ONCOLYTIC ADENOVIRAL VECTORS CODING FOR MONOCLONAL ANTI-CTLA-4 ANTIBODIES

Field of the invention

The present invention relates to the fields of life sciences and medicine. Specifically, the invention relates to cancer therapies. More specifically, the present invention relates to oncolytic adenoviral vectors and cells and pharmaceutical compositions comprising said vectors. The present invention also relates to said vectors for treating cancer in a subject and a method of treating cancer in a subject. Furthermore, the present invention relates to methods of producing monoclonal anti-CTLA4 antibodies in a cell and increasing tumor specific immune response and apoptosis in a subject, as well as to uses of the oncolytic adenoviral vectors for producing monoclonal anti-CTLA4 antibodies in a cell and increasing tumor specific immune response and apoptosis in a subject.

Background of the invention

Cancer can be treated with surgery, hormonal therapies, chemotherapies, radiotherapies and/or other therapies but in many cases, cancers, which often are characterized by an advanced stage, cannot be cured with present therapeutics. Therefore, novel cancer cell targeted approaches, such as gene therapies, are needed.

During the last twenty years gene transfer technology has been under intensive examination. The aim of cancer gene therapies is to introduce a therapeutic gene into a tumor cell. These therapeutic genes introduced to a target cell may, for example, correct mutated genes, suppress active oncogenes or generate additional properties to the cell. Suitable exogenous therapeutic genes include but are not limited to immunotherapeutic, anti-angiogenic, chemoprotective and “suicide” genes, and they can be introduced to a cell by utilizing modified virus vectors or non-viral methods including electroporation, gene gun and lipid or polymer coatings.

Requirements of optimal viral vectors include an efficient capability to find specific target cells and express the viral genome in the target cells. Furthermore, optimal vectors have to stay active in the target tissues or cells. All these properties of viral vectors have been developed during the last decades and, for example retroviral, adenoviral and adeno-associated viral vectors have been widely studied in biomedicine.

To further improve tumor penetration and local amplification of the anti-tumor effect, selectively oncolytic agents, e.g., conditionally replicating adenoviruses, have been constructed. Oncolytic adenoviruses are a promising tool for treatment of cancers and have shown good safety and some efficacy in clinical trials. Tumor cells are killed by oncolytic adenoviruses due to the replication of the virus in a tumor cell, the last phase of the replication resulting in a release of thousands of virions into the surrounding tumor tissues for effective tumor penetration and vascular re-infection. Due to engineered changes in the virus genome, which prevent replication in non-tumor cells, tumor cells allow replication of the virus while normal cells are spared.

Replication can be limited to the tumor tissue either by making partial deletions in the adenoviral E1 region or by using tissue or tumor specific promoters (TSP). Insertion of such a promoter may enhance effects of vectors in target cells and the use of exogenous tissue or tumor-specific promoters is common in recombinant adenoviral vectors.

Most clinical trials have been performed with early generation adenoviruses based on adenovirus 5 (Ad5). The anti-tumor effect of oncolytic adenoviruses depends on their capacity for gene delivery. Unfortunately, most tumors have low expression of the main Ad5 receptor, wherefore modifications have been introduced to the Ad5 capsid. For instance, a capsid modification with the serotype 3 knob has shown improved infectivity and good efficacy in ovarian cancer (Kanerva A, et al., Clin Cancer Res 2002;8:275-80; Kanerva A, et al., Mol Ther 2002;5:695-704; Kanerva A, et al., Mol Ther 2003;8:449-58). Also, as the fiber and the penton base of the Ad vectors are key mediators of the cell entry mechanism, targeting of recombinant Ad vectors may be achieved via genetic modifications of these capsid proteins (Dmitriev I., et al. 1998, Journal of Virology, 72, 9706-9713). Currently most oncolytic viruses in clinical use are highly attenuated in terms of replication due to several deletions in critical viral genes. These viruses have shown excellent safety record, but the antitumor efficacy has been limited.

Clinical and preclinical results show that treatment with unarmed oncolytic viruses is not immunostimulatory enough to result in sustained anti-tumoral therapeutic immune responses. In this regard, oncolytic viruses have been armed to be more immunostimulatory. Moreover, viral replication and expression of immunomodulatory proteins within a tumor potentiates the

immune system by inducing cytokine production and release of tumor antigens (Ries SJ, et al., Nat Med 2000;6:1128-33).

Arming oncolytic viruses combines the advantages of conventional gene delivery with the potency of replication competent agents. One goal of arming viruses is the induction of an immune reaction towards the cells that allow virus replication. As mentioned above, virus replication alone, although immunogenic, is normally not enough to induce effective anti-tumor immunity. To strengthen the induction of therapeutic immunity, viruses have been armed with stimulatory proteins, such as cytokines, for the facilitation of the introduction of tumor antigens to antigen presenting cells, such as dendritic cells, and their stimulation and/or maturation. Introduction of immune-therapeutic genes into tumor cells and, furthermore, their translation of the proteins, leads to the activation of the immune response and to more efficient destruction of tumor cells. The most relevant immune cells in this regard are natural killer cells (NK) and cytotoxic CD8+ T-cells.

A key revelation in cancer immunotherapy has been the realization that, due to tumor immune evasions mechanisms, the induction of an anti-tumor immune response is not sufficient to eradicate the disease. Instead, because of the immune suppressive nature of advanced tumors, down-regulation of inhibitory T-cells is also required (Dranoff G., Nat Rev Cancer 2004;4:11-22; de Visser KE et al., Nat Rev Cancer 2006;6:24-37). One of these key regulatory pathways involves cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152), which acts against B7/CD28 mediated stimulation. The preclinical antitumor efficacy of antibodies antagonistic to CTLA-4 has been previously shown in several tumor models (Leach DR et al., Science 1996;271:1734-6; Kwon ED et al., Proc Natl Acad Sci USA 1999;96:15074-9).

Presently, two fully human monoclonal antibodies (mAbs) against CTLA-4 are in clinical development; IgG1 ipilimumab (formerly MDX-010) and IgG2 tremelimumab (formerly CP-675,206). Several published studies attest to the biologic and clinical activity of ipilimumab and tremelimumab in patients with melanoma and other cancers (Kirkwood JM et al., Clin Cancer Res 2010;16:1042-8; Hodi FS et al., N Engl J Med. 2010; 363;8:711-23; Ribas A et al., Oncologist 2007;12:873-83). Although anti-tumor activity has been seen in many trials, also severe and even fatal side effects have been reported. Side effects relate to normal tissues exposure due to systemic administration, while

efficacy is determined by reduction of immune suppression at the tumor. Possibilities for local production of a CTLA-4 mAb are thus warranted.

CTLA-4 is an activation-induced, type I transmembrane protein of the Ig superfamily, expressed by T lymphocytes as a covalent homodimer that functions as an inhibitory receptor for the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) (Ribas A et al., Oncologist 2007;12:873-83). CTLA-4 blockade with mAbs results in increased interleukin-2 (IL-2) and interferon-gamma (IFN-γ) production by lymphocytes; and increased expression of major histocompatibility complex (MHC) class I molecules (Lee KM et al., Science 1998;282:2263-6; Paradis TJ et al., Cancer Immunol Immunother 2001;50:125-33).

One of the main mechanisms that the tumors use to escape anti-tumor immunity is through regulatory T cells (T-Reg). Among the several approaches that have been used thus far to downregulate T-Reg, the anti-CTLA4 mAb is the only one whose safety and efficacy has been proven in a large randomized study (Hodi FS et al., N Engl J Med. 2010; 363;8:711-23). Although this trial represents a breakthrough for tumor immunotherapy, it was preceded by a negative Phase 3 study with another anti-CTLA4 mAb (Ribas A et al. J Clin Oncol ASCO suppl. 2008; 287). Also, in all anti-CTLA4 mAb trials severe immune-related adverse events (irAEs) have caused mortality resulting in concern over the safety of the approach. Therefore, further approaches, such as utilization of a gene therapy platform, are needed, because it could increase local concentration for enhanced efficacy while reducing side effects related to systemic exposure.

