LacZ. The presence of the intact GAr was confirmed by restriction analysis and the integrity of the complete fusion protein was confirmed by sequencing.

To derive plasmids pGZ_{ORF}-LacZ, pGZr-LacZ and pΔr-LacZ from plasmid pGAr-LacZ, the QuickChange Site-directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) was used according to the manufacturer's protocols.

To create plasmid pGZ_{ORF}-LacZ and therefore detect translation starting at the 3rd AUG of EBNA-1, primers (sense) 5'-CAGGAGGTGGAG//CGGGGTCGAGGAGGC-3' and (antisense) 5'-GCCTCCTCGACCCCG//CTCCACCTCCTG-3' were used to delete two nucleotides (indicated with'//') at the end of the GAr-coding region of plasmid pGAr-LacZ.

This resulted in a plasmid in which the LacZ open reading frame is in frame with the GZ open reading frame. To boost the translation of the GZr containing LacZ, plasmid pGZr-LacZ was created. It was obtained by insertion of two nucleotides (insertions are underlined) near the start of the GAr coding region in plasmid pGZ_{ORF}-LacZ with the primers (sense) 5'-CACGGTGGAACA**GA**GGAGCAGGAGCAG-3' and (antisense) 5'-CTGCTCCTGCTCC**TC**TGTTCCACCGTG-3'. In this plasmid translation of the GZ open reading frame is initiated at the AUG normally used for EBNA-1.

A repeatless control was created to establish the effect of both the GAr and the GZr repeats. The codons for the repeats were deleted from plasmid pGAr-LacZ with the primers (sense) 5'-GGGGGTGATAACCATGGA|GGAGAAAAGAGGCCCAGG-3' and (antisense) 5'-CCTGGGCCTCTTTTCTCC|TCCATGGTTATCACCCCC-3', (the | mark indicates the location of the junction), yielding plasmid pΔr-LacZ. All resulting PCR products were verified by restriction analyses and complete sequence analyses for the presence of the mutations and the repeat sequences and integrity of the fusion proteins. The repeat sequences were relatively unstable in the plasmids (Ossevoort et al., 2003). We isolated spontaneously deletion plasmids with smaller repeats of both the GAr and GZr. By sequencing, the deletion was mapped and the resulting plasmids were called pMiniGAr-LacZ and pMiniGZr-LacZ.

Cell lines

The 293T and B77 (a hypoxanthine phosphoribyltransferase-negative (HPRT-) BALB/c 3T3 cell line) (Varmus et al., 1973) cell lines were cultured in DMEM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml and 0.2% glucose. P13.1 (Lammert et al., 1996) cells were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 28 μM β-mercaptoethanol and 500 μg hygromycin B per ml.

Stable B77-derived cell lines expressing the different LacZ variants were made by plasmid transfection of cell cultures in 6-well plates with 2.7 μg LacZ construct and 0.3 μg pRSV-neo per well using the Calcium Phosphate co-precipitation technique (Graham & van der Eb, 1973). After 48 h, medium was replaced with medium containing 500 μg G-418 sulfate (Geneticin, Gibco) per ml. After elimination of the G418-sensitive cells, the cultures were maintained on medium with 200 μg G418 per ml. For isolation of clonal cell lines, highly diluted single-cell suspensions were seeded and monoclonal cell populations were isolated and expanded. The cells were evaluated for the presence of *E. coli* β-galactosidase by in-situ staining and galactolight activity assay.

β-galactosidase assays

Cell cultures were assayed for β-galactosidase activity by X-gal (5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside) staining as described (Hoeben et al., 1991). Briefly, cells were

washed with ice-cold Phosphate-buffered saline containing 0.5 mM $MgCl_2$, and fixed with 5.4% formaldehyde, 0.8% glutaraldehyde in PBS at 4˚C. After washing, the cells were incubated in 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide, 2mM $MgCl_2$ and 1 mg X-gal per ml PBS and incubated at 37˚C for 4-16 h. The β-galactosidase activity was also assayed by Galactolight assay (Applied Biosystems) according to the manufacturers instructions. In brief, cultured cells were washed twice in ice-cold PBS and lysed in lysis buffer (25mM Tris-phosphate pH 7.8, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Ten µl lysate was added to 100 µl of reaction buffer containing galacton and incubated at room temperature for 30 min. Hereafter, 100 µl of accelerator was added and the β-galactosidase activity was measured with an illuminometer (Lumat LB, EG&G Bertholt).

Western analysis

Cells were lysed in RIPA buffer (50 mM Tris.Cl pH=7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40) and protein extracts were size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes and probed with mouse anti-LacZ antibodies (1/500 diluted, Roche) or mouse anti-actin antibodies (1/2000, ICN Biomedicals Inc., Aurora, OH, USA). After incubation with a peroxidase-conjugated goat-anti-mouse secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

Generation of b-galactosidase specific CTLs

β-Galactosidase-specific CTL clones were generated by injecting BALB/c mice intra-muscularly with $1*10^8$ PFU rAd5-nls-LacZ. Two weeks post-injection, the mice were sacrificed and splenocytes were harvested. As a control, splenocytes were harvested from naïve mice. The splenocytes were stimulated with 5000-rad irradiated The LacZ-expressing P13.1 cells for 1 week. Hereafter the effector cells were ready for use.

IFNγELISA

In 96-wells plates, 5000 or 2500 effectors were co-cultured with 20,000 targets/well in triplicate in the presence of 10U IL-2 per ml for 2 days at 37 ºC, at 5% CO_2. One day prior to harvesting of the supernatants, maxisorb plates (Nunc) were coated with 1 µg/ml allophycocyanin-conjugated Rat αM-IFNγ antibody (BD Biosciences) in sodium-carbonate buffer (pH=9.6) overnight at 4ºC. The coated plates were washed 4 times with PBS containing 0.05% Tween-20 and subsequently blocked with PBS containing 1% BSA and 0.05% Tween-20, for 1 hour at 37ºC. After 4 washes the plates were incubated with the supernatants for 2 h at 37ºC. A standard series starting with 10 ng IFNγ (recombinant murine IFNγ, Tebu-bio) per ml medium was diluted 1 in 2 till a final concentration of 9.7 pg per ml.

This standard series and a blank were added to the plates in duplicate. After 4 washes, 0.5 μg per ml biotinylated RatαM-IFNγ monoclonal antibody (BD Biosciences) in block solution was added as conjugate and incubated for 1 h at room temperature. Thereafter the plates were washed 4 times and subsequently streptavidin conjugated poly-Horseradish Peroxidase (Sanquin reagents, Amsterdam, The Netherlands) was added at a concentration of 133 ng per ml in block solution and incubated for 1 h at room temperature. The plates were washed again 4 times, and 4.5mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS, Sigma-Aldrich) with H_2O_2 was added to the plates, and OD was measured at 415 nm.

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Chapter 4

Characterization of an Immuno'stealth' Derivative of the Herpes Simplex Virus Thymidine Kinase gene

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Characterization of an Immuno'stealth' Derivative of the Herpes Simplex Virus Thymidine Kinase gene

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Abstract

The cellular immune response against transgene-encoded *neo*antigens is a potential hurdle in gene therapy applications where long-term expression of transgenes is desired. Here a new optimized derivative of the Herpes Simplex Virus 1-Thymidine Kinase gene is described. The HSV-TK gene is frequently used in experimental studies on gene-directed enzyme prodrug therapy. In the optimized gene, the HSV-TK coding region is fused with the codons for the Gly-Ala repeat of the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) to prevent proteasomal degradation of the HSV-TK. To measure the protective effect in vitro, a model CTL epitope derived from the ovalbumin was inserted in the TK. Cells expressing the GAr-modified TK do not present TK-derived peptides in the MHC. Furthermore, conservative nucleotide substitutions were introduced, which prevent splicing, as well as mutations that render the TK-expressing cells more sensitive to ganciclovir (GCV). The GAr HSV-TK fusion protein is fully functional in vitro. This HSV-TK gene may be especially useful in those gene therapy applications where an immune response against the transgene-encoded product would frustrate the treatment.

Introduction

The cellular immune response against transgene-encoded *neo*antigens is a major setback in gene therapy applications where long-term expression of transgenes is desired. Transgene-product immunity is prominent upon adenovirus-mediated gene transfer, e.g. against the *Escherichia coli* β-galactosidase and the Tet transactivator protein in animal models. In rodents, strong immune responses against this *neo*antigen were observed following adenovirus-mediated gene transfer into liver, muscle, lung and brain, leading to local inflammation, destruction of the transduced cells, and loss of transgene expression.[1-4] The prime candidates for this target cell destruction are the antigen-specific major histocompatibility complex class I (MHC class I) restricted cytotoxic T lymphocytes (CTLs).[1,5-7] Also in primates a CTL response directed against the transgene product has been shown to occur after retrovirus-mediated gene transfer.[8,9] In a clinical trial aiming at inducing a graft-versus-leukemia response, 8 of 24 treated patients developed a specific cytotoxic $CD8^+$ T cell-mediated immune response against the cells genetically engineered to express the herpes simplex virus 1 (HSV1) thymidine-kinase (TK) gene. This led to the selective elimination of the modified cells.[9]

HSV-TK is the archetypical enzyme used in gene-directed enzyme prodrug therapies (GDEPT). Its capacity to convert the antiherpetic nucleoside analogues ganciclovir and aciclovir to toxic nucleotides has been used effectively in gene therapy protocols to eradicate tumor cells and lymphocytes that expressed the HSV-TK transgene upon virus-mediated gene

transfer. Although effective, in some applications the use of the HSV-TK is limited by the induction of a cellular immune response against the HSV-TK protein.

The cellular immune response may also frustrate other cancer gene therapy applications. The TK/ganciclovir (GCV) combination is very potent and can be used to eradicate tumor cells. Here the metabolic bystander effect is enhancing its effectiveness. Transport of phosphorylated GCV via gap junctions to neighboring cells sensitizes also the neighboring non-transduced cells (reviewed by Van der Eb et al., 2004).[10] A preexisting immune response directed against HSV-TK may frustrate the efficacy especially if a significant time period exists between vector administration and the onset of GCV administration. This may be the case in those applications where the HSV-TK gene has been included in a vector as fail-safe.[11,12]

Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class-I molecules. Given the important role of CTLs in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen-presentation.[13-15] In general, the viruses interfere with antigen presentation by frustrating the cell's capacity to generate or present antigenic peptides. In few cases, the inhibition blocks presentation of specific polypeptides. One such example comes from the Epstein-Barr Virus (EBV) nuclear-antigen 1 (EBNA-1). EBNA-1 is expressed in all latency programs of the virus and is indispensable for the virus as it is required for the maintenance of the viral episomes. Although EBNA-1-specific CTLs have been described in infectious mononucleosis patients and healthy carriers, they cannot efficiently recognize EBV-infected cells.[16-21]

The failure to recognize endogenously expressed EBNA-1 has been attributed to the glycine-alanine repeat (GAr) domain in the EBNA-1 sequence. It is shown that the GAr blocks proteasomal degradation in-*cis* and therefore presumably the subsequent presentation in the MHC class-I context.[22,23]

In a previous study we showed that this system could be used to create a 'stealthed' version of the *E.coli*-derived LacZ gene encoding β-galactosidase. Fusions of the EBNA-1 GAr with β-gal prevented presentation of β-gal-derived peptides. This resulted in full protection of the cells against β-gal-specific CTL. The GAr-β-gal fusion protein retained its full β-gal activity. In addition, we demonstrated that the GAr fusions with the firefly luciferase and the HSV-TK yielded functional proteins [24]. Here we describe the construction and characterization of new immuno 'stealth' variants of the HSV-TK for use in experimental gene therapy.

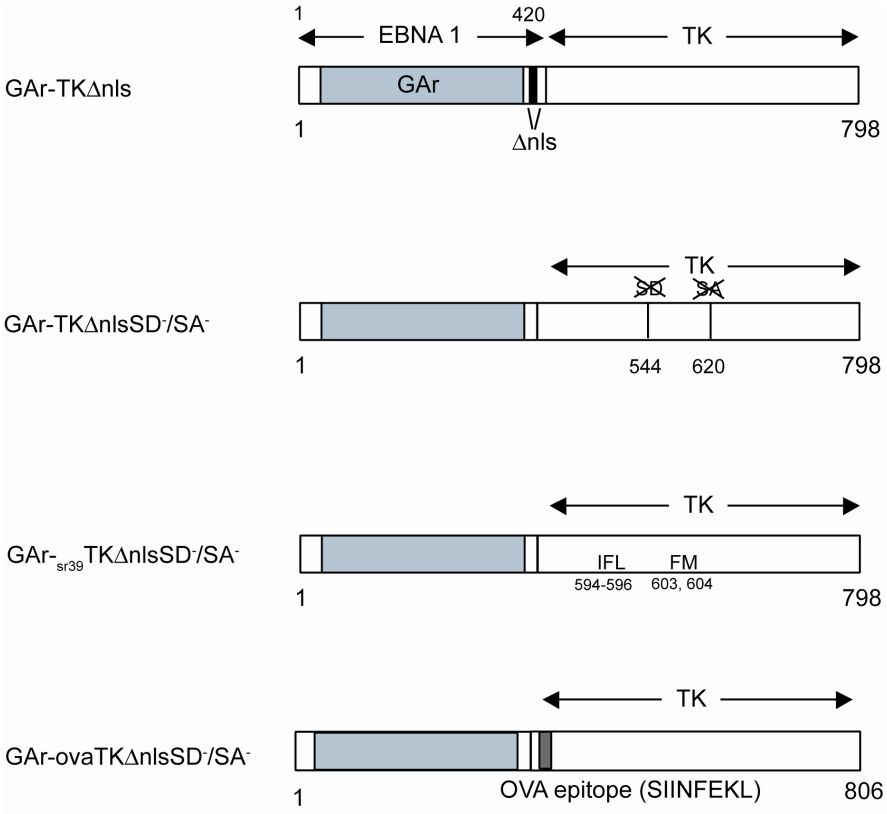

Figure 1
Schematic outline of the chimaeric Gly-Ala repeat constructs with TK. Indicated are the Gly-Ala repeat region (GAr; aminoacids 90-328), the nuclear localization signal (nls, aa 378-386, deleted from all constructs), the point mutations in the splice-donor and acceptor sites to abolish splicing (SD, SA, aa 544 and 620), the sr39TK mutations and their new aa composition (aa 594-596 and 603, 604) and the inserted OVA epitope (aa 431-438). The HSV1-TK gene starts at aa 420. Of all constructs, also variants with a deletion of the GAr region (i.c. amino acids 41 to 376) were generated.

Results

In a previous study we have shown that fusion of the HSV-TK with amino acids 1- 420 of EBNA-1, which encompasses the glycine and alanine-rich region (GAr) of EBNA-1, was compatible with enzyme function. The EBNA-1 fragment used contains the nuclear localization signal of EBNA-1 in addition to the GAr. As a result the GAr-TK fusion proteins accumulate in the nucleus, in contrast to the wild-type HSV-TK protein (fig 2). To test whether normal sub-cellular localization could be restored, the amino acids 378 to 388 encoding the nuclear localization signal (nls) were removed from plasmid pcDNA3.1-GAr-TK,[24] generating plasmid pcDNA3.1-GAr-TKΔnls. Upon transfection in 293T cells, the GAr-TKΔnls protein is indistinguishable in sub-cellular distribution from the wild-type protein. Therefore in all subsequent experiments the Δnls variants of the GAr-TK fusions were used.

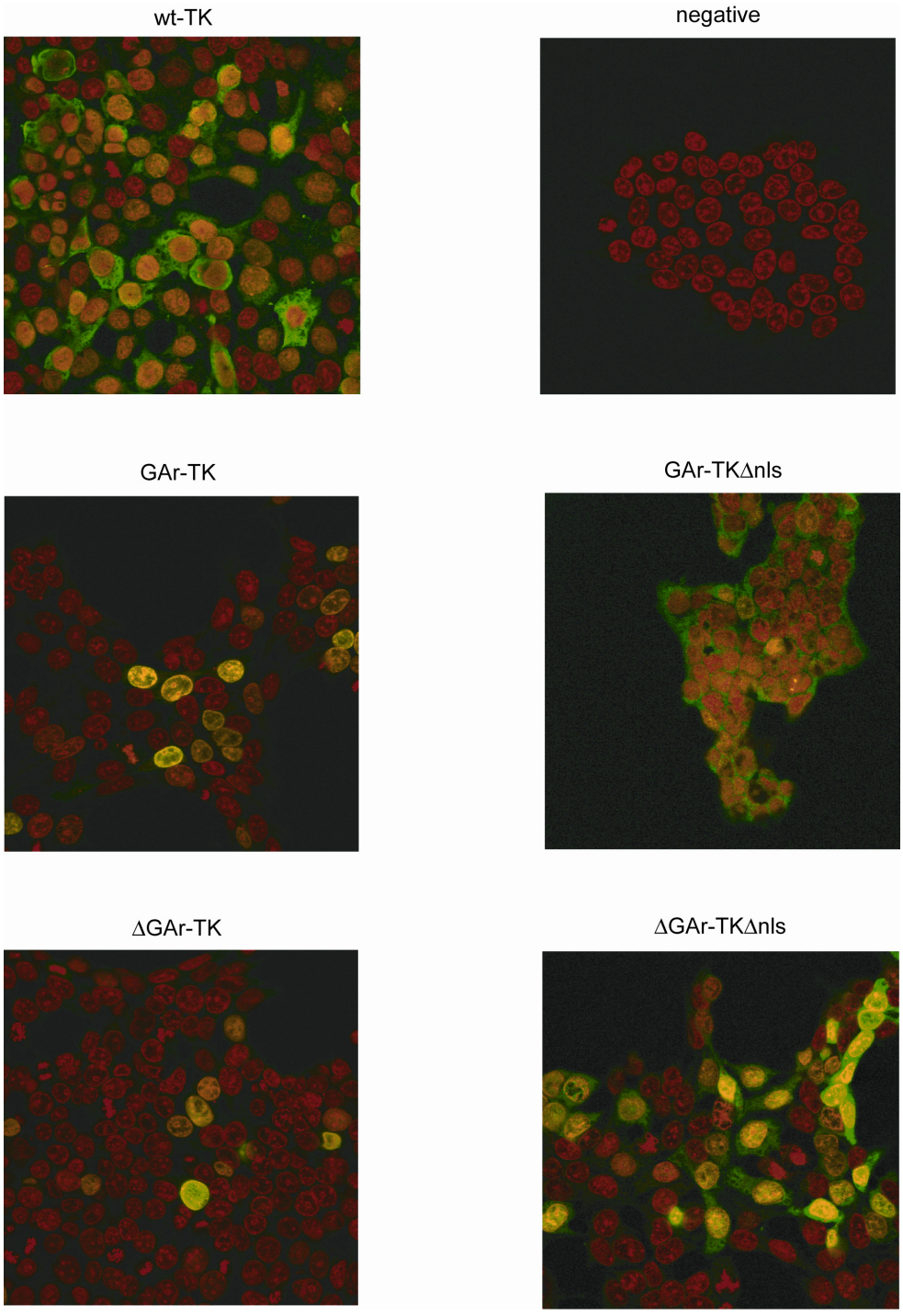

Figure 2.
Sub-cellular localization of the different TK proteins. 293T cells were transfected with wt-TK, GAr-TK, GAr-TKΔnls, ΔGAr-TK or ΔGAr-TKΔnls. Cells were incubated with rabbit anti-HSV-TK antiserum and a FITC-conjugated goat-anti-rabbit secondary antibody. The nucleus was stained with Propidium Iodide and the cells were analyzed by a confocal laserscan microscopy. Deletion of the nls but not the GAr restores the wt-TK distribution pattern.

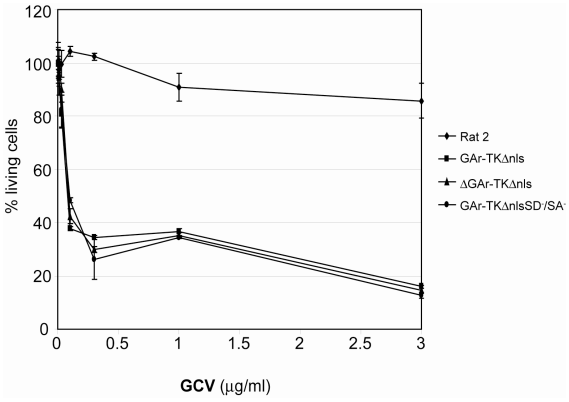

Figure 3
Deletion of the nls, GAr or SD/SA sites do not alter GCV sensitivity. Rat2 cell lines containing different TK constructs were seeded 3000 cells per well, 0.6 cm^2, with different concentrations GCV. After 4 days cell viability was assessed with the WST-1 colorimetric assay.

It has been demonstrated that the HSV-TK coding region contains cryptic splice-donor and splice-acceptor sites flanking the codons for the catalytic domain.[31] These were removed from the GAr-TKΔnls construct by conservative mutagenesis as described previously to generate the construct GAr-TKΔnlsSD$^-$/SA$^-$. This prevented splicing as described previously. Indeed, no spliced product could be detected by RT-PCR after transfection of this plasmid (data not shown). Therefore in all subsequent experiments the SD$^-$/SA$^-$ variants of the GAr-TK gene were used.

To verify the functionality of these plasmids, these were transfected into Rat2 cells and the resulting clones were tested for GCV sensitivity. Cells were seeded in 96-well plates and different concentrations GCV were added to the medium. After 5 days the viability of the cells was determined. As shown in figure 3, all the cell lines containing the various GAr, nls and SD-/SA- modifications were sensitive to the GCV, indicating that the plasmids encode functional TK variants.

So far no immunodominant CTL epitopes have been identified in the HSV-TK. To test the presentation of antigenic peptides by of the various TK constructs, a model CTL epitope was inserted in the N-terminal region of TK (Fig. 1). It has been shown that this part of the protein is dispensable for TK activity.[32] The ovalbumin epitope SIINFEKL (OVA 257-264) was used.[33,34] This allowed the use of an established CTL clone, B3Z [29], to test antigen presentation in vitro.

We first inserted the coding regions for GAr-TKΔnlsSD$^-$/SA$^-$ and ΔGAr-TKΔnlsSD$^-$/SA$^-$ into a lentiviral vector. Subsequently, the OVA epitope was inserted creating pGAr-ovaTKΔnlsSD$^-$/SA$^-$ and pΔGAr-ovaTKΔnlsSD$^-$/SA$^-$, respectively. Lentiviral particles were generated by the quadruple transfection technique and quantified by p24 ELISA. Vectors with and without GAr region could be grown to similar titers (data not shown). To transfer the variant TK genes into B16 cells (H2-K^b), we exposed the cells to approximately 80 ng of p24/10^5 cells for each vector. PCR analysis of DNA isolated from the transduced cell populations did not reveal evidence for rearrangements or deletions in the GAr region. Western-blot analysis revealed the presence of proteins of the expected sizes (Fig 4).

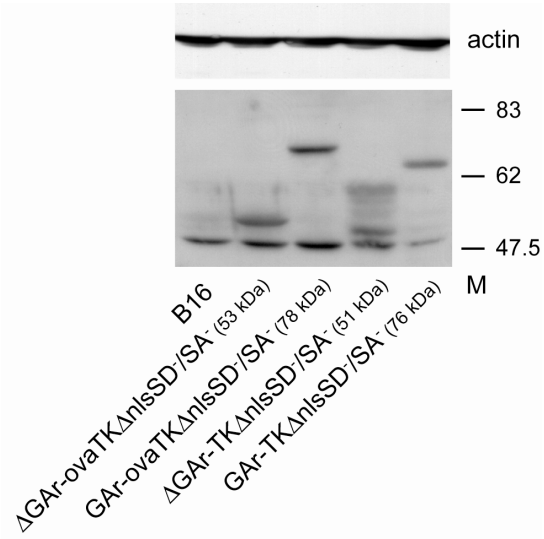

Figure 4
All modified TK genes are efficiently expressed. B16 cells and B16 cells expressing GAr-TKΔnls, ΔGAr-TKΔnls, GAr-ovaTKΔnls or ΔGAr-ovaTKΔnls were lysed and protein extracts were size fractionated by SDS-PAGE. The western blot was analyzed with a polyclonal rabbit anti-HSV-TK antibody. All bands are at the expected size and no smaller products are visible. The bands migrating at 48 and 60 kDa are resulting from the cross reactivity in the assay with a B16-derived cellular protein and the immunoglobulin light chains, respectively.

Moreover, the GAr containing proteins reveal a homogeneous band, demonstrating the integrity and the stability of the vectors with the GAr element. The GCV-sensitivity assay did not reveal differences between the TK variants with and without the OVA peptide (Fig 5). This demonstrates that insertion of the OVA epitope into the N-terminal part of TK does not significantly affect its capacity to convert GCV.

To test the presentation of the OVA peptide, the variant-TK expressing cell populations were exposed to B3Z CTL hybridoma cells. In these cells, recognition of the OVA epitope induces the expression of an IL2-promoter that drives expression of a LacZ gene. The accumulation of LacZ was assayed by CPRG assay (Fig. 6). In the absence of the OVA peptide no β-gal activity can be detected. Expression of ΔGAr-ovaTKΔnlsSD$^-$/SA$^-$ leads to a strong induction of the reporter. However, if the GAr region is linked to the epitope as in the GAr-ovaTKΔnlsSD$^-$/SA$^-$ expressing cells, the peptide is not presented as is evident from the lack of induction of the β-gal reporter. This demonstrates that the GAr repeat can prevent presentation of the OVA epitope.

To enhance the applicability of the GAr-modified TK we introduced the activating mutations of the sr39 mutant described by Black et al. in GAr-TKΔnlsSD$^-$/SA$^-$ to generate GAr-TK $_{sr39}$ΔnlsSD$^-$/SA$^-$. The sr39 mutant was isolated after semi-random mutagenesis and has a Km value of 14.3-fold lower then wt-TK.[35,36] However, under the conditions used here we could show no enhancement for GCV sensitivity (data not shown).

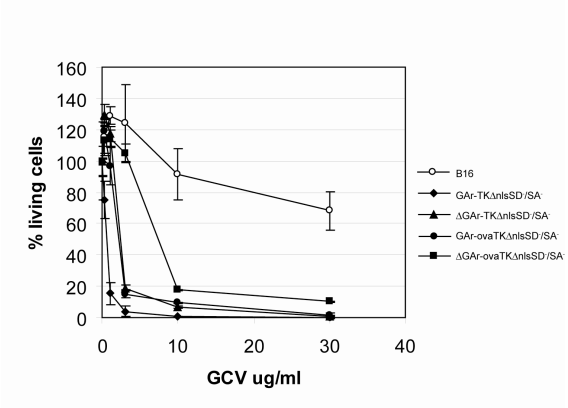

Figure 5
Insertion of the OVA epitope does not essentially alter GCV sensitivity at higher GCV concentrations. B16 cell lines containing different TK constructs were seeded 3000 cells per well, 0.6 cm^2, with different concentrations GCV. After 4 days cell viability was assessed with the WST-1 colorimetric assay.

Discussion

Herpes Simplex Virus-derived thymidine kinase (HSV-TK) is the archetypical enzyme used in gene-directed enzyme prodrug therapies (GDEPT). Its capacity to convert the antiherpetic nucleoside analogues ganciclovir and acyclovir to toxic nucleotides has been used effectively in gene therapy protocols to eradicate tumor cells and lymphocytes that expressed the HSV-TK upon virus mediated gene transfer. Although effective, the use of the HSV-TK is limited by the induction of a cellular immune response against the HSV-TK protein. Upon infusion of HSV-TK-expressing T lymphocytes, 8 of 24 recipients developed an immune response against the transgene product. One avenue to circumvent this problem is to develop new 'human' suicide genes.[9,37,38]

As an alternative approach to evade the antigen-specific immune response we evaluated the use of the Glycine-Alanine repeat (GAr) region of the nuclear antigen 1 (EBNA-1) of the Epstein Barr Virus (EBV) to protect HSV-TK derived peptides from being presented. Here we provide evidence that fusing the GAr region of the EBNA-1 to HSV-TK reduces the presentation of transgene-product-derived peptides in vitro. Furthermore, we introduce some extra features that could be optimizing the TK gene even more. Fusions of the entire N-terminal sequence (aa 1- 420) of EBNA-1 with TK lead to a markedly changed subcellular localization TK. We were able to restore the normal cell distribution pattern of the modified-TKs by deleting the nls from the EBNA-1 derived sequences. In addition, we introduced mutations to prevent splicing, something that is known to occur in up to 5% of the cells expressing TK after retrovirus-mediated gene transfer. Previously we have shown that fusing the GAr with TK does not alter the effectiveness of the enzyme. In this study we show that the mutations we made to restore the distribution pattern and to prevent the splicing maintained the enzymatic activity of the encoded TK. Furthermore, we could show that

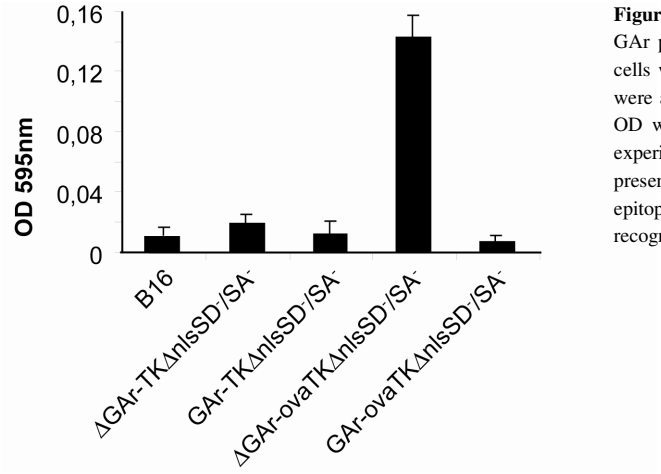

Figure 6
GAr protects the OVA epitope from being recognized. Target cells were seeded 6250 per 0.6 cm^2 well and 50000 B3Z cells were added (n = 3). After o/n incubation CPRG was added and OD was measured at 595 nm. Depicted is one representative experiment of four experiments. The B3Z cells react on the presence of the OVA epitope in absence of the GAr and OVA epitope. The presence of the GAr completely abolishes the recognition.

insertion of the OVA epitope did not overtly alter the capacity of TK to convert GCV. When we monitored for presentation of the OVA epitope using B3Z indicator cells, we observed a clear difference between ovaTK with or without the GAr, indicating that fusing the GAr to TK does indeed frustrate presentation even in highly stable enzymes as TK. So far, we have been unable to test the effect of the GAr on TK presentation in mice. We did not see significant differences in tumor take between naive mice and mice that were vaccinated with a first generation adenovirus vector that carriers the HSV-TK gene, upon challenge with B16 cells, B16 GAR-TKΔnls cells, and B16 ΔGAR-TKΔnls cells (data not shown). These data suggest that expression of the HSV-TK does not lead to the induction of antigen-specific CTL in C57/BL6 mice.