It has been reported that combination of tumor-localized anti–CTLA4 scFv expression with methods of systemic T-Reg depletion has a synergistic effect (Tuve S, et al. Cancer Res 2007;67:5929-39). Further, the authors did not observe autoimmune reactions in a murine model when anti–CTLA-4 scFv antibody is continuously expressed by the tumors and anti-CD25 antibodies are given i.p (Tuve S, et al. Cancer Res 2007;67:5929-39). In contrast, when anti-CTLA4 mAb is given systemically together with T-Reg depletion autoimmune reactions are observed (Sutmuller RP et al., J Exp Med 2001;194:823-32; Takahashi T et al. J Exp Med 2000;192:303-10). One possible reason for the synergy is that depletion of T-Regs reduces the number of regulatory cells, while anti-CTLA-4 reduces the activity of

suppressive cells. Moreover, anti-CTLA4 can also reduce the suppression of antigen presenting cells.

Adenoviruses are medium-sized (90-100nm), non-enveloped icosahedral viruses, which have double stranded linear DNA of about 36 000 base pairs in a protein capsid. The viral capsid has fiber structures, which participate in attachment of the virus to the target cell. First, the knob domain of the fiber protein binds to the receptor of the target cell (*e.g.*, coxsackievirus adenovirus receptor, CAR), secondly, the virus interacts with an integrin molecule and thirdly, the virus is endocytosed into the target cell. Next, the viral genome is transported from endosomes into the nucleus and the replication machinery of the target cell is utilized also for viral purposes (Russell W.C., J General Virol 2000;81:2573-2604).

The adenoviral genome has early (E1-E4), intermediate (IX and IVa2) and late genes (L1-L5), which are transcribed in a sequential order. Early gene products affect defense mechanisms, the cell cycle and the cellular metabolism of the host cell. Intermediate and late genes encode structural viral proteins for the production of new virions (Wu and Nemerow, Trends Microbiol 2004;12:162-168; Russell W.C., J General Virol 2000;81;2573-2604; Volpers C. and Kochanek S. J Gene Med 2004;6, suppl 1: S164-71; Kootstra N.A. and Verma I.M. Annu Rev Pharmacol Toxicol 2003;43: 413-439).

More than 50 different serotypes of adenoviruses have been found in humans. Serotypes are classified into six subgroups A-F and different serotypes are known to be associated with different conditions, i.e., respiratory diseases, conjunctivitis and gastroenteritis. Adenovirus serotype 5 (Ad5) is known to cause respiratory diseases and it is the most common serotype studied in the field of gene therapy. In the first Ad5 vectors E1 and/or E3 regions were deleted enabling insertion of foreign DNA to the vectors (Danthinne X, Imperiale MJ., Gene Therapy. 2000;7:1707-1714). Furthermore, deletions of other regions as well as further mutations have provided extra properties to viral vectors. Indeed, various modifications of adenoviruses have been suggested for achieving efficient anti-tumor effects.

Still, more efficient and accurate gene transfer as well as increased specificity and sufficient tumor killing ability of gene therapies are warranted. Safety records of therapeutic vectors must also be excellent. The present invention provides a cancer therapeutic tool with these aforementioned

properties by utilizing both oncolytic and immunotherapeutic properties of adenoviruses in a novel and inventive way.

Brief description of the invention

The object of the invention is to provide novel methods and tools for achieving the above-mentioned properties of adenoviruses and thus, solving the problems of conventional cancer therapies. More specifically, the invention provides novel methods and tools for gene therapy.

The present application describes the construction of recombinant viral vectors, methods related to the vectors, and their use in tumor cells lines, animal models and blood cells of cancer patients and normal donors.

The present invention relates to an oncolytic adenoviral vector comprising

1) an adenovirus serotype 5 (Ad5) nucleic acid backbone comprising a capsid modification, preferably a capsid modification with an adenovirus serotype 3 (Ad3) knob (Ad5/3 capsid chimerism)

2) a 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and

3) a nucleic acid sequence encoding a fully human monoclonal antibody specific for CTLA-4 in the place of the deleted adenoviral genes *gp19k/6.7K* in the E3 region.

The present invention further relates to a cell comprising the adenoviral vector of the invention.

The present invention also relates to a pharmaceutical composition comprising the adenoviral vector of the invention.

The present invention also relates to the adenoviral vector of the invention for treating cancer in a subject.

The present invention also relates to a method of treating cancer in a subject, wherein the method comprises administration of the vector or the pharmaceutical composition of the invention to a subject suffering from cancer, especially from cancer refractory to conventional chemotherapeutic and/or radiation treatments.

Furthermore, the present invention relates to a method of producing a fully human monoclonal antibody specific for CTLA-4 in a cell, wherein the method comprises:

carrying a vehicle comprising an oncolytic adenoviral vector of the invention to a cell, and

expressing a fully human monoclonal antibody specific for CTLA-4 of the vector in the cell.

Furthermore, the present invention relates to a method of increasing tumor specific immune response in a subject, wherein the method comprises:

carrying a vehicle comprising an oncolytic adenoviral vector of the invention to a target cell or tissue,

expressing a fully human monoclonal antibody specific for CTLA-4 (anti-CTLA4 mAb) of the vector in the cell,

increasing the amount of anti-CTLA4 mAb production in tumors (but not normal tissues) by virtue of tumor-specific replication of the genome of the virus.

Still, the present invention also relates to a use of an oncolytic adenoviral vector of the invention for producing a fully human monoclonal antibody specific for CTLA-4 in a cell.

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The present invention provides a tool for treatment of cancers, which are refractory to or incurable by current therapeutic approaches. Also, restrictions regarding tumor types suitable for treatment remain few compared to many other treatments. In fact all solid tumors may be treated with the proposed invention. The present invention may help to destroy larger tumors by mass and more complex tumors than by previous technologies. The treatment can be given intratumorally, intracavitary, intravenously and in a combination of these. The approach can give systemic efficacy despite local injection. The approach can also eradicate cells proposed as tumor initiating ("cancer stem cells") (Eriksson M et al. Mol Ther 2007; 15(12):2088-93).

Besides enabling the transport of the vector to the site of interest the vector of the invention also assures the expression and persistence of the transgene. The present invention solves a problem related to therapeutic resistance of conventional treatments. Furthermore, the present invention

provides tools and methods for selective treatments, with less toxicity or damage to healthy tissues. Advantages of the present invention include also different and reduced side effects in comparison to other therapeutics. Importantly, the approach is synergistic with many other forms of therapy including chemotherapy, small molecular inhibitors and radiation therapy, and can therefore be used in combination regimens.

Induction of an immune reaction towards cells that allow replication of unarmed adenoviruses is normally not strong enough to lead to development of therapeutic tumor immunity. In order to overcome this weakness, the present invention provides armed adenoviruses with a fully human monoclonal antibody specific for CTLA-4, which strengthens antitumor immunity by activating T cytotoxic cells and antigen presenting cells, and by downregulating regulatory T-cells and other suppressive cells. The anti-CTLA-4 mAbs can also directly induce apoptosis of tumor cells which often express CTLA-4.Through the adenovirus vectors of the invention, several anti-tumor mechanisms mediated by CTLA-4 blocking antibodies, as described above, are realized:

(A): Anti–CTLA-4 mAbs can block immune suppressive signaling derived from CTLA-4 molecule on the surface of activated T cells (Chambers CA et al., Annu Rev Immunol 2001;19:565-94).

(B): An important inhibitory subset of T-cells (regulatory T-cells, T-Reg) constitutively expresses CTLA-4 and can bind B7 molecules on dendritic cells which are key antigen presenting cells (Paust S et al., Proc Natl Acad Sci USA 2004;101:10398-403). Subsequent upregulation of indoleamine 2,3-dioxygenase and other suppressive circuits results in tolerization of T cells in the microenvironment, instead of cytotoxic action (Munn DH et al., J Clin Invest 2004;114:280-90.

(C): CTLA-4-expressing T cells (including T-Reg) may also bind directly to activated T cells, because B7 costimulatory molecules are expressed on the surface of activated human T cells. CTLA-4–blocking mAbs interfere with this suppressive signaling and result in the local expansion of tumor antigen-specific T cells (Paust S et al., Proc Natl Acad Sci USA 2004;101:10398-403).

(D): CTLA-4 is expressed on the surface of many tumor cells (Contardi E et al., Int J Cancer 2005;117:538-50), presumably reflecting direct immune suppressive action on B7 of cytotoxic T-cells and DCs. Interestingly, CTLA-4–blocking mAbs can induce direct killing of tumor cells by triggering

apoptosis (Ribas A et al., Oncologist 2007;12:873-83; Contardi E et al., Int J Cancer 2005;117:538-50) and *in vivo* this could be enhanced further by antibody dependent cellular cytotoxicity (Jinushi M et al. Proc Natl Acad Sci U S A 2006;103:9190-5).

(E): Tumor-expressed CTLA-4 may trigger increased indoleamine 2,3-dioxygenase in tumor-infiltrating DCs, and CTLA-4–specific monoclonal Abs would also block this effect (Ribas A et al., Oncologist 2007;12:873-83).

Thus, 5 different mechanisms make the adenoviruses of the invention comprising monoclonal anti-CTLA-4 a potent anti-tumor approach, but without side effects associated with systemic exposure, which have resulted in safety concerns (Hodi FS et al., N Engl J Med. 2010; 363;8:711-23; Sanderson K et al., J Clin Oncol 2005;23:741-50. In addition to these 5 transgene mediated mechanisms, oncolytic replication of the virus will add to anti-tumor efficacy. Given the potent immunostimulatory activity mediated by adenovirus *per se* (Tuve S, et al. Vaccine. 2009;27(31):4225-39), oncolysis adds to the overall immunological utility of the approach. In Example 9 (Figures 1, 11, 12) below we describe a further improvement in this regard; adding CpG moieties into the adenovirus genome makes the virus even more immunostimulatory.

Compared to adenoviral tools of the prior art, the present invention provides a more simple, more effective, inexpensive, non-toxic and/or safer tool for cancer therapy. Furthermore, helper viruses or co-administration of recombinant molecules are not needed.

The present invention provides a new generation of infectivity enhanced and highly effective adenoviruses that retain the good safety of older viruses but results in higher levels of efficacy. Importantly, the present invention describes oncolytic adenoviruses which provide immunological factors critical with regard to the efficacy of oncolytic viruses.

The novel products of the invention enable further improvements in cancer therapy.

Brief description of the Figures

Figure 1 shows the construction of single- and double-targeted and anti-CTLA4-armed oncolytic adenoviruses. A schematic illustration of the oncolytic adenoviruses Ad5/3-Δ24aCTLA4 (single-targeted; others are double-targeted), Ad5/3-hTERT-Δ24aCTLA4, Ad5/3-hTERT-Δ24aCTLA4-CpG, Ad5/3-E2F-Δ24aCTLA4, and Ad5/3E2F-Δ24aCTLA4-CpG, the replication-deficient

Ad5/3-aCTLA4 as well as the unarmed oncolytic Ad5/3-Δ24 (positive control) and the replication-deficient Ad5/3-Luc1 (negative control).

Figures 2A-2F show evaluation of the virus produced anti-CTLA4 monoclonal antibody expression and function.

Figure 2A: Serum free supernatants of virus infected A549 (lanes 1 and 2 from left to right) or PC3-MM2 (lanes 3 and 4) cells analyzed by Western blot for specific detection of the human Ig (both heavy and light chains) in native (upper row) or in denaturating conditions (lower row). Lanes 1 and 3: cells infected with Ad5/3-Δ24aCTLA4; lanes 2 and 4: cells infected with Ad5/3-aCTLA4.

Figure 2B: A functional assay demonstrating the activity of virus produced anti-CTLA4 monoclonal antibody. Jurkat cells were incubated with ionomycin, phorbol 12-myristate 13-acetate (PMA) and recombinant human B7, resulting in CD28 and CTLA-4 positive cells resembling T-cells stimulated by antigen presenting cells but inhibited by suppressor cells, a situation often found in advanced tumors. Supernatant from Ad5/3-Δ24aCTLA4 or Ad5/3-aCTLA4 infected cells resulted in inhibition of B7/CTLA-4 mediated suppression. This is seen as increased production of IL-2, a T-cell activation marker. Recombinant anti-CTLA4 monoclonal antibody was included as a positive control.

Figure 2C: A loss of function assay demonstrating the affinity of recombinant CTLA-4 (rCTLA-4) to virus produced anti-CTLA4 monoclonal antibody. Jurkat cells were activated as before but now also rCTLA-4 was added to bind B7, preventing CTLA-4 activation. When a supernatant containing aCTLA4 was added, rCTLA-4 was blocked, and B7 was able to bind CTLA-4 for reduced IL-2 production. Columns, mean of three independent experiments; bars, SE *, $P < 0.05$; ***, $P < 0.001$.

Figure 2D: CTLA4 expression levels in A549, SKOV3-ip1, PC3-MM2 tumor cell lines and a UT-SCC8 low-passage tumor explant. All tumor cell lines were positive for CTLA4 in fluorescence assisted cell sorting.

Figures 2E and 2F: Adenoviral qPCR showing that the large size of anti-CTLA4 expression cassette or the production of anti-CTLA4 protein does not affect the replicativity of Ad5/3-Δ24aCTLA4. Briefly, A549 cells were infected with PBS, Ad5/3-Δ24 and Ad5/3-Δ24aCTLA4 and the number of viral particles was measured by qPCR at different time points from the supernatant

and from the cells. No significant difference was found between the virus groups.

Figure 3 shows the cell killing efficiency of anti-CTLA4 armed adenoviruses and control vectors. The tumor cell lines (A) PC3-MM2, (B) SKOV3-ip1, (C) A549 and (D) the low-passage tumor explant UT-SCC8 were infected. Ad5/3-Δ24aCTLA4 had an oncolytic potency similar to the positive control virus Ad5/3-Δ24 in all cell lines. Also the replication deficient Ad5/3-aCTLA4 had anti-tumor activity, since tumor cells express CTLA4. Columns, mean of triplicate assays; bars, SE. **, $P < 0.01$; ***, $P < 0.001$.

Figure 4 shows tumor growth suppression and apoptosis in immune deficient mice with human prostate cancer xenografts (PC3-MM2 tumors) and human lung cancer xenografts (A549 tumors) treated with an anti-CTLA-4 monoclonal antibody expressing oncolytic adenovirus. PC3-MM2 tumors (n = 8/group) were established in nude mice, where human anti-CTLA-4 does not have immunological activity. Therefore, the model measures only oncolysis and the proapoptotic effect of anti-CTLA-4. After 7 days, tumors (5-8 mm in diameter) were treated intratumorally with 1×10^8 virus particles on days 0, 2, and 4 (arrows). Mock mice were injected with growth media only.

Figure 4A: Ad5/3-Δ24aCTLA4 had the best anti-tumor activity in this highly aggressive model. Tumor size is presented relative to mean initial size. Points, mean; bars, SE; **, $P < 0.01$ (Student's t test on day 7).

Figure 4B: On day 5, tumor cryosections were stained for expression of anti-CTLA4 (Human IgG, upper row) or apoptosis (active caspase-3, lower row) by immunohistochemistry (brown). Images taken at ×40 magnification.

Figure 4C: On day 7, human IgG levels in tumors and plasma of mice treated with Ad5/3-Δ24aCTLA4 or Ad5/3-aCTLA4 were measured. In Ad5/3-Δ24aCTLA4 treated tumors 81-fold more anti-CTLA4 mAb was found in comparison to Ad5/3-aCTLA4 treated tumors ($p<0.05$). In addition, 43.3-fold more anti-CTLA4 mAb was found in tumors treated with Ad5/3-Δ24aCTLA4 than in the plasma of the same animals ($p<0.05$). The average plasma concentration was 392.6 µg/g (SE 312.0), which is below concentrations reported tolerated in humans treated with ipilimumab (561 µg/mL) and tremelimumab 450 µg/mL, respectively (REFS) (1 mL of plasma weighs circa 1 g). In Ad5/3-Δ24aCTLA4 tumors, mAb concentration was 16 977 µg/g and

therefore much higher than in plasma. Columns, mean of triplicate assays; bars, SE.

Figure 4D. Ad5/3-Δ24aCTLA4 caused significant antitumor activity compared to mock ($P < 0.01$) in a lung cancer model. Significant difference between Ad5/3-Δ24aCTLA4 and Ad5/3-Δ24 was not seen. Groups are shown until the first mouse from the group had to be killed as per animal regulations. Points, mean; bars, SE.

Figure 5 shows increased IL-2 and IFN-γ production in stimulated peripheral blood mononuclear cells (PBMC) of cancer patients mediated by Ad5/3-Δ24aCTLA4. Cancer patients (patient I244, patient C261, patient M158 or patient X258) PBMCs were stimulated with ionomycin, PMA and recombinant human B7 to mimic tumor induced immune suppression and treated with filtered supernatants from virus infected PC3-MM2 cells. IL-2 (A) and IFN-γ (B) are markers of T-cell activation and were measured by FACS array. A recombinant anti-CTLA-4 monoclonal antibody was included as a positive control. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 6 shows IL-2 and IFN-γ production in peripheral blood mononuclear cells (PBMC) of healthy donors by Ad5/3-Δ24aCTLA4. PBMCs from 2 healthy donors were stimulated with ionomycin, PMA and recombinant human B7 and treated with filtered supernatants from virus infected PC3-MM2 cells. IL-2 (A) or IFN-γ (B) levels in the growth media were measured by a FACS array. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$; ***, $P < 0.001$.