Although an EBNA-1 derived glycine and alanine-rich sequence of only 8 amino acids in length was found to significantly inhibit proteasomal degradation of instable reporter protein,[39,40] it remains to be established if such short sequences are sufficient to fully prevent antigen-presentation and CTL-mediated cell destruction. We will therefore direct further research into the reduction of the repeat length while maintaining the beneficial effect of the GAr in our system.

A recent study demonstrated that the GAr does not fully prevent the generation of antigenic peptides of full-length EBNA-1.[41] This is attributed to the formation of so-called defective ribosomal products or DRiPs. Several differences between the latter study and ours should be noted. The full length EBNA-1 was studied in human B-cells and lymphoblastoid cell lines (LCLs). Whereas specific lysis is observed in the GAr-containing EBNA-1, more efficient lysis could be obtained when the GAr was deleted from EBNA-1, confirming a stealthing effect of the GAr in the natural context. This is in accordance with the data from Lee et al.,[42] who also noted a partial protective effect of GAr in its natural context.

Furthermore, deletion of the GAr greatly reduces the half-life of EBNA-1 in B cells. This is consistent with a negative effect of the GAr on the efficiency of proteasomal degradation.[22] In addition, we have studied the effect of the GAr with HSV-TK (this study), LacZ and luciferase,[24] and GFP (M.O. & A.Z., unpublished data). These proteins are rather stable already and therefore may require less stabilization by the GAr, to sufficiently inhibit antigenic-peptide formation.

Nonetheless, our data describe an immuno 'stealthy' HSV-TK gene. We demonstrated that the 'stealthy' variant could confer GCV sensitivity to the transduced cells. It also evades recognition by established CTL. However, formal proof of its efficacy in humans awaits demonstration of the protective effects against human TK-directed CTL. This could be tested with CTL isolated from patients that have received TK-modified cells and mounted a TK-specific CTL response. We kindly invite anyone who has such material to initiate such studies using the vectors described here.

Materials and Methods

Construction of the GAr fusion genes

The HSV-TK gene containing plasmids are all derived from pGAr-TK.[24] To create pΔGAr-TK, pGAr-TKΔnls, pΔGAr-TKΔnls, pGAr-TKΔnlsSD$^-$/SA$^-$ and pGAr-TK $_{sr39}$ΔnlsSD$^-$/SA$^-$ the QuikChange Site-Directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) was used according to the manufacturer's protocols with the primers listed in Table 1.

First, the leucine-rich nuclear localization signal (nls) was deleted from the pcDNA3.1-GAr-TK with primer set 1 creating pcDNA3.1-GAr-TKΔnls. Following the manufacturer's suggestion both primers were chosen to have completely complementary sequences. In the sequence, the mark indicates the junction. Primer set 2 was used to delete the GAr (from amino acids 41 to 376) from the pcDNA3.1-GAr-TK, yielding pcDNA3.1-ΔGAr-TK. To obtain pcDNA3.1-ΔGAr-TKΔnls that lacks both the nls and the GAr, primer set 3 was used on plasmid pcDNA3.1-GAr-TKΔnls to remove the GAr.

The plasmid lacking the cryptic splice-donor and splice-acceptor sites (SD$^-$/SA$^-$) was created from pcDNA3.1-GAr-TKΔnls. First primer set 4 was used to insert conservative changes that remove the splice-donor site (the mutation is shown in bold). After mutagenesis the presence of this mutation was verified by DNA sequence analysis. The resulting plasmid was used as template to create the pcDNA3.1-GAr-TKΔnlsSD$^-$/SA$^-$ by mutagenesis with primer set 5 (the mutation that is introduced is shown in bold) to inactivate the cryptic splice-acceptor site. A similar strategy was used to create pcDNA3.1-ΔGAr-TKΔnlsSD$^-$/SA$^-$ by starting with plasmid pcDNA3.1-ΔGAr-TKΔnls.

Table 1: Primers used for the construction of the TK mutants.

Primers		Sequence 5'-3'
1	Δnls-$_{fw}$	GGTCGTGGACGTGGAGAA|CAGTCATCATCATCCGGG
	Δnls-$_{rv}$	CCCGGATGATGATGACTG|TTCTCCACGTCCACGACC
2	ΔGAr-$_{fw}$	GGGGGTGATAACCATGGA|GGAGAAAAGAGGCCCAGG
	ΔGAr-$_{rv}$	CCTGGGCCTCTTTTCTCC|TCCATGGTTATCACCCCC
3	ΔGAr$_{2\text{-}fw}$	GGGGGTGATAACCATGGA|GGAGAACAGTCATCATCATCC
	ΔGAr$_{2\text{-}rv}$	GGATGATGATGACTGTTCTCC|TCCATGGTTATCACCCCC
4	SD$^{-}_{fw}$	CCGCCTCGACCA**A**GGTGAGATATC
	SD$^{-}_{rv}$	GATATCTCACC**T**TGGTCGAGGCGG
5	SA$^{-}_{fw}$	CAGCATGACCCCCCA**A**GCCGTGCTGGCGTTC
	SA$^{-}_{rv}$	GAACGCCAGCACGGC**T**TGGGGGGTCATGCTG
6	OVA-$_{fw}$	CGCGAGCATCATTAATTTCGAGAAGCTGGC
	OVA-$_{rv}$	CGCGGCCAGCTTCTCGAAATTAATGATGCT
7	sr39$_{1\text{-}fw}$	CCGGCCCTCACC**A**TC**T**TC**C**TCGACCGCCATGGG
	sr39$_{1\text{-}rv}$	CCCATGGCGGTCGA**G**GA**A**GA**T**GGTGAGGGCCGG
8	sr39$_{2\text{-}fw}$	CGCCATCCCATCGCC**TT**C**A**TGCTGTGCTACCCGGCC
	sr39$_{2\text{-}rv}$	GGCCGGGTAGCACAGCA**T**G**AA**GGCGATGGGATGGCG
9	TK splice-$_{fw}$	CTCGACCAGG|GCCGTGCT
	TK-$_{rv2}$	CGACGAAGCTTAGTTAGCCTCCCCCATCTCCCG

The mark in primer sets 1-3 indicated where the nls or the GAr was deleted. The bold and underlined letters in the other primers indicate mutations. The mark in primer 9 indicates the junction of the spliced TK.

In the resulting plasmids, the ovalbumin (OVA)-derived CTL epitope (SIINFEKL, 257-264) was inserted in frame in the thymidine kinase coding region. The plasmids were digested with *Mlu*I. A unique site for this enzyme is present in the region of the TK gene coding for the N-terminus. Primer set 6 was annealed generating *Mlu*I overhangs, and the double-stranded product was cloned into the *Mlu*I site of the (Δ)GAr-TKΔnlsSD^{-}/SA^{-} plasmids, creating pcDNA3.1-(Δ)GAr-OVA-TKΔnlsSD^{-}/SA^{-} .

All PCR products were characterized by restriction analysis and the presence of the mutations was verified by DNA sequence analysis. For the lentiviral system, the complete GAr-TKΔnlsSD-/SA^{-} and ΔGAr-TKΔnlsSD^{-}/SA^{-} coding regions were excised by *Spe*I and *Xho*I digestion and cloned into the pRRL vector digested with the same enzymes,[25] yielding pRRL-GAr-TKΔnlsSD-/SA^{-} and pRRL-ΔGAr-TKΔnlsSD^{-}/SA^{-}.

On the latter plasmids, primer set 7 was used to create the mutations of the TK$_{sr39}$ at amino acids 594-596 (Fig 1). After confirmation of the presence of these mutations, primer set 8 was used on this template to create the mutations of the TK$_{sr39}$ at the amino acids 603 and 604, yielding pRRL-GAr-TK$_{sr39}$ΔnlsSD-/SA^{-} and pRRL-ΔGAr-TK$_{sr39}$ΔnlsSD^{-}/SA^{-}. The PCR products were confirmed by restriction analysis and sequencing for the presence of the mutations and the GAr.

Cell lines

The 293T cells [26], B16 cells [27], and the thymidine-kinase deficient Rat2 [28] cells were cultured in DMEM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml and 0.2% glucose. The B3Z indicator cells [29] were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 28 µM β-mercaptoethanol and 500 µg hygromycin B per ml.

Stable Rat2 cell lines expressing the different TK variants were made by transfection of 10 cm dishes with 9 µg TK construct and 1 µg pRSVneo using the Ca-Phosphate coprecipitation technique.[30] After 48 h, medium was replaced with medium containing 800 µg G418 sulphate (Geneticin, Gibco) per ml. After elimination of the G418-sensitive cells, the cultures were maintained on medium with 200 µg G418 per ml and used as polyclonal cultures. The cells were evaluated for the presence of the TK protein by immunofluoresence and ganciclovir (GCV) sensitivity assays.

B16 cell lines stable expressing TK variants were made by transduction with lentiviruses at 1 ng p24/5000 cells o/n in the presence of 8 µg/ml polybrene. The next day, cells were seeded sparsely and monoclonal cell lines were isolated and expanded. Monoclonal cell lines were assayed for TK expression by western analysis and GCV sensitivity.

Virus production

Lentiviruses were produced using the quadruple transfection technique as described.[25] Briefly, 293T cells (60-70 % confluent) were transfected using the Ca-Phosphate co-precipitation technique. In a Ø 10 cm dish, 3.5 µg pCMV-VSVG, 6.6 µg pMDLg/RRE, 5 µg pRSV-REV and 10 µg transfer vector plasmid were added. After 48 h and 72 h, the virus-containing supernatant was harvested by collecting the medium and filtrating through a 45-µm filter (Pall Gelman, Portsmouth, UK). The virus-containing supernatant was stored at –80ºC until use. The p24 amounts were determined by ELISA (Gentaur, Brussels, Belgium) and served as a surrogate measurement of the transducing titer, by assuming that 1 ng p24 is the equivalent of 2500 transducing particles.

Enzymatic assays

Ganciclovir sensitivity assays to detect HSV-TK activity were performed in Rat2 cells or B16 cells as indicated. Briefly, 3000 cells were seeded per 0.6 cm^2 well, with different concentrations of GCV (Cymevene®, Roche diagnostics, Almere, The Netherlands) in triplicate. Cultures were grown for 4 days at 37ºC, 5% CO_2. Cell viability was assessed with the WST-1 colorimetric assay (Roche) according to the manufacturer's description.

Chlorophenolred-β-D-galactopyranoside (CPRG, Calbiochem) assays to detect presentation of OVA, target cells were seeded 12000-6000-3000 per 0.6 cm^2 well and 50000 B3Z cells

were added. After o/n incubation at 37ºC, 5% CO_2, CPRG was added and OD was measured at 595 nm.

Western analyses

B16 cells or B16 cells expressing GAr-TKΔnls, ΔGAr-TKΔnls, GAr-ovaTKΔnls or ΔGAr-ovaTKΔnls were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40) and protein extracts were size fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes and probed with rabbit anti-HSV-TK antiserum (1/250 diluted), kindly provided by Dr M Janicot (Aventis-Gencell, Vitry-Sur-Seine, France). After incubation with a peroxidase-conjugated goat-anti-rabbit secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

RT-PCR

293T cells were transfected with GAr-TKΔnls or GAr-TKΔnlsSD$^-$/SA$^-$ using the Ca-Phosphate coprecipitation technique. After 48 h, RNA was extracted using the Absolutely RNA®, RT-PCR miniprep kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's protocol. Superscript II (Clontech) was used to reverse transcribe 4 μg of RNA (DNaseI treated). After reverse transcription, a PCR was performed with primer set 9. The forward primer was chosen to overlap the junction after splicing occurred, to show exclusively spliced, and therefore inactive, TK. The PCR products were analyzed on a 1% agarose slab gel.

Localization study

293T cells were transfected with wt-TK, GAr-TK, GAr-TKΔnls, ΔGAr-TK or ΔGAr-TKΔnls with the Ca-Phosphate coprecipitation technique. After 48 h, cells were fixed with methanol and subsequently incubated with rabbit anti-HSV-TK antiserum (1/250 diluted). After incubation with a FITC-conjugated goat-anti-rabbit secondary antibody and Propidium Iodide staining of the nucleus, the cells were analyzed by a Confocal LaserScan Microscope (CLSM, Leica DM IRBE, Leica microsystems B.V., Rijswijk, The Netherlands) using the Leica confocal software.

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Chapter 5

In-Cis Inhibition of Antigen Processing by the Latency-Associated Nuclear Antigen I of Kaposi Sarcoma Herpes Virus

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In Cis Inhibition of Antigen Processing by the Latency-Associated Nuclear Antigen I of Kaposi Sarcoma Herpes Virus.

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Abstract

Kaposi Sarcoma Herpes Virus (KSHV), also known as Human Herpes Virus 8 (HHV8), can persist as an episome in target cells. The Latency-Associated Nuclear Antigen 1 (LANA-1) is a key component of the latency process, and may be a functional equivalent of the EBNA-1 protein of Epstein-Barr Virus. EBNA-1 can subdue immune recognition by virtue of a long glycine and alanine-rich repeat, which interferes with the proteasomal degradation of EBNA-1 and in this way averts the presentation of antigenic peptides. LANA-1 contains a strongly acidic repeat region of approximately 580 amino acids, which consists almost exclusively of aspartic-acid, glutamine, and glutamic-acid residues. Despite the LANA-1 repeat is not homologous to the EBNA-1 Gly-Ala-rich repeat, we demonstrate that this acidic region interferes with antigen processing *in-cis*. Upon transfection of expression vectors containing LANA-1-eGFP fusion genes the cells did not present an ovalbumin-derived $H2K^b$-restricted CTL epitope inserted at the C-terminus of the fusion protein. Deletion of the central acidic-repeat region of LANA-1 abolished the capacity of LANA-1 to block antigen presentation. Similar to the EBNA-1-derived Gly-Ala-rich repeat, the LANA-1 repeat does not inhibit presentation *in trans:* co-transfection of LANA-1 expression vectors does not inhibit presentation of the OVA epitope from the GFP_{ova} fusion protein. These data demonstrate that the acidic repeat region of LANA-1 can function as *in-cis* acting inhibitor of antigen presentation. This may contribute to the immune evasion of cells latently infected by KSHV.

Introduction

Kaposi Sarcoma Herpes Virus (KSHV) also called Human herpes virus 8 (HHV8), is a gamma herpes virus associated with AIDS-related lymphoproliferative disorders [20,25]. Like other herpes viruses KSHV can persist for life in a latent form in infected cells [5,29,33].

One of the most studied proteins implicated in herpes virus latency is the Epstein-Barr Virus (EBV) Nuclear Antigen 1 (EBNA-1). EBNA-1 binds to the viral origin of replication and to metaphase chromosomes [14 17,30,37], thus allowing EBV episomal maintenance within the infected cell and equal partitioning to the daughter cells. EBNA-1 is the only viral protein present in all EBV-associated tumours. By a mechanism not totally understood yet, the Gly-Ala repeat can interfere with the proteasomal degradation and prevent cytotoxic T lymphocyte epitope generation [15,16,21]. The current model explaining this phenomenon dictates that a long stretch of alanines interspaced by 1, 2 or 3 glycines would give to EBNA-1 the appropriate conformation to interact with a proteasomal component that contain a hydrophobic pocket [27]. Although this 30 to 200 GA repeat (depending on virus isolates) confers a benefit mechanism

for the virus, it appears that this sequence has not been evolutionary conserved, since none of the other herpes virus proteins contain a homologous repeat.

Like EBV, KSHV infects human B cells, macrophages, endothelial cells, and epithelial cells. The infected cells are not eradicated from host cells by the immune system, suggesting that KSHV developed a strategy to evade the immune system [5,12,13,24]. During latency, KSHV persists as a multicopy circular episomal DNA in the nucleus, and here it expresses a small subset of viral genes. One of the proteins encoded by these genes is the Latency-Associated Nuclear Antigen 1, LANA-1 (also called LNA or LNA-1), the EBNA-1 homologue in KSHV [1]. LANA-1 is a large multi-functional nuclear protein of 1162 amino acids, expressed from ORF73, and is involved in numerous cellular processes [9]. It has been reported to improve dissemination of KSHV by modulating expression of oncosuppressor proteins [6,8,23]. The central region of LANA-1 is occupied by a long acidic sequence that can be arbitrary divided in 3 parts: aspartic acid/glutamic acid (DE), glutamine/glutamic acid (QE), and aspartic acid/glutamin (DQ) [2] repeats. The function of the central domain is not well described yet, but seems to be required for the activation of the latent EBV promoter Cp[34]. LANA-1 contains two nuclear localisation signals (nls), one located in the N-terminal part (aa 24 to aa 30) [22] and one in the C-terminal part [26]. LANA-1 is associated with heterochromatin during interphase and with chromosomes during mitosis [19,31].

Here we demonstrate that the Latency-Associated Nuclear Antigen-1 of KSHV can inhibit the presentation of antigenic peptides. In this respect it resembles latency-associated proteins in EBV and MHV68 [1]. The mechanism involved is not clear but it is evident that the long central acidic region of LANA-1 is required for this inhibition.

Results

To examine the effect of LANA-1 on antigen presentation in vitro, we generated a Green fluorescent protein gene that carries the codons for a heterologous CTL epitope (Fig.1A). The resulting protein GFP_{ova} can be used to monitor the antigen presentation with the well-characterized B3Z CTL hybridoma cell line [28]. Briefly, B3Z can recognize Ova peptide (SIINKFEL) when the peptide is presented in the context of $H2K^b$ MHC I. Recognition induces expression of the IL-2 gene, which can be monitored by the induction of an heterologous IL-2 promoter linked to the E.coli *lacZ* gene. Responsiveness of B3Z to GFP/GFP_{ova} transfected cells, evaluated by LacZ quantification, shows 8 fold increase in $H2K^b$ context, whereas no induction is observed without the appropriate MHC I molecules (Fig.1B), demonstrating the specificity of the assay.

To assess whether LANA-1 or EBNA-1 can affect this presentation, $H2K^b$ 293T cells were co-transfected with GFP_{ova} and increasing amounts (0, 0.05, 0.1, 0.2, 0.8 µg DNA) of LANA-

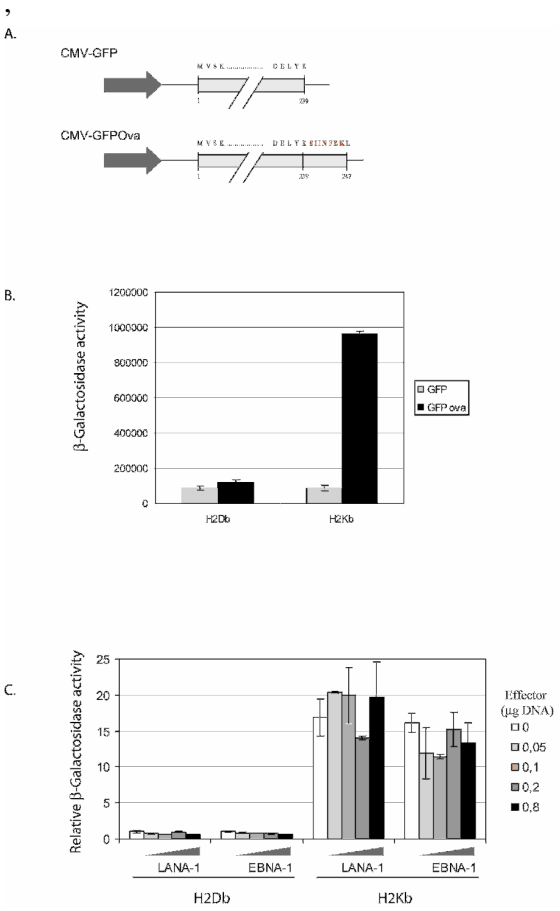

Figure 1
EBNA-1 and LANA-1 have no *in-trans* acting inhibitory effect on OVA presentation. (A) Schematic diagram of GFP and GFP_{ova} showing the insertion of the octameric antigenic peptide (OVA) SIINFEKL. (B) B3Z assay on OVA expressing cells. 293T cells were transfected with 0.8 µg of pRRL-CMV-GFP/GFP_{ova} and 0.2 µg MHC I encoding plasmid (pcDNA $H2K^b$/$H2D^b$). β-galactosidase activity was measured 24h after co-culture (48h post transfection). (C) Trans-inhibition B3Z assay. 293T cells were cotransfected like previously (1B) with increasing amounts of pCMV-EBNA-1 or pcDNA3.1-LANA-1. Point "0" $H2D^b$ is used to normalized *LacZ* activity and arbitrary set to 1.

1 or EBNA-1 expressing vector. In the negative control (GFP_{ova} only), we observed, like previously, a strong induction of β-galactosidase activity in the presence of $H2K^b$ indicating that the Ova epitope is efficiently recognized by the B3Z hybridomas (Fig.1C). As expected, EBNA-1 does not affect the antigen presentation *in-trans*, as is evident from the absence of significant differences in *LacZ* expression upon EBNA-1 co-transfection (p=0.0901 compared to mock-transfected cells at 0.8 µg DNA). Also LANA-1 did not affect antigen presentation *in-trans* in this assay (p=0.2702).

To evaluate whether LANA-1 could inhibit antigen presentation *in-cis* we generated new GFP_{ova} fusion proteins (Fig.2A). We and others have shown previously that the Gly-Ala repeat of EBNA-1 can interfere with the antigen processing [21]. We fused the N-terminal part of EBNA-1 to GFP_{ova}, generating the EBNA-GFP_{ova} to serve as a positive control in our experiments. A similar construct was generated in which the Gly-Ala repeat of EBNA-1 was replaced by LANA-1, resulting in LANA-GFP_{ova}. From the latter clone a deletion construct was made from which the codons for the central acidic region, i.e. aa 360 to aa 911, were

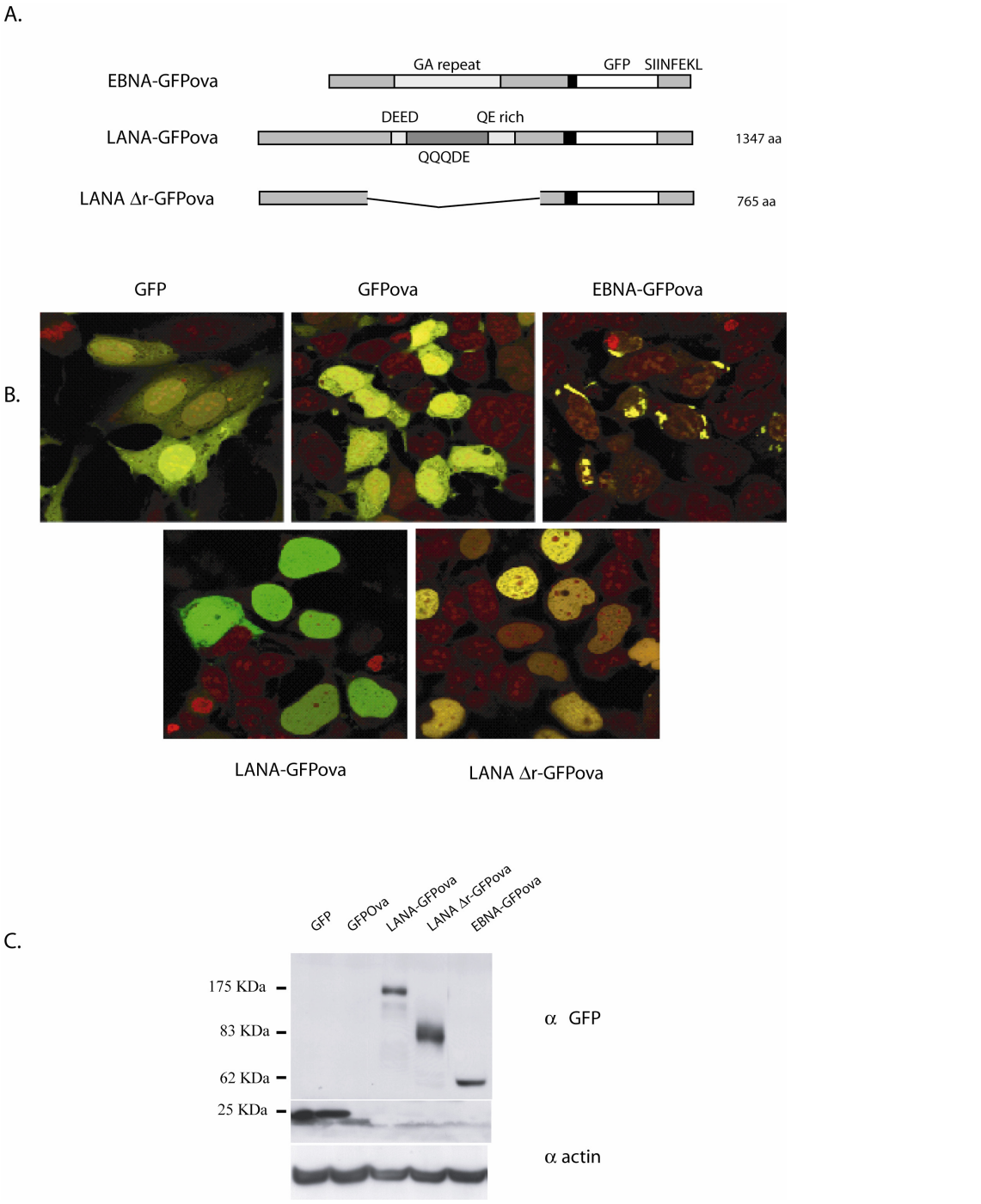

Figure 2

GFP fusion proteins. (A) Schematic diagram of pRRL-CMV-EBNA-GFP$_{ova}$, pRRL CMV-LANA-GFP$_{ova}$ and pRRL-CMV-LANAΔr-GFP$_{ova}$. (B) Cellular localization of modified GFP. Confocal microscopy pictures taken 60h after transfection in 911 cells. (C) Western blot analysis of protein extracts from 911 transfected cells expressing GFP / GFP$_{ova}$ / LANA-GFP$_{ova}$ / LANAΔr-GFP$_{ova}$ / EBNA-GFP$_{ova}$. Anti-GFP antibody is used for the detection, and loading control is provided by anti-Actin.

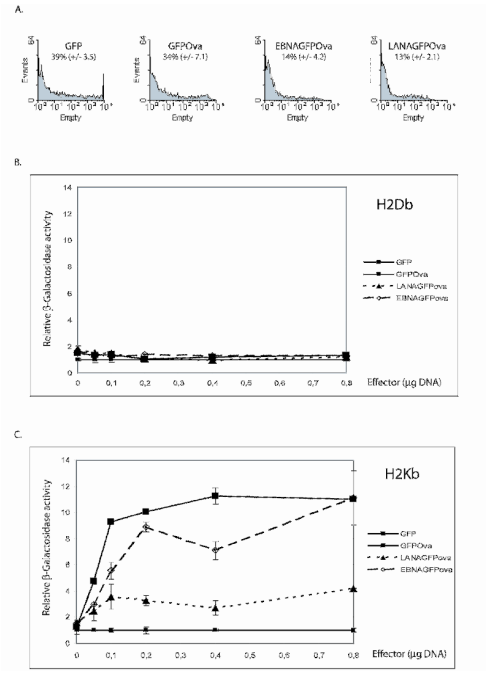

Figure 3
LANA acts as an *in-cis*-inhibitor of antigen processing. (A) FACS analysis of 293T transfected cells. 15h after transfection with 0.8 µg of GFP / GFP$_{ova}$ / EBNA-GFP$_{ova}$ / LANA-GFP$_{ova}$ expressing vector, percentage of GFP positive cells is determined in order to evaluate the amount of target cells. Ratio 1:2 is used between GFP positive cells and B3Z hybridomas. (B)&(C) *LacZ* activity expressed from the B3Z CTL after co-culture with 293T transfected cells. GFP is used to normalized *LacZ* activity and arbitrary set to 1.

removed (Fig. 2A). In the LANA-1 fusion genes the codons for the nls sequences have been retained leading to predominant nuclear localization of the fusion proteins (Fig. 2B). EBNA-GFP$_{ova}$ is present as the specific stippled perinuclear pattern described before. We checked the expression level of each fusion protein by western analysis. All constructs (GFP, GFP$_{ova}$, LANA-GFP$_{ova}$, LANAΔr-GFP$_{ova}$, EBNA-GFP$_{ova}$) are expressed at equal levels and the fusion proteins migrate at the position consistent with their expected molecular weights of 27, 28, 170, 85, and 64 kDa, respectively (Fig.2C).

To evaluate the ability of LANA-1 to inhibit the ova-peptide presentation *in-cis*, we co-transfected 293T cells with increasing amounts (0, 0.05, 0.1, 0.2, 0.4, 0.8 µg DNA) of the expression vectors for GFP, GFP$_{ova}$, EBNA-GFP$_{ova}$, and LANA-GFP$_{ova}$ and either H2K^b or H2D^b MHC I expression vectors. The percentages of target cells expressing the GFP protein was determined by FACS analysis (Fig.3A). A ratio 1:2 between GFP-positive cells and B3Z hybridomas was used. Sixteen hours post transfection, no significant differences were noted either in percentage of GFP positive cells or in mean fluorescence intensity (MFI) (data not shown) between the different constructs. In the presence of H2K^b, we observed a strong response of B3Z against the GFP$_{ova}$ expressing cells. The activation is specific since no *LacZ* activity was detected in the H2D^b context or in the GFP expressing cells. Furthermore, the dose-response curve reaches a plateau already at 0.1 µg DNA with a maximal induction comprised between 8 and 12 times compared to the background signal (i.e. GFP without the OVA epitope). When LANA-1 is fused to GFP$_{ova}$ we found a significant (60%-80%)

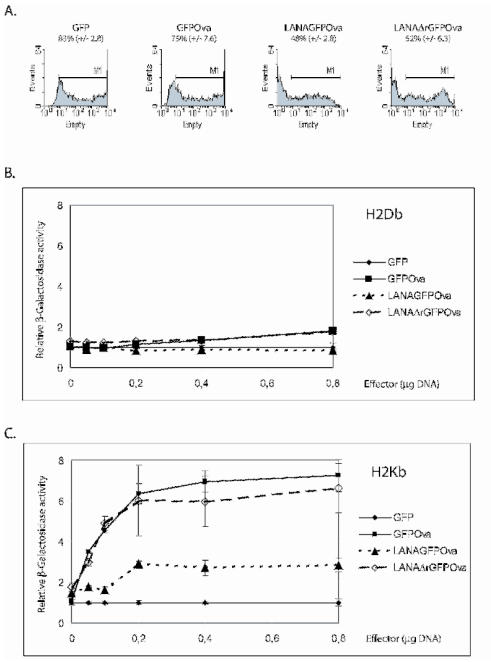

Figure 4
The acidic region from LANA-1 is required for a maximal inhibitory effect. (A) FACS analysis of 293T transfected cells 30h after transfection. (B) & (C) β–galactosidase activity. Similar experiment as described in figure 3 with 293T cells transfected with GFP / GFP_{ova} / LANA-GFP_{ova} / LANAΔr-GFP_{ova} expressing vectors

inhibition of *LacZ* activity (Fig.3C). Surprisingly, we noticed that the CTL were able to recognize the EBNA-GFP_{ova} expressing cells (5 to 10-fold *LacZ* induction and no statistical differences were observed at 0.8 µg between GFP_{ova} and EBNA-GFP_{ova} expressing cells).