Figure 7 shows a schematic of the anti-CTLA-4 functionality assays performed for Examples shown in Figures 2, 5 and 6. *In vivo*, dendritic cells present antigens via major histocompatibility complex (MHC) to the T cell receptor (TCR) on the T cell surface. For immune response instead of tolerance, co-stimulation is also needed. This is mediated by binding of B7.1 or B7.2 (of on DCs) to CD28 on the T cell. T cell activation can be quantitated by production of IL-2 and IFN-γ.

Figure 7A: In the absence of an intact immune system, T-cell activation can be simulated *in vitro* by incubating PBMCs with phorbol 12-myristate 13-acetate (PMA) and ionomycine resulting in activation independent of TCR and CD28 signaling.

Figure 7B: Upon T cell activation, as a negative feedback control mechanism, CTLA-4 expression is also upregulated.

Figure 7C: CTLA-4 has 100-fold higher affinity than CD28 to B7 resulting in T cell arrest.

Figure 7D: Blockade of CTLA-4 signaling by anti-CTLA-4 antibodies blocks inhibition resulting in activation of T Cells.

Figure 7E: When soluble recombinant human CTLA-4 is added, the anti-CTLA-4 mAb is sequestered leaving B7 free to bind to CTLA-4 for T cell arrest.

Figure 7F: If recombinant CTLA-4 is added to the growth media in the absence of anti-CTLA4 mAb, it sequesters B7 leaving the T cell in the activated status.

Figure 8 shows the specific anti-CTLA4 mAb activity by Ad5/3-Δ24aCTLA4 in stimulated peripheral blood mononuclear cells (PBMC) of cancer patients. Cancer patients' (patient I244, patient C261, patient M158 or patient X258) PBMCs were stimulated with ionomycin, PMA, recombinant human B7 and recombinant human CTLA-4; and treated with filtered supernatants from virus infected PC3-MM2 cells. IL-2 (A) or IFN-γ (B) levels in the growth media were measured by the FACS array. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$.

Figure 9 shows the specific anti-CTLA4 mAb activity by Ad5/3-Δ24aCTLA4 in stimulated peripheral blood mononuclear cells (PBMC) of healthy donors. PBMCs were incubated with ionomycin, PMA, recombinant human B7 and recombinant human CTLA-4 and treated with filtered supernatants from virus infected PC3-MM2 cells. IL-2 (A) or IFN-γ (B) levels in the growth media were measured by a FACS array. Columns, mean of three independent experiments.

Figure 10 demonstrates the utility of the replication competent platform in increasing anti-CTLA4 mAb expression in comparison to the replication deficient virus. PC3-MM2 cells were seeded at 20 000 cells per well and infected at 10VP per cell with the respective viruses. 24h, 48h and 72h post-infection the supernatants were collected and analyzed by ELISA for the amount of human IgG. A 3 fold increase was observed with the oncolytic virus Ad5/3-Δ24aCTLA4 in comparison to the replicative deficient Ad5/3-aCTLA4. Columns, mean of quintuplicate assays; bars, SE. ***, $P < 0.001$.

Figure 11 shows the effect of an oncolytic adenovirus containing toll-like receptor 9 (TLR-9) stimulating CpG molecules in a xenograft mouse model of lung cancer. Nude mice (5 mice per group, two tumors per mouse) were implanted with A549 cells and treated as follows: saline (black triangle), CpG-rich oncolytic adenovirus (Ad5-Δ24CpG, white cirle), oncolytic adenovirus (Ad5-Δ24, black square), oncolytic adenovirus + recombinant CpG oligos (white square). The CpG rich virus was most effective in mediating antitumor immunity,and it was even more effective than oncolytic adenovirus given in combination with a recombinant CpG molecule.

Figure 12A shows the results of a MTS cell killing assay performed on splenocytes harvested from treated mice at 72 hours (same mice as in Figure 11). The percentage of A549 still alive at the indicated time point is reported. Figure 12B suggests that the murine anti-CTLA4 does not affect the anti-virus immunity.

Figure 12B shows the results of an interferon gamma ELISPOT assay suggesting that the murine anti-CTLA4 does not affect the anti-virus immunity. Immunocompetent C57Bl/6 mice (n=5) were treated three times with PBS, Ad5/3-Δ24, Ad5/3-Δ24 and mouse aCTLA4 antibody or with mouse aCTLA4 antibody only. Two weeks later spleens were collected and PBMCs analyzed by interferon gamma ELISPOT after stimulation with UV-inactivated Ad5/3-Δ24 or by functional virus. SPU = spot producing units.

Detailed description of the invention

Adenoviral vector

In Ad5, as well as in other adenoviruses, an icosahedral capsid consists of three major proteins: hexon (II), penton base (III), and a knobbed fiber (IV), along with minor proteins: VI, VIII, IX, IIIa, and IVa2 (Russell W.C., J General Virol 2000;81:2573-2604). Proteins VII, small peptide *mu*, and a terminal protein (TP) are associated with DNA. Protein V provides a structural link to the capsid via protein VI. Virus encoded protease is needed for processing some structural proteins.

The oncolytic adenoviral vector of the present invention is based on an adenovirus serotype 5 (Ad5) nucleic acid backbone comprising a capsid modification, such as an adenovirus serotype 3 (Ad3) knob (Ad5/3 capsid chimerism), a 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and a nucleic acid sequence encoding a fully human monoclonal antibody

specific for CTLA-4 (anti-CTLA4 mAb or aCTLA4) in the place of the deleted *gp19k/6.7K* in the E3 region.

In a preferred embodiment of the invention, the adenoviral vector is based on a human adenovirus.

The Ad5 genome contains early (E1-4), intermediate (IX and IVa2) and late (L1-5) genes flanked by left and right inverted terminal repeats (LITR and RITR, respectively), which contain the sequences required for the DNA replication. The genome also contains packaging signal (ψ) and major late promoter (MLP).

Transcription of the early gene E1A starts the replication cycle followed by expression of E1B, E2A, E2B, E3 and E4. E1 proteins modulate cellular metabolism in a way that makes a cell more susceptible to virus replication. For example they interfere with NF-κB, p53, and pRb-proteins. E1A and E1B function together in inhibiting apoptosis. E2 (E2A and E2B) and E4 gene products mediate DNA replication and E4 products also effect the virus RNA metabolism and prevent the host protein synthesis. The E3 gene products are responsible for defending against the host immune system, enhancing cell lysis, and releasing of virus progeny (Russell W.C., J General Virol 2000;81:2573-2604).

Intermediate genes IX and IVa2 encode minor proteins of the viral capsid. Expression of the late genes L1-5, which lead to production of the virus structural components, encapsidation and maturation of the virus particles in the nucleus, is influenced by MLP (Russell W.C., J General Virol 2000;81:2573-2604).

Compared to a wild type adenovirus genome, the adenoviral vector of the invention lacks 24 base pairs from CR2 in E1 region, specifically in E1A region, and *gp19k* and *6.7K* in E3 region and comprises a capsid modification in the fiber of the virus. In some embodiments, compared to a wild type adenovirus genome, the adenoviral vector of the invention additionally comprises hTERT promoter or an E2F promoter in the E1 region, specifically upstream of the E1A region, and lacks *gp19k* and *6.7K* in the E3 region. In some embodiments, the invention also includes an adenoviral backbone enriched with TLR-9 binding CpG islands, which have been placed in the E3 region (Figure 1).

In a preferred embodiment of the invention, in addition to amended/partial regions E1 and E3, the oncolytic adenoviral vector of the

invention further comprises one or more regions selected from a group consisting of E2, E4, and late regions. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises the following regions: a left ITR, partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5, E4, and a right ITR. The regions may be in any order in the vector, but in a preferred embodiment of the invention, the regions are in a sequential order in the 5' to 3' direction. Open reading frames (ORFs) may be in the same DNA strand or in different DNA strands. In a preferred embodiment of the invention, the E1 region comprises a viral packaging signal.

As used herein, expression "adenovirus serotype 5 (Ad5) nucleic acid backbone" refers to the genome or partial genome of Ad5, which comprises one or several regions selected from a group consisting of partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5 and E4 of Ad5 origin. In a preferred embodiment, the vector of the invention comprises a nucleic acid backbone of Ad5 with a portion of Ad3 (e.g., a part of the capsid structure).

As used herein, expression "partial" region refers to a region, which lacks any part compared to a corresponding wild type region. For instance "partial E3" refers to E3 region lacking *gp19k/6.7K*.

As used herein, expressions "VA1" and "VA2" refer to virus associated RNAs 1 and 2, which are transcribed by the adenovirus but are not translated. VA1 and VA2 have a role in combating cellular defense mechanisms.

As used herein, expression "a viral packaging signal" refers to a part of virus DNA, which consists of a series of AT-rich sequences and governs the encapsidation process.

24 base pair deletion (D24) of E1 affects CR2 domain, which is responsible for binding the Rb tumor suppressor/cell cycle regulator protein and thus, allows the induction of the synthesis (S) phase i.e. DNA synthesis or replication phase. pRb and E1A interaction requires eight amino acids 121 to 127 of the E1A protein conserved region (Heise C. et al. 2000, Nature Med 6, 1134-1139), which are deleted in the present invention. The vector of the present invention comprises a deletion of nucleotides corresponding to amino acids 122-129 of the vector according to Heise C. et al. (2000, Nature Med 6, 1134-1139). Viruses with the D24 are known to have a reduced ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this

interaction is not necessary, e.g. in tumor cells defective in the Rb-p16 pathway (Heise C. et al. 2000, Nature Med 6, 1134-1139; Fueyo J et al. 2000, Oncogene 19, 2-12).

The E3 region is nonessential for viral replication *in vitro*, but the E3 proteins have an important role in the regulation of host immune response i.e. in the inhibition of both innate and specific immune responses. The *gp19k/6.7K* deletion in E3 refers to a deletion of 965 base pairs from the adenoviral E3A region. In a resulting adenoviral construct, both *gp19k* and *6.7K* genes are deleted (Kanerva A et al. 2005, Gene Therapy 12, 87-94). The *gp19k* gene product is known to bind and sequester major histocompatibility complex I (MHC1) molecules in the endoplasmic reticulum, and to prevent the recognition of infected cells by cytotoxic T-lymphocytes. Since many tumors are deficient in MHC1, deletion of *gp19k* increases tumor selectivity of viruses (virus is cleared faster than wild type virus from normal cells but there is no difference in tumor cells). 6.7K proteins are expressed on cellular surfaces and they take part in downregulating TNF-related apoptosis inducing ligand (TRAIL) receptor 2.

In the present invention, the cDNA coding for CTLA4 mAb transgene is placed into a *gp19k/6.7k* deleted E3 region, under the E3 promoter. This restricts transgene expression to tumor cells that allow replication of the virus and subsequent activation of the E3 promoter. The E3 promoter may be any exogenous or endogenous promoter known in the art, preferably endogenous promoter. In a preferred embodiment of the invention, a nucleic acid sequence encoding anti-CTLA4 mAb is under the control of the viral E3 promoter.

The *gp19k* deletion is particularly useful in the context of anti-CTLA4 mAb expression as it can enhance MHC1 presentation of tumor epitopes in such tumors that retain this capacity. In this context, stimulation T-cytotoxic cells by anti-CTLA4 mAb can yield the optimum benefit.

Anti-CTLA4 mAb potentiates the immune response by acting through various mechanisms including activation of T- cytotoxic cells, downregulating regulatory T cells (T-Regs) and inhibiting the direct immunosuppression of cytotoxic T-cells and antigen presenting cells (eg dendritic cells) by CTLA-4 expressing tumor cells. In addition to these 5 transgene mediated mechanisms, explained in detail above, oncolytic replication of the virus will add to anti-tumor efficacy.

The nucleotide sequence encoding CTLA4 mAb may be from any animal, such as a human, ape, rat, mouse, hamster, dog or cat, depending on the subject to be treated, but preferably CTLA4 mAb is encoded by a fully human sequence in the context of treatment of humans. The nucleotide sequence encoding CTLA4 mAb may be modified in order to improve the effects thereof, or unmodified i.e. of a wild type. In a preferred embodiment of the invention, a nucleic acid sequence encoding CTLA4 mAb is unmodified.

Insertion of exogenous elements may enhance effects of vectors in target cells. The use of exogenous tissue or tumor-specific promoters is common in recombinant adenoviral vectors and they can also be utilized in the present invention. In some embodiments the adenoviruses of the invention comprise hTERT or variants of hTERT or E2F to control the E1A region, preferably placed upstream of E1A. hTERT directs the vector to cells expressing telomerase whereas E2F directs the vector to cells with with Rb/p16 pathway defects. Such defects result in high expression levels of free E2F and high activity of the E2F promoter. However, viral replication can be restricted to target cells by any other suitable promoter, which include but are not limited to CEA, SLP, Cox-2, Midkine, CXCR4, SCCA2 and TTS. They are usually added to control E1A region, but in addition to or alternatively, other genes such as E1B or E4 can also be regulated. Exogenous insulators i.e. blocking elements against unspecific enhancers, the left ITR, the native E1A promoter or chromatin proteins may also be included in recombinant adenoviral vectors. Any additional components or modifications may optionally be used but are not obligatory in the vectors of the present invention.

In further embodiments of the invention, the adenovirus vectors of the invention feature a TLR-9 binding CpG-rich DNA region in the adenoviral backbone (Figures 1, 11, 12).

The oncolytic adenoviral vector of the invention comprises a capsid modification. Most adults have been exposed to the most widely used adenovirus serotype Ad5 and therefore, the immune system can rapidly produce neutralizing antibodies (NAb) against them. In fact, the prevalence of anti-Ad5 NAb may be up to 50%. It has been shown that NAb can be induced against most of the multiple immunogenic proteins of the adenoviral capsid, and on the other hand, it has been shown that even small changes in the Ad5 fiber knob can allow escape from capsid-specific NAb (Särkioja M, et al. Gene Ther. 2008;15(12):921-9). Modification of the knob is therefore important for

retaining or increasing gene delivery in the contact of adenoviral use in humans.

Furthermore, Ad5 is known to bind to the receptor called CAR via the knob portion of the fiber, and modifications of this knob portion or fiber may improve the entry to the target cell and cause enhanced oncolysis in many or most cancers (Ranki T. et al., Int J Cancer 2007;121:165-174). Indeed, capsid-modified adenoviruses are advantageous tools for improved gene delivery to cancer cells.

As used herein "capsid" refers to the protein shell of the virus, which includes hexon, fiber and penton base proteins. Any capsid modification i.e. modification of hexon, fiber and/or penton base proteins known in the art, which improves delivery of the virus to the tumor cell, may be utilized in the present invention. Modifications may be genetic and/or physical modifications and include but are not limited to modifications for incorporating ligands, which recognize specific cellular receptors and/or block native receptor binding, for replacing the fiber or knob domain of an adenoviral vector with a knob of other adenovirus (chimerism) and for adding specific molecules (e.g., fibroblast growth factor 2, FGF2) to adenoviruses. Therefore, capsid modifications include but are not limited to incorporation of small peptide motif(s), peptide(s), chimerism(s) or mutation(s) into the fiber (e.g., into the knob, tail or shaft part), hexon and/or penton base. In a preferred embodiment of the invention, the capsid modification is Ad5/3 chimerism, insertion of an integrin binding (RGD) region and/or heparin sulphate binding polylysine modification into the fiber. In a specific embodiment of the invention, the capsid modification is Ad5/3 chimerism.

As used herein, "Ad5/3 chimerism" of the capsid refers to a chimerism, wherein the knob part of the fiber is from Ad serotype 3, and the rest of the fiber is from Ad serotype 5.

The vector of the invention may also comprise other modifications, such as modifications of the E1B region.

As used herein, "RGD" refers to the arginine-glycine-aspartic acid (RGD) motif, which is exposed on the penton base and interacts with cellular α-v-β-integrins supporting adenovirus internalization. In a preferred embodiment of the invention, the capsid modification is a RGD-4C modification. "RGD-4C modification" refers to an insertion of a heterologous integrin binding RGD-4C motif in the HI loop of the fiber knob domain. 4C

refers to the four cysteins, which form sulphur bridges in RGD-4C. Construction of recombinant Ad5 fiber gene encoding the fiber with the RGD-4C peptide is described in detail for example in the article of Dmitriev I. et al. (Journal of Virology 1998;72:9706-9713).