Similar assays were conducted to further examine this inhibitory effect of LANA-1. 293T cells were co-transfected with increasing amounts of GFP, GFP_{ova}, LANA-GFP_{ova} or LANAΔr-GFP_{ova} expression vectors and mixed with B3Z cells (Fig 4). Again B3Z cells did not recognize the Ova epitope in $H2D^b$ context, and LANA-1 can inhibit the ova presentation (more than 60% of inhibition compared to GFP_{ova} transfected cells with 0.1 and 0.8 µg DNA) (Fig.4B). Interestingly, Fig 4C shows that after deletion of the central region, LANA-1 loses its capacity to inhibit the response of the hybridoma against OVA. Indeed, the difference between GFP_{ova} and LANAΔr-GFP_{ova} transfected cells is not significantly different ($p = 0.0772, 0.2745, 0.8154, 0.4121$, and 0.6345, respectively, at 0.05, 0.1, 0.2, 0.4, and 0.8 µg DNA).

Taken together these results suggest that LANA-1 from the KSHV can function as an *in-cis* acting inhibitor of antigen processing. This activity is dependent on the presence of the large central acidic-repeat region.

Discussion

In KSHV LANA-1 is one of the subset proteins expressed during the latency phase. Also, it is expressed at high level in all Kaposi sarcoma (KS) associated malignancies [38] [39]. Here we demonstrate that LANA-1 can prevent the presentation of linked antigens by MHC I. This may facilitate LANA-1 to prevent eradication of LANA-1 producing cells by the cellular immune system. So far, only the K3 and K5 gene products of KSHV were know to be involved in immune evasion[7]. The K3 and K5 gene products block the endocytosis of MHC I molecules[3] and the down-regulate accessory proteins involved in T-cell stimulation[4]. The fact that LANA-GFP$_{ova}$ can escape recognition by the OVA-peptide specific B3Z hybridoma suggests that LANA-1 is immunologically invisible. The central role of LANA-1 in latency and long-persistence of KSHV genome in mammalian cells, is supported by LANA disruption studies after transposon-based mutagenesis on a KSHV BAC[38].

Functional similarities between the latency-associated proteins of EBV (EBNA-1) and MHV (ORF73), demonstrate that KSHV kept a similar but still unknown mechanism to control latency. However, it appears that the presence of Gly-Ala repeat is not a prerequisite to facilitate evasion of the immune system during latency since MHV-68 ORF73 lacks the long alanine stretch. Although surprisingly, our results indicate that the Gly-Ala repeat, in this system, is unable to protect the OVA presentation, these results are consistent with IFNγ release assay performed with EBNA1-GFP or EBNA1-GAr-del-GFP[35] (GAr deleted form).

Despite the controversy around this inhibitory effect[32] [36], we and others, have already demonstrated, under certain conditions, that the Gly-Ala repeat can be very efficient as well[21], suggesting that the effectiveness of immune evasion is transgene and context-dependent. Another hypothesis we cannot exclude is the proteolysis of our fusion protein that then generates unprotected OVA epitopes. Interestingly, it seems that in addition to the 80kDa band observed on the western-blot, EBNA-GFP$_{ova}$ can generate a smaller product (around 30kDa) that could carry the OVA epitope but would not be linked to the protective Gly-Ala repeat (data not shown).

Even if the proteasome plays a central role in class I antigen generation, more than 70% of the antigenic peptides secreted comes from Defective Ribosomal Products (DRiPs) constituting the primary source of CD8$^+$T cell peptides [39]. This could indicate that the inhibition in the case of LANA-1 could also rely on a more accurate control (quantitative or qualitative) of the protein synthesis –described before for EBNA-1[40]. On the other hand, some particular characteristics previously reported, as a possible posttranslational modification like sumoylation[18,34], that could interfere with antigen generation by stabilizing LANA-1, or the specific charge structure of the protein (basic N and C-terminal ends and acidic central region) [18]could be some other pathways to investigate.

Remarquably, it is interesting to note that even if EBV is so far the only characterized gamma herpes virus that kept a GA repeat during the evolution, a frame shift on this repeat

can generate a new repeat that presents more than 65% of homology with the central region of LANA-1 (Fig.5). These results combined to the one recently described by *Bennett et al*, on ORF73 from MHV68 [1] showed that these viruses developed different strategies aiming at a similar goal, the escape of the immune system.

A last attracting prospect for this work, besides the fundamental understanding of Herpes virus actions, is the possible utilization of such a sequence in gene therapy approaches. One of the major obstacles in gene therapy is T cell mediated destruction of transgene expressing cells. Until now one of the potential candidates that can be used to stealth proteins for the immune system is the Gly-Ala repeat of EBNA-1. Unfortunately, as mentioned previously, recent studies on EBNA-1 reveal its weakness, so it becomes more and more important to find new inhibitory sequences of antigen processing in order to develop "stealth-gene library".

In this perspective LANA-1 can be an alternative to EBNA-1 as *in-cis*-inhibitor of antigen processing since these two proteins does not have any effect *in-trans* on the OVA presentation.

Materials and Methods

Constructs

To generate GFP_{ova} expressing vector, we introduced the Ova epitope (SIINFEKL) by mutagenic PCR (stratagene Kit) on pRRL-CMV-GFP vector using following primers:

Forward: 5'-ACGAGCTGTACAAGAGCATAATTAATTTCGAAAAGCTCTAAGCGGCCGCGTC-3'

Reverse: 5'-GACGCGGCCGCTTTAGAGCTTTTCGAAATTAATTATGCTCTTGTACAGCTCGTC-3'

To check the *in-cis*-inhibitory effect of EBNA-1 and LANA-1 repeats, we fused respectively amino acids 1 to 430 and 1 to 1082 to GFP_{ova}.

pRRL-CMV-EBNA-GFP_{ova} has been generated using a EBNA fragment from pLXRN-EBNA-Luciferase (previously described [21]) in pRRL-CMV-GFP_{ova}.

pRRL-LANA-GFP_{ova} has been generated replacing a SalI/SalI EBNA fragment from pRRL-CMV-EBNA-GFP_{ova} by a HindIII/XcmI fragment from pCDNA3.1 myc-LANA-1 (gift from Kenneth Kaye), creating pRRL-CMV-LANA-GFP_{ova}.

pRRL-CMV-LANAΔr-GFP_{ova} construct was generated by deleting the central region by mutagenic PCR using following primers:

Forward: 5'-GATGACAATGACAATAAGGATATCTTAGAGGAGGTGGAAGAG-3'

Reverse: 5'-CTCTTCCACCTCCTCTAAGATATCCTTATTGTCATTGTCATC-3'

All the fusions were checked by complete sequencing and immunoblot (Fig 2B).

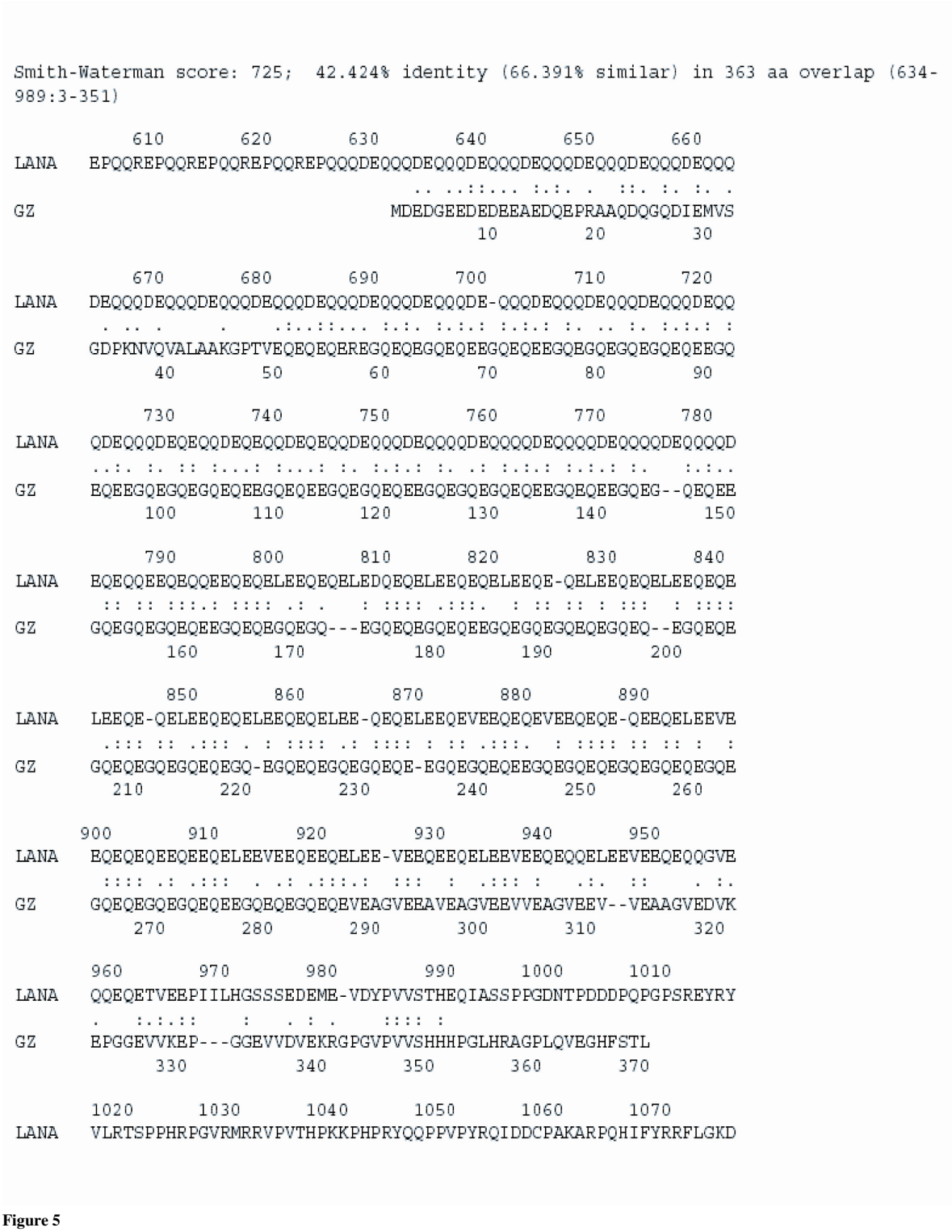

Figure 5

Amino acid alignment of LANA-1 and EBNA-1 frame shift. The EBNA-1 messenger contains a large nested open reading frame. Translation starting at an alternative initiator AUG downstream of the canonical initiation codon would yield an acidic protein (GZ) that presents 42% identity and 66% homology with LANA-1 protein according to SSearch analysis software.

Cell lines

293T, U2OS, and 911 were grown in high glucose DMEM supplemented with 10% fetal bovine serum (Gibco BRL) and penicillin/Streptomycin, supplemented with 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml and 0.2% glucose

The B3Z indicator cells[11] (Kindly provided by R.E.M. Toes) were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 28 µM β-mercaptoethanol and 500 µg hygromycin B per ml.

All cells were maintained at 37 C in a humidified atmosphere of 5% CO2 in air.

Transfection and FACS analysis

293T, U2OS and 911 cells were transfected at 70% confluency using the Calcium Phosphate co-precipitation technique[10]. Transfection in 6-well dishes and 24-well dishes are respectively performed with a total of 5µg and 2µg DNA/well.

24h or 48h after transfection as indicated, cells were resuspended in PBS-/- and the percentage of GFP positive cells was measured by FACS analysis (FACScan Becton-Dickinson). GFP fluorescence was detected using a 530/30 nm bandpass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. Using a forward-scatter/side-scatter representation of events, a region was defined to exclude cellular debris from the analysis. A number of events/FL1 (which reflects the fluorescence intensity) histogram was then established according to this region, and percentages of GFP-positive cells were determined in comparison to the negative control (untreated cells). Data analysis was performed using CellQuest 3.1 software (Becton–Dickinson). For each sample, 10,000 events were collected.

Localization study

911 cells were cultured in 6 well-plates on coverslips. 48h after transfection, cells were fixed with paraformaldehyde (PFA) 2% Tween-20 0.5% and washed 3 times with PBS/tween 0.05%. Nuclear staining was performed using Propidium Iodide solution for 15 min. Slides were then analysed by Confocal Laser Scan Microscopy (CLSM, Leica DM-IRBE).

Western blot

Cells were treated with RIPA lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40 + Protease Inhibitors). Proteins were quantified using BCA kit and 50 µg protein was loaded. The samples were analysed on 15% (GFP/GFP_{ova}) or 8% (EBNA-GFP_{ova}/LANA-GFP_{ova}) polyacrylamide-SDS. Proteins were transferred to Immobilon-P (Immobilon-P transfer membrane (PVDF); Millipore, Etten-Leur, The Netherlands) and treated with anti-GFP (1:1000), and anti-Actin (1:5000, clone C4; ICN Biomedicals, Inc., Zoetemeer, The Netherlands). After incubation with a peroxidase-conjugated goat-anti-mouse

secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). All antibodies were diluted in TBST (0.2% Tween 20, 150mM NaCl, and 10mM Tris) with 5% nonfat dried milk (Protifar Plus; Nutricia BV, Zoetermeer, The Netherlands).

B3Z assay

Determination of Ova presentation was determined as previously described [28]. Briefly, 293T transfected cells were exposed to B3Z CTL. After o/n coculture plates were centrifuged 5 min at 1500 rpm, and lysed in luciferase Lysis Buffer (125 mM Tris-phosphate, pH 7.8, 10 mM CDTA, 10 mM DTT, 50% glycerol, 5% Triton X-100).

β-Galactosidase activity was determined by luminometry as well (Lumat LB9501 luminometer (Berthold, Wildbad, Germany)) using galactolight dual light kit (Tropix).

Each experiment was performed in duplicate and repeated at least 3 times. Statistical analyses were realized using unpaired T test on graphpad.com.

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In cis inhibition of antigen processing by LANA-1 of kaposi sarcoma herpes virus

Chapter 6

General Discussion

General Discussion

The studies presented in this thesis aimed at developing and evaluating a new technique to make transgene products used in gene-therapy applications "invisible" (i.e. stealthed) to the immune system. To this end we used the Gly-Ala repeat (GAr) domain of the Epstein-Barr virus nuclear antigen-1 (EBNA-1). We provided four examples of fusions with the GAr that did not impede the function of the reporter, i.e. the *E.coli*-derived β-galactosidase, herpes simplex virus-derived thymidine kinase (HSV-TK), firefly luciferase, and jellyfish green fluorescent protein (GFP).