As used herein, "heparan sulphate binding polylysine modification" refers to addition of a stretch of seven lysines to the fiber knob c-terminus.

Expression cassettes are used for expressing transgenes in a target, such as a cell, by utilizing vectors. As used herein, the expression "expression cassette" refers to a DNA vector or a part thereof comprising nucleotide sequences, which encode cDNAs or genes, and nucleotide sequences, which control and/or regulate the expression of said cDNAs or genes. Similar or different expression cassettes may be inserted to one vector or to several different vectors. Ad5 vectors of the present invention may comprise either several or one expression cassettes. However, only one expression cassette is adequate. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises at least one expression cassette. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises only one expression cassette.

A cell comprising the adenoviral vector of the invention may be any cell such as a eukaryotic cell, bacterial cell, animal cell, human cell, mouse cell etc. A cell may be an *in vitro*, *ex vivo* or *in vivo* cell. For example, the cell may be used for producing the adenoviral vector *in vitro, ex vivo* or *in vivo*, or the cell may be a target, such as a tumor cell, which has been infected with the adenoviral vector.

In a method of producing CTLA4 mAbs in a cell, a vehicle comprising the vector of the invention is carried into a cell and the CTLA4 mAb gene is expressed and the protein is translated and secreted in a paracrine manner. "A vehicle" may be any viral vector, plasmid or other tool, such as a particle, which is able to deliver the vector of the invention to a target cell. Any conventional method known in the art can be used for delivering the vector to the cell.

Tumor specific immune response may be increased in a subject by the present invention. Activation of T- cytotoxic cells, downregulating regulatory T cells (T-Regs) and inhibiting the direct immunosuppression of cytotoxic T-cells and dendritic by the CTLA-4 expressing tumor cells occurs as a consequence of CTLA4 mAb expression.

In order to follow or study the effects of the invention, various parameters of immune response may be determined. The most common markers include but are not limited to changes in tumor or adenovirus specific cytotoxic T-cells in the blood or in tumors. The recruitment and activation of antigen presenting cells can be studied at tumors or in lymphoid tissue. Further, regulatory cell subsets (eg. Regulatory T-cells) can be studied with regard to number or activity. Serum cytokine profiles can shed light on the Th1/Th2 environment which is also important for immunity versus tolerance. The levels of these markers may be studied according to any conventional methods known in the art, including but not limited to those utilizing antibodies, probes, primers etc., such as ELISPOT assay, tetramer analysis, pentamer analysis, intracellular cytokine staining, analysis of antibodies in blood and analysis of different cell types in blood or in tumors.

Cancer

The recombinant Ad5/3 vectors of the invention have been constructed for replication competence in cells, which have defects in the Rb-pathway, specifically Rb-p16 pathway. These defective cells include all tumor cells in animals and humans (Sherr C.J. 1996, Science 274, 1672-1677). In a preferred embodiment of the invention, the vector is capable of selectively replicating in cells having defects in the Rb-pathway. As used herein “defects in the Rb-pathway” refers to mutations and/or epigenetic changes in any genes or proteins of the pathway. Due to these defects, tumor cells overexpress E2F and thus, binding of Rb by E1A CR2, that is normally needed for releasing E2F for effective replication, is unnecessary.

Some oncolytic adenoviral vectors of the invention have additionally been constructed for replication competence in cells, which express human telomerase reverse transcriptase (hTERT), which is the catalytic subdomain of human telomerase. These include over 85% of human tumors, which are found to upregulate expression of the hTERT gene and its promoter, whereas most normal adult somatic cells are devoid of telomerase or transiently express very low levels of the enzyme (Shay and Bacchetti 1997, Eur J Cancer 33:787-791). Such Rb-p16 pathway deficient/hTERT promoter combinations may target any cancers or tumors, including both malignant and benign tumors as well as primary tumors and metastasis may be targets of gene therapies. E2F transcription factors regulate the expression of a diverse set of genes involved in key cellular events related to growth control (Johnson and Schneider-

Broussard 1998, Role of E2F in cell cycle control and cancer, Front Biosci. 1998 Apr 27;3:d447-8; Muller and Helin 2000, The E2F transcription factors: key regulators of cell proliferation, Biochim Biophys Acta. 2000 Feb 14;1470(1):M1-12).

In non-cycling normal cells E2F is sequestered in pRb/E2F complexes and thus little E2F is freely available. Demonstrating its relevance in physiological growth control, the pRb pathway is disrupted in nearly all human cancers, resulting in free E2F in most cancers. The pathway can be disrupted by mutation of any of a several different molecules. However, a common feature is subsequent activation of the E2F promoter for increased E2F levels. E2F binds the promoter of many target genes, but important to its function is also autoactivation. Therefore, cells dysfunctional in p16/Rb feature high E2F levels which are amplified further through E2F binding to its promoter (Hanahan and Weinberg 2000, Cell 7;100(1):57-70; Johnson et al. 2002, Cancer Cell 1(4):325-37).

However, if adenoviral E1A is controlled by the E2F promoter, (as in, e.g., US 2008118470 A1) there is a risk for a self-amplifying vicious loop, unless the E1A/Rb ablating D24 deletion is employed simultaneously. Specifically, even low levels of E2F present in normal cells would bind to the E2F promoter, leading to expression of E1A, leading to release of more E2F from pRb/E2F complexes, leading to more E2F promoter activation and more E1A. Thus, without the D24 deletion which ablates binding of E1A to pRb, the E2F promoter leads to oncolytic adenoviruses which can replicate also in normal cells, which might have safety consequences.

Unmethylated double strand DNA can stimulate TLR9, an endosomal receptor that bridges the innate and the adaptive immune response. The insertion of CpG-rich regions in the adenovirus backbone increase the capability of adenovirus to stimulate TLR9 in antigen presenting cells hence increasing T cell stimulation and maturation as well as NK activation (Nayak S, Herzog RW. Gene Ther. 2010 Mar;17(3):295-304.).

In a specific embodiment of the invention the cancer is any solid tumor. In a preferred embodiment of the invention, the cancer is selected from a group consisting of nasopharyngeal cancer, synovial cancer, hepatocellular cancer, renal cancer, cancer of connective tissues, melanoma, lung cancer, bowel cancer, colon cancer, rectal cancer, colorectal cancer, brain cancer, throat cancer, oral cancer, liver cancer, bone cancer, pancreatic cancer,

choriocarcinoma, gastrinoma, pheochromocytoma, prolactinoma, T-cell leukemia/lymphoma, neuroma, von Hippel-Lindau disease, Zollinger-Ellison syndrome, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, ureter cancer, oligodendroglioma, neuroblastoma, meningioma, spinal cord tumor, osteochondroma, chondrosarcoma, Ewing's sarcoma, cancer of unknown primary site, carcinoid, carcinoid of gastrointestinal tract, fibrosarcoma, breast cancer, Paget's disease, cervical cancer, esophagus cancer, gall bladder cancer, head cancer, eye cancer, neck cancer, kidney cancer, Wilms' tumor, Kaposi's sarcoma, prostate cancer, testicular cancer, Hodgkin's disease, non-Hodgkin's lymphoma, skin cancer, mesothelioma, multiple myeloma, ovarian cancer, endocrine pancreatic cancer, glucagonoma, pancreatic cancer, parathyroid cancer, penis cancer, pituitary cancer, soft tissue sarcoma, retinoblastoma, small intestine cancer, stomach cancer, thymus cancer, thyroid cancer, trophoblastic cancer, hydatidiform mole, uterine cancer, endometrial cancer, vagina cancer, vulva cancer, acoustic neuroma, mycosis fungoides, insulinoma, carcinoid syndrome, somatostatinoma, gum cancer, heart cancer, lip cancer, meninges cancer, mouth cancer, nerve cancer, palate cancer, parotid gland cancer, peritoneum cancer, pharynx cancer, pleural cancer, salivary gland cancer, tongue cancer, and tonsil cancer.

Pharmaceutical composition

A pharmaceutical composition of the invention comprises at least one type of the vectors of the invention. Furthermore, the composition may comprise at least two, three or four different vectors of the invention. In addition to the vector of the invention, a pharmaceutical composition may also comprise any other vectors, such as other adenoviral vectors, such as those described in US2010166799 A1, other therapeutically effective agents, any other agents, such as pharmaceutically acceptable carriers, buffers, excipients, adjuvants, antiseptics, filling, stabilizing or thickening agents, and/or any components normally found in corresponding products.

The pharmaceutical composition may be in any form, such as in a solid, semisolid or liquid form, suitable for administration. A formulation can be selected from a group consisting of, but not limited to, solutions, emulsions, suspensions, tablets, pellets and capsules.

In a preferred embodiment of the invention, the oncolytic adenoviral vector or pharmaceutical composition acts as an *in situ* cancer vaccine. As used herein "*in situ* cancer vaccine" refers to a cancer vaccine, which both kills

tumor cells and also increases the immune response against tumor cells. Virus replication is a strong danger signal to the immune system useful for a TH1 type cytotoxic T-cell response), and thus acts as a powerful co-stimulatory factor maturation and activation of APCs, and recruitment of NK cells.

A critical discovery in the field of tumor immunology is that induction of an anti-tumor immune response is not sufficient for therapeutic efficacy. Instead it is critical to also reduce tumor mediated immune suppression. Tumor cell lysis also helps to present tumor fragments and epitopes to APCs and further co-stimulation is produced by inflammation. Thus, an epitope independent (i.e., not HLA restricted) response is produced in the context of each tumor and therefore takes place *in situ*. Tumor specific immune response is activated in the target cells allowing thereafter antitumor activities to occur on the whole subject level, e.g., in distant metastases.

The effective dose of vectors depends on many factors including the subject in need of the treatment, the tumor type, the location of the tumor and the stage of the tumor. The dose may vary for example from about 10^8 viral particles (VP) to about 10^{14} VP, preferably from about $5x10^9$ VP to about 10^{13} VP and more preferably from about $8x10^9$ VP to about 10^{12} VP. In one specific embodiment of the invention the human dose is in the range of about $5x10^{10}$ - $5x10^{11}$ VP.

The pharmaceutical compositions may be produced by any conventional processes known in the art, for example by utilizing any one of the following: batch, fed-batch and perfusion culture modes, column-chromatography purification, CsCl gradient purification and perfusion modes with low-shear cell retention devices.

Administration

The vector or pharmaceutical composition of the invention may be administered to any eukaryotic subject selected from a group consisting of plants, animals and human beings. In a preferred embodiment of the invention, the subject is a human or an animal. An animal may be selected from a group consisting of pets, domestic animals and production animals.

Any conventional method may be used for administration of the vector or composition to a subject. The route of administration depends on the formulation or form of the composition, the disease, the location of tumors, the patient, co-morbidities and other factors. In a preferred embodiment of the invention, the administration is conducted through an intratumoral, intramuscu-

lar, intra-arterial, intravenous, intrapleural, intravesicular, intracavitary or peritoneal injection, or an oral administration.

Only one administration of oncolytic adenoviral vectors of the invention may have therapeutic effects. However, in a preferred embodiment of the invention, oncolytic adenoviral vectors or pharmaceutical compositions are administered several times during the treatment period. Oncolytic adenoviral vectors or pharmaceutical compositions may be administered for example from 1 to 10 times in the first 2 weeks, 4 weeks, monthly or during the treatment period. In one embodiment of the invention, administration is done three to seven times in the first 2 weeks, then at 4 weeks and then monthly. In a specific embodiment of the invention, administration is done four times in the first 2 weeks, then at 4 weeks and then monthly. The length of the treatment period may vary, and for example may last from two to 12 months or more.

Additionally, the administration of the oncolytic adenoviral vectors of the invention can preferably be combined to the administration of other oncolytic adenoviral vectors, such as those described in US2010166799 A1. The administration can be simultaneous or sequential.

In order to avoid neutralizing antibodies in a subject, the vectors of the invention may vary between treatments. In a preferred embodiment of the invention, the oncolytic adenoviral vector having a different fiber knob of the capsid compared to the vector of the earlier treatment is administered to a subject. As used herein “fiber knob of the capsid” refers to the knob part of the fiber protein (Figure 1a). Alternatively, the entire capsid of the virus may be switched to that of a different serotype.

The gene therapy of the invention is effective alone, but combination of adenoviral gene therapy with any other therapies, such as traditional therapy, may be more effective than either one alone. For example, each agent of the combination therapy may work independently in the tumor tissue, the adenoviral vectors may sensitize cells to chemotherapy or radiotherapy and/or chemotherapeutic agents may enhance the level of virus replication or affect the receptor status of the target cells. Alternatively, the combination may modulate the immune system of the subject in a way that is beneficial for the efficacy of the treatment. For example, chemotherapy could be used to downregulate suppressive cells such as regulatory T-cells. Alternatively, chemotherapy can be used after oncolytic virus therapy to boost the immunelogical response by killing of tumor cells and subsequent release of epitopes

and or viruses. Chemotherapy can also sensitize tumor cells to oncolytic viruses and vice versa. The agents of combination therapy may be administered simultaneously or sequentially. In a preferred embodiment of this invention, patients receive simultaneous cyclophosphamide to enhance the immunological effect of the treatment.

In a preferred embodiment of the invention, the method or use further comprises administration of concurrent radiotherapy to a subject. In another preferred embodiment of the invention, the method or use further comprises administration of concurrent chemotherapy to a subject. In yet another preferred embodiment of the invention, the method or use further comprises administration of other oncolytic adenovirus or vaccinia virus vectors to a subject. The administration of vectors can be simultaneous or sequential.

As used herein “concurrent” refers to a therapy, which has been administered before, after or simultaneously with the gene therapy of the invention. The period for a concurrent therapy may vary from minutes to several weeks. Preferably the concurrent therapy lasts for some hours. In one embodiment, cyclophosphamide is administered both as an intravenous bonus and orally in a metronomic fashion.

Agents suitable for combination therapy include but are not limited to All-trans retinoic acid, Azacitidine, Azathioprine, Bleomycin, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Cytarabine, Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, Epirubicin, Epothilone, Etoposide, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Imatinib, Mechlorethamine, Mercaptopurine, Methotrexate, Mitoxantrone, Oxaliplatin, Paclitaxel, Pemetrexed, Temozolomide, Teniposide, Tioguanine, Valrubicin, Vinblastine, Vincristine, Vindesine and Vinorelbine.

In a preferred embodiment of the invention, the method or use further comprises administration of verapamil or another calcium channel blocker to a subject. “Calcium channel blocker” refers to a class of drugs and natural substances which disrupt the conduction of calcium channels, and it may be selected from a group consisting of verapamil, dihydropyridines, gallopamil, diltiazem, mibefradil, bepridil, fluspirilene and fendiline.

In a preferred embodiment of the invention, the method or use further comprises administration of autophagy inducing agents to a subject. Autophagy refers to a catabolic process involving the degradation of a cell's

own components through the lysosomal machinery. “Autophagy inducing agents” refer to agents capable of inducing autophagy and may be selected from a group consisting of, but not limited to, mTOR inhibitors, PI3K inhibitors, lithium, tamoxifen, chloroquine, bafilomycin, temsirolimus, sirolimus and temozolomide. In a specific embodiment of the invention, the method further comprises administration of temozolomide to a subject. Temozolomide may be either oral or intravenous temozolomide. Autophagy inducing agents may be combined with immunomodulatory agents. In one embodiment, oncolytic adenovirus coding for anti-CTLA4 mAb is combined with both temozolomide and cyclophosphamide.

In one embodiment of the invention, the method or use further comprises administration of chemotherapy or anti-CD20 therapy or other approaches for blocking of neutralizing antibodies. “Anti-CD20 therapy” refers to agents capable of killing CD20 positive cells, and may be selected from a group consisting of rituximab and other anti-CD20 monoclonal antibodies. “Approaches for blocking of neutralizing antibodies” refers to agents capable of inhibiting the generation of anti-viral antibodies that normally result from infection and may be selected from a group consisting of different chemo-therapeutics, immunomodulatory substances, corticoids and other drugs. These substances may be selected from a group consisting of, but not limited to, cyclophosphamide, cyclosporin, azathioprine, methylprenisolone, etoposide, CD40L, FK506 (tacrolismus), IL-12, IFN-gamma, interleukin 10, anti-CD8, anti-CD4 antibodies, myeloablation and oral adenoviral proteins.

The approach described in this application can also be combined with molecules capable of overcoming neutralizing antibodies. Such agents include liposomes, lipoplexes and polyethylene glycol, which can be mixed with the virus. Alternatively, neutralizing antibodies can be removed with an immunopheresis column consisting of adenoviral capsid proteins.