We showed that the GAr does not affect induction of transgene directed CTL activity upon adenovirus-mediated transfer and expression of a GAr-LacZ fusion gene. However, recognition of transgene-expressing cells by antigen-specific CTL is abolished sufficiently to prevent cytolysis by β-galactosidase-specific CTL. The observation that the GAr does not inhibit priming of CTL is important and consistent with reports describing the presence of EBNA-1 specific-CTL in EBV-seropositive individuals (Blake et al., 1997; Blake et al., 2000; Rickinson and Moss, 1997). This inability of completely preventing the generation and presentation of antigenic peptides, may be attributed to the fact that the GAr seems not entirely prevent formation of defective ribosomal products (DRiPs) (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). These DRiPs play an important role in antigenic peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001). However, there are marked differences between our studies and the DRiPs study of Tellam and collaborators. They studied the full length EBNA-1 in human B-cells and lymphoblastoid cell lines (LCLs). Whereas specific lysis is observed in the GAr-containing EBNA-1, more efficient lysis could be obtained when the GAr was deleted from EBNA-1, confirming a stealthing effect of the GAr in the natural context. This is in accordance with the data from Lee et al., who also noted a partial protective effect of GAr in its natural context. Furthermore, deletion of the GAr greatly reduces the half-life of EBNA-1 in B cells. This is consistent with a negative effect of the GAr on the efficiency of proteasomal degradation (Levitskaya et al., 1995). In addition, we have studied the effect of the GAr with rather stable proteins and therefore they may require less stabilization by the GAr, to sufficiently inhibit antigenic-peptide formation.

We showed that the GAr does not affect induction of transgene directed CTL activity upon adenovirus-mediated transfer and expression of a GAr-LacZ fusion gene. As antigen presentation by professional APC, most likely DC, is crucial to the initiation of virus-specific CTL responses, the presence of EBNA-1-specific CTL in EBV-positive donors suggests that antigen processing for MHC class I by specialized APC is not hampered by the GAr. Our data strongly suggest that cross-priming does occur, since we could show priming in an indirect

manner (non-matching MHC class I molecules). The presence of this EBNA-1-specific CTLs points to the possibility that GAr does not affect the processing of exogenously acquired EBNA-1 antigens. This can be explained by the fact that in the cross-priming pathway upon phagocytosis of exogenous proteins there is limited proteolysis and these degradation products are exported to the cytosol (Lizee et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Ackerman et al., 2003). This limited proteolysis probably results in the separation of the GAr from the antigenic CTL epitope. In this way, the processing of the CTL epitope is liberated from the inhibitory influence of the GAr on proteasomal antigen degradation, explaining the observation that CTL priming proceeds in an uninhibited fashion.

In addition to the GAr, we showed that an alternative ORF in EBNA-1 can also inhibit presentation of linked antigens. This ORF contains a repeat of the same length as GAr and is rich in Gly (G), Glu and Gln (Z) residues. Therefore we named it the GZ-rich repeat. Remarkably, this repeat is strongly similar to the last one-third of the latency-associated nuclear antigen-1 (LANA-1) from kaposi sarcoma herpes virus (KSHV) a.k.a. human herpes virus 8 (HHV-8). This protein is, like EBNA-1, involved in episomal maintenance of the virus genome. Also this repeat region is implicated with inhibition of antigenic presentation. This together suggests that (long) repeats in general have an inhibitory effect on antigen presentation. It is speculated by Sharipo and collaborators (2001) that the inhibitory effect of the GAr requires at least three alanine residues in a β-strand conformation with adjacent hydrophobic binding pockets of a putative receptor (Sharipo et al., 2001). In the case of the GZr and the LANA-1, there are no alanine residues present and since the whole repeat is strongly acidic, it is highly unlikely that there will be hydrophobic pockets involved in the receptor part. This suggests that the GZr and LANA-1 repeats may function in a different way.

In addition to the full-length repeats of GAr and GZr we were able to obtain mini-repeats. These mini-repeats were tested as well since it is known that short EBNA-1 derived glycine and alanine-rich sequence of only 8 amino acids are able to significantly inhibit proteasomal degradation of instable reporter protein (Sharipo et al., 2001; Sharipo et al., 1998; Dantuma et al., 2000). Surprisingly, the mini-repeats blocked presentation more efficiently than the full length repeats. The mechanism for the more potent inhibition is unclear. Maybe these shorter proteins form less DRiPs then longer proteins, on the basis of size and inherent difficulties in folding or assembly (Schubert et al., 2000).

Together, these results make out a small library of inhibitory sequences for the generation of “immunostealth” genes. This is useful in gene therapy approaches where a life-long restoration of the defects is required. Furthermore, it can be used to create safer suicide genes from bacterial or viral origin. The most widely used suicide gene is the HSV-TK. Since this is a viral gene, problems can occur with its use. Indeed, upon infusion of HSV-TK-expressing T lymphocytes, 8 of 24 recipients developed an immune response against the transgene product (Thomis et al., 2001). To circumvent this problem attempts to use human

genes as suicide system, have been done. Of course in the use of human genes for suicide gene therapy, as is the case with Fas (Thomis et al., 2001) or caspase 8 (Carlotti et al., 2005) stealthing may not be necessary. However, the TK and ganciclovir combination has a very useful bystander effect, which makes this combination the system of choice for many gene therapy applications.

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Chapter 7

Summary & Samenvatting

Summary

Gene therapy is a potentially powerful form of molecular medicine and potentially broadly applicable. It would provide treatments for a large number of inherited and acquired diseases. Ideally, the affected gene should be replaced, but most protocols aim at adding a correct copy of the afflicted gene. Often viruses are used to get the gene transferred into the cells. This can lead to an immune response against the vehicle, i.e. the virus. Expression of a "new" gene may also lead to synthesis of proteins foreign to the immune system. Cells producing these proteins will therefore be recognized as infected or aberrant and targeted for destruction. This is of course an unwanted effect in those applications where a life-long repair of the defect is desired.

In **Chapter 1**, an overview is given of both immune responses against one of the most frequently used viral vectors, the adenovirus, and immune responses against transgene products. For better understanding this issue is discussed in the context of the current state of cancer- and gene therapy. In addition, a brief description is provided on how the immune system recognizes the presented peptides as well as viral mechanisms to evade the system.

In **Chapter 2**, we use one of the known immune evasion mechanisms to "stealth" transgenes products. Here, we fuse the codons for the Gly-Ala repeats (GAr) of the Epstein-Barr Virus (EBV) nuclear antigen 1 (EBNA-1) with three different transgenes, i.e. *E.coli* LacZ, firefly Luciferase and herpes simplex virus 1 (HSV1) thymidine kinase (TK). We show that fusion of the GAr does not lead to significant reductions in the activities of these proteins. Interestingly and to our surprise, the GAr-LacZ fusion protein is capable of inducing CTLs. However, when we tested cells infected with GAr-LacZ for recognition by LacZ specific CTLs, these cells were protected against lysis. This apparent paradox can be explained by cross-priming. We showed that in the absence of the correct MHC class I molecules, CTLs can be primed, indicating that cross-priming does occur. Moreover, we showed in animal experiments that GAr-LacZ-expressing cells resided longer in the body than the non-modified LacZ expressing cells. From these data we concluded that the EBV GAr system could well be used in gene therapy settings to prevent the immune system of reacting on transgenes.

Upon closer examination of the EBNA-1 open reading frame (ORF), we found a nested (frame-shifted) ORF. This alternative ORF would yield a strongly acidic protein with a calculated molecular weight of 40.7 kDa. This protein would contain a 238 amino-acids long glycine, glutamine, and glutamic acid-rich repeat. The polypeptide tract rich in Gly and Glx residues was named GZ- repeat (GZr) region. In **Chapter 3**, we fused this nested ORF (GZr) with the LacZ gene and were able to show β-galactosidase activity upon transfer of the fusion gene into cultured cells. We set out to test this new repeat next to the original GAr, two mini constructs consisting of one third of the normal GAr or GZr and a construct lacking all repeats. All constructs were equally active for β-galactosidase and tested for recognition by LacZ specific CTLs. This showed that, in contrast to the repeat deleted construct, all the

constructs bearing repeats were at least partially protected for recognition by LacZ specific CTLs. Interestingly, the mini repeats, both miniGAr and miniGZr, were better in protecting the cells against recognition. Since the repeat-deleted construct and the normal LacZ-expressing cells were recognized, we were able to show that this is a repeat-dependent mechanism. This shows that the GZ repeats might be another candidate to shield transgenes for the immune system.

One of the examples of a transgene where an immune response was mounted against, is the herpes simplex virus 1 (HSV1) thymidine kinase (TK). In **Chapter 4**, TK was fused to the GAr to try to blunt the immune response against it. Since the EBNA-1 protein resides in the nucleus, it contains a nuclear localization signal (nls). Although there are no clues that a nuclear localization of TK is detrimental to the activity, we did remove the nls. This indeed restored the TK localization to normal and the GAr alone did not influence the localization. We introduced also some mutations in the TK to obtain a more sensitive and unsplicable TK. In our cell system the mutations did not have any effect on the activity. To evaluate the protective effect of the GAr, we introduced a marker CTL epitope in the constructs. Insertion of the ovalbumin (OVA) epitope did not alter the activity of TK as well. When cells expressing TK harboring the OVA epitope and GAr-TK with the OVA epitope, were cocultured with B3Z cells, capable of recognizing the OVA epitope, it was clear that the GAr could shield OVA for these cells. We therefore suggest that GAr-TK might be a good candidate to use in bone marrow transplantation protocols.

The Kaposi sarcoma herpes virus (KSHV) / human herpes virus 8 (HHV-8) has a protein that is, like EBNA-1, involved in maintenance of the episome and therefore also in establishing latency. This protein, latency-associated nuclear antigen-1 (LANA-1), has a long acidic repeat where the last part is similar to the newly found GZ repeat. In **Chapter 5** we investigated LANA-1's capabilities in immune evasion. When tested *in-trans* with a GFPOva fusion, both the EBNA-1 and LANA-1 failed to protect OVA for recognition. Then, a fusion was made of LANA-1 and GFPOva and similarly a GArGFPOva fusion protein. Both were expressed equally and localization studies confirmed that also LANA-1 has an nls. When the constructs then were tested for recognition by B3Z cells, LANAGFPOva showed clear reduction in recognition compared to GFPOva. In this setting however, the GArGFPOva fusion was not as efficient as we have previously seen. The repeat units in LANA-1 are also responsible for the protective effect like the GAr and GZr in EBNA-1. This was shown using a repeat-deleted construct, LANAΔrGFPOva, which showed similar characteristics as the GFPOva. In conclusion, this study describes for the first time an immune evasion effect of another herpes virus then EBV. Like EBNA-1 it also depends on repeats, but the mechanism by which it inhibits presentation of linked antigens remains to be established.

Samenvatting

Gentherapie is een potentieel effectieve vorm van moleculaire geneeskunde en potentieel wijd inzetbaar. Het zou een oplossing kunnen bieden voor zowel erfelijke en andere ziekten. In het gunstigste geval zou het beschadigde gen moeten worden vervangen door een gezond gen, maar de meeste gentherapie protocollen richten zich op het toevoegen van een correct gen. Voor het toevoegen van het gen in de zieke cellen wordt een vector gebruikt. Die vector, vaak een virus, kan ervoor zorgen dat er een immuun respons ontstaat tegen de gen-drager. Ook de expressie van op deze manier toegevoegde correcte genen kan leiden tot het synthetiseren van eiwitten die onbekend zijn voor het immuunsysteem. De cellen die deze eiwitten produceren zullen daarom als geïnfecteerd of afwijkend worden beschouwd en worden opgeruimd door het immuunsysteem. Dit is natuurlijk een ongewild effect in de toepassing van gentherapie waar een levenslange reparatie van het defecte gen is gewenst.

In **Hoofdstuk 1** wordt een overzicht gegeven van de immuun respons tegen zowel een van de meest gebruikte virale vectoren, het adenovirus, als de gebruikte transgenen en hun transgenproducten. Dit is ter wille van de duidelijkheid geplaatst in een context van de huidige stand van zaken bij kanker- en gentherapie. Daarbij is een korte beschrijving gegeven hoe het immuunsysteem antigene peptiden herkent en presenteert. Ook worden virale mechanismen beschreven om dit systeem te vermijden.

In **Hoofdstuk 2** gebruiken we een bestaand immuunsysteem ontwijkend mechanisme om transgen producten onzichtbaar te maken (te “stealthen”). Hier fuseren we de codons van de Gly-Ala repeats (GAr) van het Epstein-Barr Virus (EBV) nuclear antigen (EBNA-1) met drie verschillende transgenen. We gebruiken het LacZ gen van de darmbacterie *E. coli*, het Luciferase gen van vuurvliegjes en het thymidine kinase (TK) gen van het herpes simplex virus 1 (HSV1). We laten zien dat fusie van deze GAr aan deze transgenen niet leidt tot een reductie in de activiteiten van deze eiwitten. Interessant genoeg en tot onze verbazing is het fusie-eiwit GAr-LacZ wel in staat om CTLs te induceren. Maar als we testen of GAr-LacZ geïnfecteerde cellen worden herkend door LacZ specifieke CTLs, zien we dat ze worden beschermd tegen lysis. Deze ogenschijnlijke paradox kan worden verklaard door het mechanisme van cross-priming. We laten zien dat zelfs in afwezigheid van de correcte MHC class I molecules CTLs kunnen worden geïnduceerd, wat er op wijst dat cross-priming inderdaad gebeurd. We laten in dier-experimenten ook nog zien dat cellen die GAr-LacZ tot expressie brengen langer in het lichaam blijven dan cellen die het ongemodificeerde LacZ tot expressie bengen. Uit deze data hebben wij de conclusie getrokken dat het EBV-GAr-systeem inderdaad kan worden gebruikt om een immuun respons tegen de gebruikte transgenproducten te voorkomen in gentherapeutische protocollen.

Toen we het EBNA-1 open reading frame (ORF) beter bekeken, konden we ook een intern (frame-shifted) ORF vinden. Dit alternative ORF zou een eiwit tot expressie brengen dat sterk zuur was en een berekend moleculair gewicht heeft van 40.7 kDa. Dit eiwit zou een

lange zure repeat hebben van 238 aminozuren bestaande uit glycine (Gly), glutamine (Gln) en glutamine zuur (Glu). Deze repeat noemden we de GZ-repeat (GZr), omdat die bestaat uit alleen Gly (G) en Glx (Z) aminozuren. In **Hoofdstuk 3** fuseren we deze nieuwe repeat (GZr) uit het nested ORF met het gen voor LacZ. Wanneer we dit fusie gen tot expressie brachten in cellen konden we duidelijke β-galactosidase activiteit aantonen. We testten deze nieuwe repeat samen met de eerder gebruikte GAr, twee mini repeat constructen die maar 1/3 van de normale GAr en GZr bevatten en een construct waar we alle repeats hebben uitgehaald. Al deze constructen lieten gelijke niveaus van β-galactosidase activiteit zien. Daarna testten we ze voor herkenning van het intracellulaire LacZ door LacZ specifiek CTLs. Alle constructen die repeats hadden, waren tenminste gedeeltelijk beschermd tegen herkenning door LacZ specifieke CTLs. De mini repeats, zowel miniGAr als miniGZr, waren beter in het beschermen tegen herkenning. De cellen die normaal LacZ of het repeatloze LacZ tot expressie brachten, werden wel herkend door LacZ speciefieke CTLs. Hierdoor kunnen we concluderen dat het mechanisme van het beschermen van de cellen berust op het hebben van repeats. Bovendien kan de GZr ook gebruikt worden voor het beschermen van transgenen tegen het immuunsysteem.

Een van de voorbeelden waarbij een transgen wordt herkend en opgeruimd door het immuun systeem is het herpes simplex virus 1 (HSV1) thymidine kinase (TK). In **Hoofdstuk 4** hebben we TK aan de GAr gefuseerd om de immuun respons te stoppen. Omdat het EBNA-1 eiwit normaal in de kern aanwezig is, bezit het een nuclear localization signal (nls). Hoewel we geen aanwijzingen hebben dat TK in de celkern minder goed functioneert, hebben we toch de nls uit het construct verwijderd. Die verwijdering zorgde er inderdaad voor dat het fusie eiwit niet meer in de celkern tot expressie kwam. Een fusie van GAr alleen met TK (zonder nls) had geen enkel effect op de localisatie. We hebben mutaties aangebracht in het TK om het gevoeliger te maken voor de pro-drug ganciclovir (GCV). Ook hebben we mutaties aangebracht om ervoor te zorgen dat het actieve deel van TK er niet meer kan worden uitgehaald door middel van splicing. Deze mutaties hadden geen effect op de activiteit van TK. Om beter te kunnen beoordelen wat het beschermende effect van GAr is, hebben we een bekend CTL epitoop van ovalbumine (OVA) als marker ingebouwd in het TK. Het inbouwen van dit OVA epitoop zorgde ook niet voor een verminderde activiteit van TK. We hebben cellen die het GAr-TK met OVA epitoop of normaal TK met OVA epitoop tot expressie brengen samen gekweekt met B3Z cellen. Deze B3Z cellen kunnen het OVA epitoop herkennen waarna ze β-galactosidase to expressie brengen. In onze experimenten konden we na het samen kweken duidelijk zien dat wanneer het OVA epitoop is ingebouwd in GAr-TK er geen herkenning was door de B3Z cellen. Daaruit konden we concluderen dat GAr het TK kan beschermen en zouden wij het GAr gemodificeerde TK aanbevelen om te gebruiken in beenmerg-transplantatie protocollen.

Het Kaposi sarcoma hepes virus (KSHV)/humaan herpes virus 8 (HHV-8) heeft ook een eiwit dat net als EBNA-1 is betrokken bij het handhaving van het episoom en daarom ook

in het vestigen van latentie. Dit eiwit, latency-associated nuclear antigen-1 (LANA-1), heeft een lange zure repeat waarvan het laatste deel lijkt op de nieuw gevonden GZ repeat. In **Hoofdstuk 5** hebben we LANA-1 getest in het vermijden van de immuun respons. Wanneer we EBNA-1 en LANA-1 *in trans* testten met een GFPOva fusie eiwit, zagen we dat beide eiwitten faalden in het beschermen van OVA. Daarna hebben we een fusie gemaakt van LANA-1 en GFPOva en op dezelfde manier GAr en GFPOva. Beiden kwamen tot hetzelfde expressie niveau en lokalisatie studies toonden aan dat LANA-1 ook een nls heeft. Wanneer de cellen werden getest voor herkenning door B3Z cellen liet LANAGFPOva een duidelijke reductie zien in herkenning ten opzichte van GFPOva. Hier was alleen het beschermende effect van GArGFPOva minder groot dan we eerder hebben laten zien. Ook in LANA-1 zijn de repeats verantwoordelijk voor het beschermende effect net zoals in het geval van de GAr en GZr in ENBA-1. Dit werd duidelijk nadat we konden aantonen dat een construct waar de repeats waren uitgehaald, LANAΔrGFPOva, hetzelfde reageerde dan normaal GFPOva. Dit leidt tot de conclusie dat we voor het eerst hebben kunnen aantonen dat er een immuunsysteem vermijdend effect is van een ander herpes virus dan het EBV. Net zoals in EBNA-1 zijn repeats hiervoor verantwoordelijk. Het mechanisme van de bescherming voor de antigenen die eraan gekoppeld zijn, is nog niet ontrafeld.

List of Abbreviations

aa	amino acid
AAV	adeno-associated virus
Ab	antibody
Ad	adenovirus
Ala	alanine
APC	antigen presenting cell
CLIP	class II-associated Ii peptide
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DRiPs	defective ribosomal products
EBNA-1	Epstein-Barr virus nuclear antigen-1
EBV	Epstein-Barr virus
eGFP	enhanced green fluorescent protein
ER	endoplasmatic reticulum
eYFP	enhanced yellow fluorescent protein
GAr	glycine-alanine repeat region
GCV	ganciclovir
GDEPT	gene-directed enzyme prodrug therapy
Gln	glutamine
Glu	glutamic acid
Glx	glutamine or glutamic acid
Gly	glycine
GVDH	graft-versus-host disease
GZr	glycine-glutamine-glutamic acid repeat region
HCMV	human cytomegalovirus
HHV-7	human herpes virus-7
HHV-8	human herpes virus-8
HIV-1	human immunodeficiency virus-1
HSC	hematopoietic stem cells
HSV-1	herpes simples virus-1
Hyg	hygromycin resistance gene
IFN-γ	interferon-γ
KSHV	kaposi sarcoma herpes virus
LAMP-1	lysosomal-associated membrane protein
LANA-1	latency-associated nuclear antigen-1

MIIC	MHC II compartment
MCMV	murine cytomegalovirus
MEC	mouse embryo cells
MHC	major histocompatibility complex
MLV	murine leukemia virus
MOI	multiplicity of infection
NK	natural killer cell
nls	nuclear localization signal
ORF	open reading frame
OVA	ovalbumin
PFU	plaque-forming units
RCC	renal cell carcinoma
SCID-X1	X-linked severe combined immunodeficiency
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TK	thymidine kinase

List of Publications

1. Ossevoort, M., Visser, B.M.J., Van den Wollenberg, D.J.M., Van der Voort, E.I.H., Offringa, R., Melief, C.J.M., Toes, R.E.M., Hoeben, R.C. Creation of 'stealth' genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1. **Gene Ther.** (2003) 10, 2020-2028

2. Schagen, F.H.E., Ossevoort, M., Toes, R.E.M., Hoeben, R.C. Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion. **Crit. Rev. Oncol. Hemat.** (2004) 50, 50-70

3. Ossevoort, M., Zaldumbide, A., Cramer, S.J., van der Voort, E.I.H., Toes, R.E.M., Hoeben, R.C. Characterization of an Immuno'stealth' Derivative of the Herpes Simplex Virus Thymidine Kinase gene. Accepted for publication in **Cancer Gene Ther.** Advanced online publication, 3 February 2006; doi:10.1038/sj.cgt.7700925

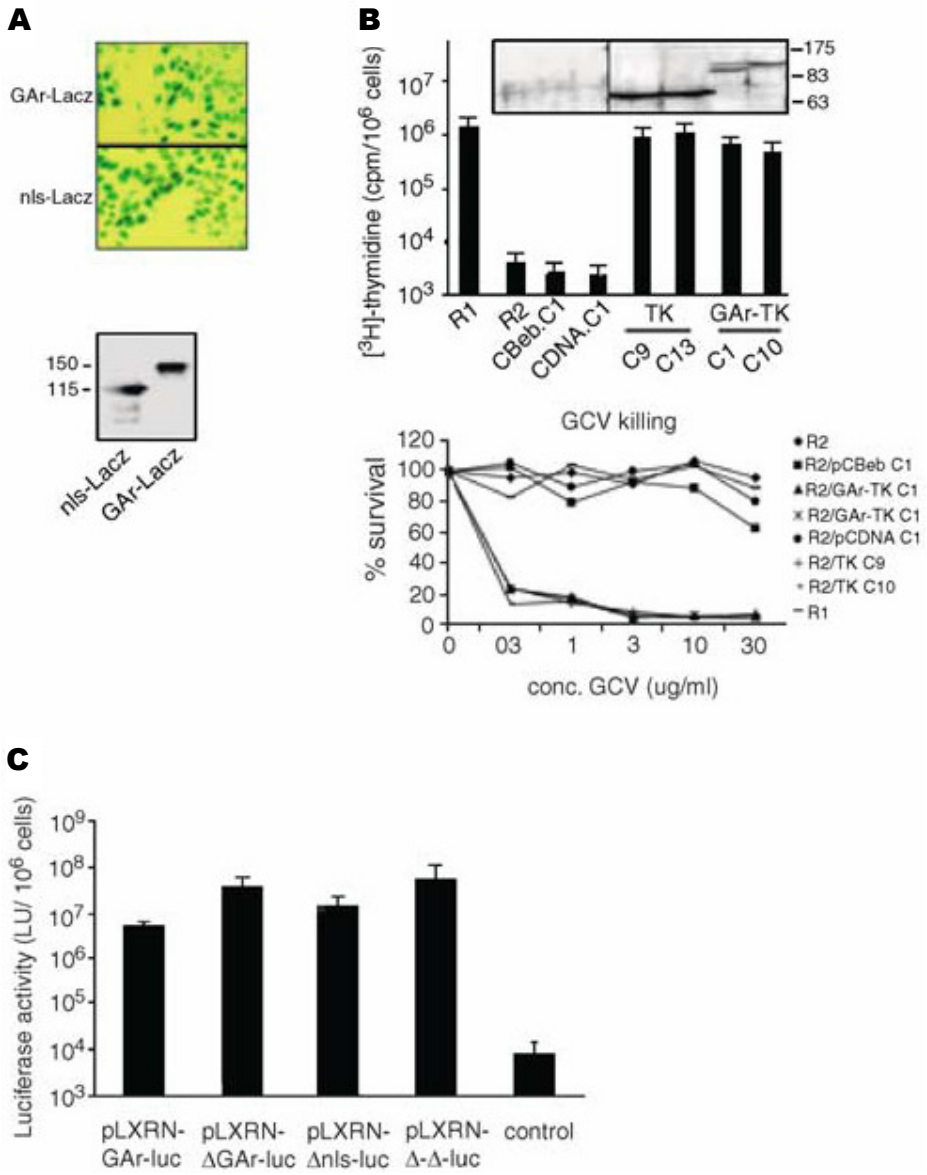
4. Ossevoort, M., Zaldumbide, A., te Veldhuis, A., Cramer, S.J., Hoeben, R.C. The Nested Open Reading Frame in the Epstein Barr Virus Nuclear Antigen 1 mRNA Encodes a Glycine, Glutamic-acid, and Glutamine-Rich Protein, Which Functions As an In Cis-Acting Inhibitor of Antigen Processing. ***Submitted***

5. Zaldumbide, A., Ossevoort, M., Toes, R.E.M., Hoeben, R.C. *In-cis* inhibition of antigen processing by the latency-associated nuclear antigen I of Kaposi Sarcoma herpes virus. ***Submitted***

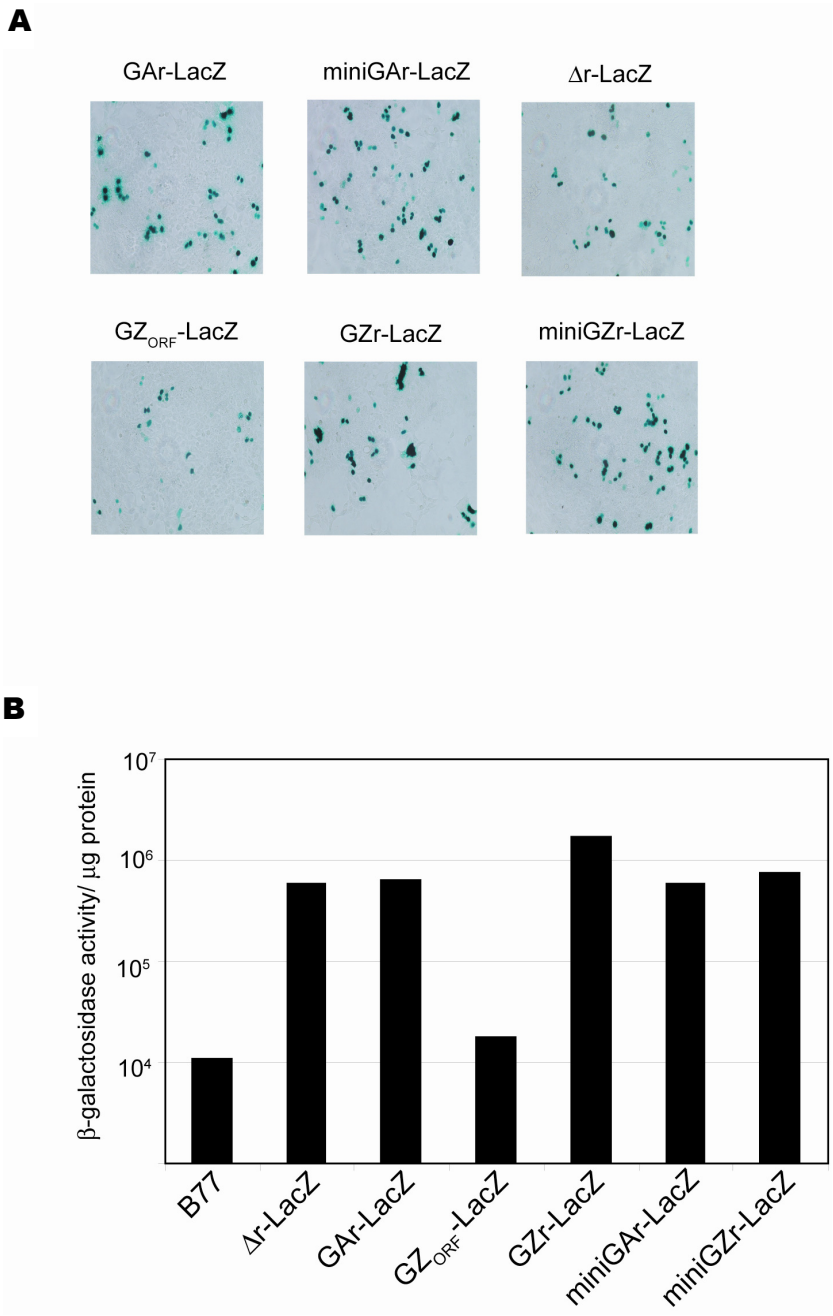
Curriculum vitae

De auteur van dit proefschrift werd geboren op 17 maart 1976 in Waalre. In het jaar 1994 behaalde zij het diploma gymnasium aan het Van Maerlantlysceum in Eindhoven. In datzelfde jaar startte zij met de studie Medische Biologie aan de Universiteit van Utrecht. Tijdens deze studie heeft zij als bijvakstage onderzoek gedaan naar microbiële virulentie factoren op het Eijkman-Winkler Instituut voor Medische Microbiologie (Prof. Dr. H. Van Dijk en Drs. H. Bootsma). Deze stage werd gevolg door de hoofdvakstage op de afdeling Virologie van de faculteit Diergeneeskunde in Utrecht (Prof. Dr. P. Rottier en Dr. H. Glansbeek). In 1999 studeerde zij af en ging zij met behulp van een VSB beurs naar Zweden, waar zij een jaar onderzoek deed aan het Karolinska Instituut (Prof. Dr. H. Garoff). In 2000 werd begonnen met een promotieonderzoek bij de afdeling Moleculaire Celbiologie van het Leids Universitair Medisch Centrum. Onder leiding van Prof. Dr. R. C. Hoeben vond het in dit proefschrift beschreven onderzoek plaats. Tegenwoordig is zij werkzaam als onderzoeker in het Leiden/Amsterdam Center for Drug Research (LACDR) bij de vakgroep Toxicologie van Prof. Dr. B. van de Water in Leiden.

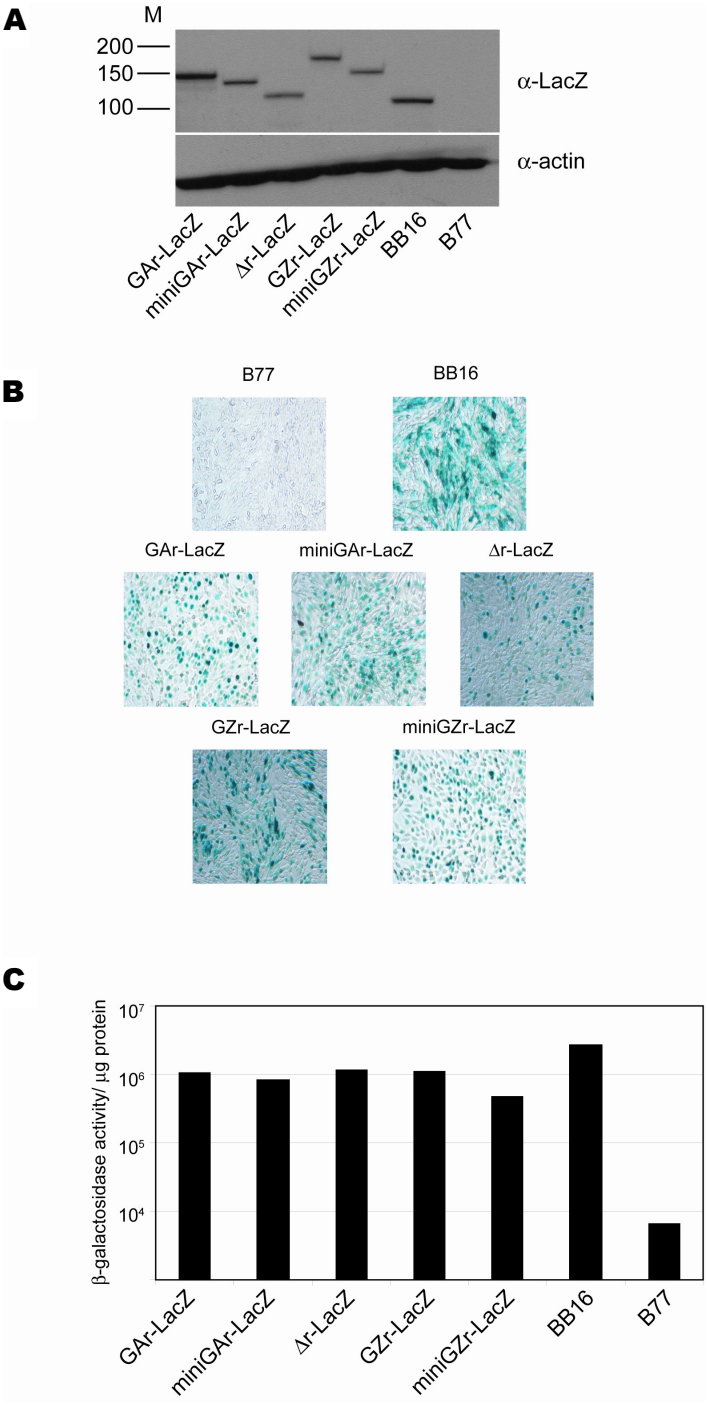
Appendix: color figures



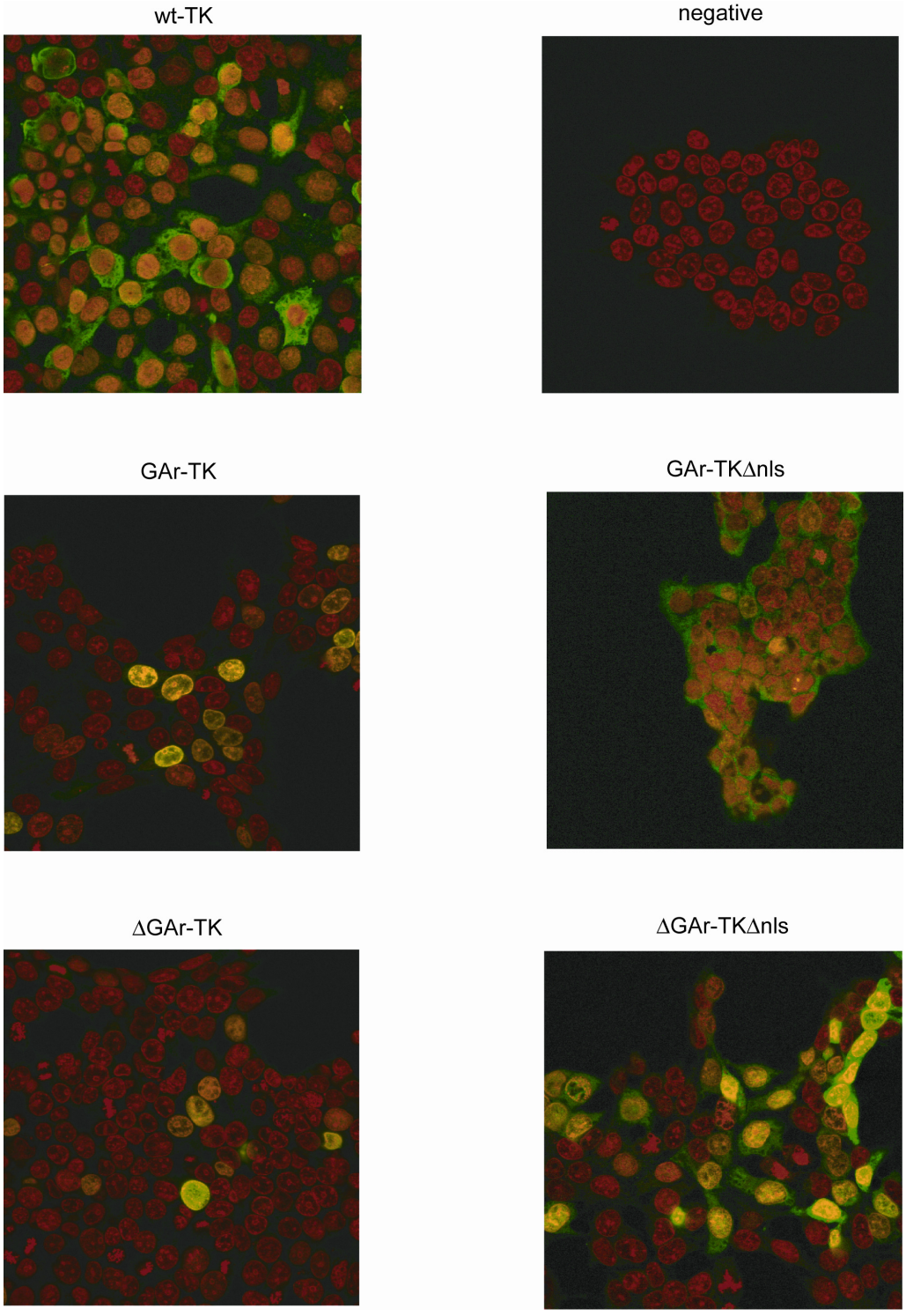
Chapters 2, figure 2. GAr fusion enzymes retain their activity. (a) Activity of GAr-β-galactosidase. Hep2 cells were infected with rAd5-nls-LacZ or rAd5-GAr-LacZ (4 PFU/cell). At 48 h postinfection, the cells were fixed and stained for β-galactosidase activity. In addition, H_{1299} cells grown in 10-cm dishes were infected with rAd5-nlsLacZ or rAd5-GArLacZ with 10 PFU/cell. After 48 h, protein extracts were made and size-fractionated by SDSPAGE, transferred to nitrocellulose membranes and probed with an anti-LacZ mouse monoclonal antibody (Roche, Basel, Switzerland). (b) Activity of GAr-TK. The TK-deficient cell line Rat2 (R2, TK^-) was used to generate stable cell lines containing the various plasmids. CBeb.C1 and CDNA.C1 are G418-resistant Rat2 cells obtained after transfection of the empty pCBeb and pCDNA3.1 plasmids, respectively. GArTK.C1 and C10 are independent G418-resistant clones of Rat2 cells stably expressing the pGAr-TK plasmid. TK.C9 and C13 are two independent G418-resistant Rat2 clones derived by transfection of the plasmid pCDNA-TK. Rat1 (R1) is the TK^+ parental cell line from which Rat2 had been derived. [^{3}H]thymidine incorporation (± s.d.) is represented per 10^6 cells. The inset depicts a Western analysis of the Rat2 clones with a polyclonal goat anti-HSV-TK antibody. The faster-migrating band in clone GAr-TK C1 may result from splicing within the TK coding region.[59] The same cell lines were analyzed for their ganciclovir sensitivity by growing these cells for 48 h in the presence of varying concentrations of ganciclovir. Cell viability was determined with the WST-I colorimetric assay. (c) Activity of GArluciferase. Cultures of 911 cells were transfected with pCBeb (as a negative control), pLXRN-GAr-luc and the GAr- and nls-deletion derivatives. Cells were lysed 18 h post-transfection and the luciferase activity was measured in the lysates. The mean of three experiments is shown, expressed as light units/10^6 cells ± 1 s.d.



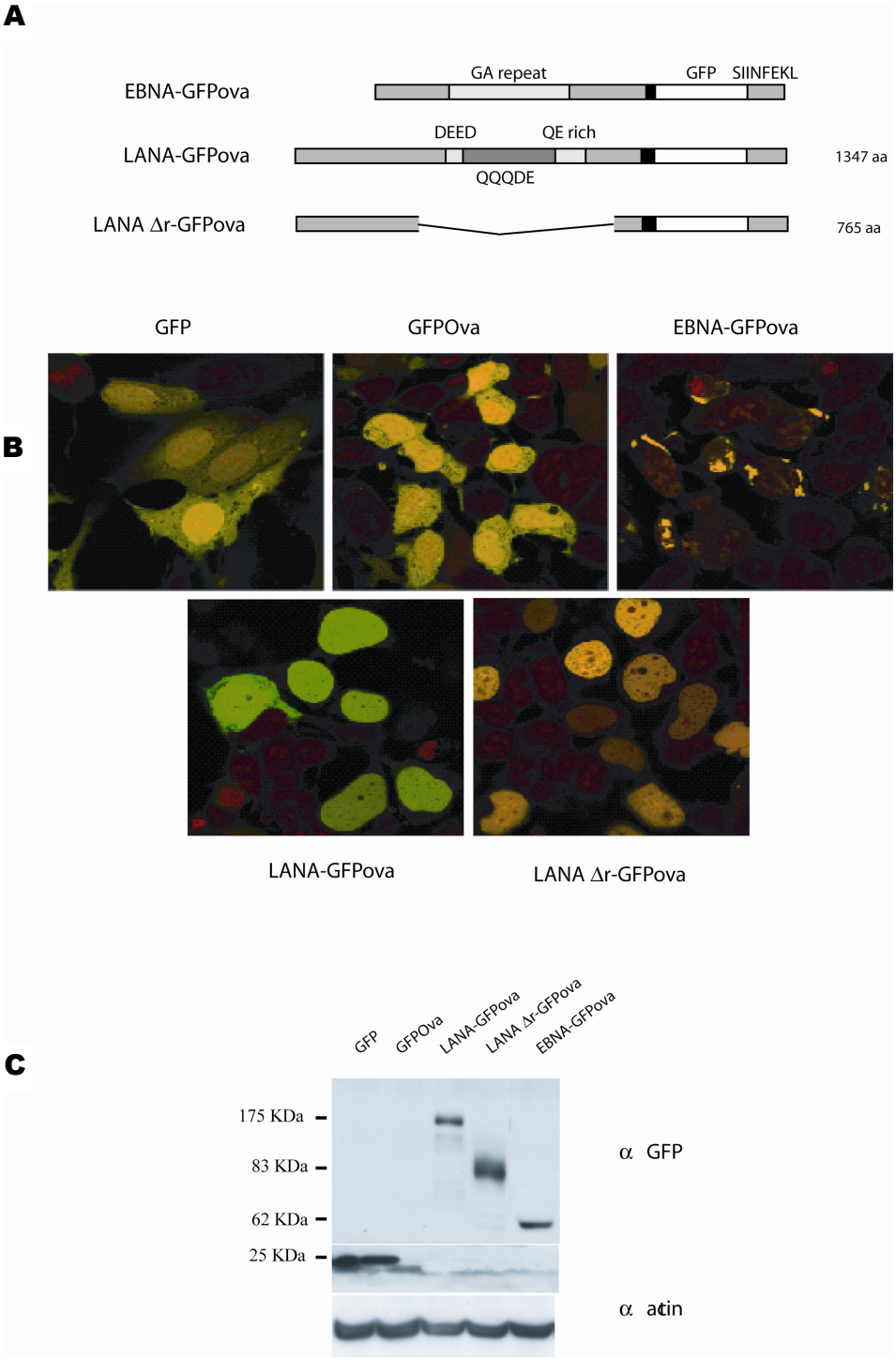
Chapter 3, figure 3. *In-situ* β-galactosidase activity assay (A) and galactolight assay (B) of the transfected 293T cells. A 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ-encoding plasmids. After 48 hours, the cells were fixed, and stained overnight. (B) 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ encoding plasmids. After 48 hours, the cells were lysed and galacton was added. The β-galactosidase activity is normalized for the protein concentration in the lysate.



Chapter 3, figure 4. Characterization of the stable B77 cell lines. B77 cells were transfected with plasmids encoding miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ proteins respectively. After establishing stable cell lines, the cells expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ, as well as B77 and BB16 cells (B77 cells expressing LacZ) were lysed and protein extracts were size fractionated by SDS-PAGE (A). The western blot was analyzed with a monoclonal mouse anti-LacZ antibody. All proteins migrate at the expected size, and no smaller products are visible. (B) The same cell lines, as well as B77 and BB16 were fixed stained *in-situ*. (C) Stable B77 cell lines expressing the miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ constructs, and B77 and BB16 cells were lysed and galacton was added. Plotted is the normalized β-galactosidase activity.



Chapter 4, figure 2. Sub-cellular localization of the different TK proteins. 293T cells were transfected with wt-TK, GAr-TK, GAr-TKΔnls, ΔGAr-TK or ΔGAr-TKΔnls. Cells were incubated with rabbit anti-HSV-TK antiserum and a FITC-conjugated goat-anti-rabbit secondary antibody. The nucleus was stained with Propidium Iodide and the cells were analyzed by a confocal laserscan microscopy. Deletion of the nls but not the GAr restores the wt-TK distribution pattern.



Chapters 5, figure 2. GFP fusion proteins. (A) Schematic diagram of pRRL-CMV-EBNA-GFP$_{OVA}$, pRRL CMV-LANA-GFP$_{OVA}$ and pRRL-CMV-LANAΔr-GFP$_{OVA}$. (B) Cellular localization of modified GFP. Confocal microscopy pictures taken 60h after transfection in 911 cells. (C) Western blot analysis of protein extracts from 911 transfected cells expressing GFP / GFP$_{OVA}$ / LANA-GFP$_{OVA}$ / LANAΔr-GFP$_{OVA}$ / EBNA-GFP$_{OVA}$. Anti-GFP antibody is used for the detection, and loading control is provided by anti-Actin.