The oncolytic adenoviral vector of the invention induces virion mediated oncolysis of tumor cells and activates human immune response against tumor cells. In a preferred embodiment of the invention, the method or use further comprises administration of substances capable of further downregulation of regulatory T-cells in a subject. “Substances capable to downregulating regulatory T-cells” refers to agents that reduce the amount of cells identified as T-suppressor or Regulatory T-cells. These cells have been identified as featuring one or many of the following immunophenotypic

markers: CD4+, CD25+, FoxP3+, CD127- and GITR+. Such agents reducing T-suppressor or Regulatory T-cells may be selected from a group consisting of anti-CD25 antibodies or chemotherapeutics. These substances may be useful for reducing the number of Regulatory T-cells which aCTLA4 is effective chiefly in suppressing their activity.

In a preferred embodiment of the invention, the method or use further comprises administration of cyclophosphamide to a subject. Cyclophosphamide is a common chemotherapeutic agent, which has also been used in some autoimmune disorders. In the present invention, cyclophosphamide can be used to enhance viral replication and the effects of aCTLA4 induced stimulation of NK and cytotoxic T-cells for enhanced immune response against the tumor. It can be used as intravenous bolus doses or low-dose oral metronomic administration or their combination.

In the present invention, to ensure that CTLA-4–blocking mAb does not kill activated cytotoxic T cells, which may be useful for the therapy, the human IgG2 subtype was chosen. IgG2 induces minimal complement activation and Ab-dependent cell-mediated cytotoxicity (Bruggemann M. et al. J Exp Med 1987;166:1351-1361) and also decreases the possibility of cytokine release syndrome in the context of human use (Ribas A et al., Oncologist 2007;12:873-83).

Any method or use of the invention may be either *in vivo*, *ex vivo* or *in vitro* method or use.

In the present invention an oncolytic adenovirus is armed with fully human monoclonal antibody specific for CTLA-4. In this approach, tumor cells are killed due to virus replication and due to anti-CTLA4 mAb anti-tumor immune activation and direct pro-apoptotic tumor cell killing. Additional benefit may result from tumor antigen release due to virus replication that can improve the efficacy of anti-CTLA-4 mAb therapy by potentially allow for a more specific immune response against tumor targets (Hodi FS et al., N Engl J Med 2010; 363;8:711-23; Mokyr MB et al., Cancer Res 1998;58:5301-4; Wolchok JD and Saenger Y., Oncologist 2008;13 Suppl 4:2-9). Also, side effects of the treatments are nonoverlapping, which might facilitate increased efficacy without increasing toxicity. It is shown that oncolytic adenoviruses can effectively express functional anti-CTLA4 mAb as a transgene in cancer cell lines (Fig.2). Also, it was found that it is possible to combine oncolytic adenovirus replication with anti-CTLA4 mAb and obtain increased cell killing

(Fig.3-4). This has been of concern, since CTLA-4 blockade with mAbs results in increased production of IFN-γ and major histocompatibility complex (MHC) class I molecules that potentially can inhibit virus replication (McCart JA et al., Gene Ther 2000;7:1217-23; Nakamura H et al., Cancer Res 2001;61:5447-52). Viral oncolysis together with anti-CTLA4 mAb expression resulted in higher antitumor activity *in vivo* than either treatment alone (Fig.4). There are previous reports of the treatment of cancer patients with oncolytic viruses expressing Granulocyte–macrophage colony–stimulating factor (GMCSF) in combination with low-dose metronomic cyclophosphamide to reduce T-Regs (Cerullo V et al., Cancer Res;2010; 70:4297-309; Koski A et al., Mol Ther. 2010 Jul 27. [Epub ahead of print]. PMID: 20664527). In addition, cell-mediated delivery of mouse anti-CTLA-4 from a GM-CSF-secreting tumor cell immunotherapy activated potent anti-tumor responses and prolonged overall survival with reduced evidence of systemic autoimmunity (Simmons AD et al., Cancer Immunol Immunother 2008;57:1263-70). Therefore, the efficacy of the approach can be further improved in a multimodal approach with cancer conventional therapies e.g. radiochemotherapy, vaccines, e.g. GM-CSF or/and reduction of T-Regs with e.g. cyclophosphamide.

This is the first time when it is shown that a full length mAb can be produced from an oncolytic adenovirus. Also, it is the first fully human anti-CTLA4 mAb expressed by a tumor targeted replicative competent platform. There were no side effects seen in the mouse experiment that was performed. The data from cancer patients PBMCs provides a rationale for the clinical translation of Ad5/3-Δ24aCTLA4 as an oncolytic viral vector for treating of patients with advanced cancer. Since the p16-Rb pathway is defective in many if not all solid tumors (Sherr CJ., Science 1996;274:1672-724), Ad5/3-Δ24aCTLA4 and other Δ24 defective adenoviruses of the invention are suitable for the treatment of many most types of cancer refractory to available treatments. Those embodiments of the invention which comprise, in addition to Δ24, also hTERT or E2F promoters, enlarge the utility of the present invention practically to all cancers. Also, adding the promoter may allow larger treatment doses, reduced side effects and enhanced systemic utility. Importantly, adding CpG moieties into the virus genome may enhance the anti-tumor immune response.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Examples

Animals

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. NMRI nude mice were obtained from Taconic (Ejby, Denmark) at 4 to 5 weeks of age and quarantined at least for 1 week prior to the study. Health status of the mice was frequently monitored and soon as any sign of pain or distress was evident they were killed.

Cell culture

Human head and neck squamous cell carcinoma (HNSCC) low passage tumor cell culture UT-SCC8 (supraglottic larynx) (27) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (PromoCell GmbH, Heidelberg, Germany), 1% nonessential amino acids (Gibco, Invitrogen, Carlsbad, California) 2 mmol/L glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (all from Sigma, St. Louis, MO). The UT-SCC cells were used in low passage, typically passage 15-30.

Human transformed embryonic kidney cell line 293, human lung cancer cell line A549, human ovarian cancer cell line SKOV3-ip1 and human prostate cancer cell line PC-3MM2; and Jurkat (Clone E6-1) human leukemic T cell lymphoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in the recommended conditions.

Human samples

Peripheral blood mononuclear cells (PBMC) of healthy individuals and of patients with advanced metastatic tumors refractory to conventional therapies were obtained with informed consent.

Statistical analysis

Two tailed Student's t-test was used and a p-value of <0.05 was considered as significant.

Example 1. Construction of adenoviruses

Chimeric adenoviruses bearing the cDNA sequence coding for an IgG2 type anti-CTLA4 mAb were generated (Fig. 1). The coding sequence of

anti-CTLA4 mAb was introduced into the *6.7K/gp19K* deletion of adenoviral E3A to create replication competent adenoviruses Ad5/3-Δ24aCTLA4 (SEQ ID. NO:1), Ad5/3-hTERT-Δ24aCTLA4 (SEQ ID. NO:2), Ad5/3-hTERT-Δ24aCTLA4-CpG (SEQ ID. NO:3), Ad5/3-E2F-Δ24aCTLA4 (SEQ ID. NO:4), and Ad5/3-E2F-Δ24aCTLA4-CpG (SEQ ID. NO:5) or into the deleted E1 driven by CMV promoter to create replication deficient adenovirus Ad5/3-aCTLA4 (SEQ ID. NO:6).

The oncolytic adenoviruses were generated and amplified using standard adenovirus preparation techniques (Kanerva A, et al., Mol Ther 2002;5:695-704; Bauerschmitz GJ, et al., Mol Ther 2006;14:164-74; Kanerva A and Hemminki A., Int J Cancer 2004;110:475-80; Volk AL, et al., Cancer Biol Ther 2003;2:511-5). Briefly, either an E1 or E3 shuttle vector with the transgene and other moieties (promoters, CpG, poly-A) was first constructed and the recombined with a rescue plasmid in bacterial cells featuring human recombinases. The main features of the viruses, including those of the control viruses Ad5Luc1 (Kanerva A et al., Clin Cancer Res 2002;8:275-80) and Ad5/3-Δ24 (Kanerva A et al., Mol Ther 2003;8:449-58) are described in Figure 1.

To generate Ad5/3-Δ24aCTLA4, the plasmid pTHSN-aCTLA4 was generated. pTHSN-aCTLA4 contains the heavy and light chains of IgG2 type anti-CTLA4 mAb in the E3 region of the adenoviral genome deleted for *6.7K/gp19K*. The equimolarity of the heavy and light chains is achieved with an internal ribosome entry site (IRES) between the chains. pAdEasy-1.5/3-Δ24-aCTLA4 was generated by homologous recombination in *Escherichia coli* BJ5183 cells (Qbiogene Inc., Irvine, CA, USA) between *Fsp*I-linearized pTHSN-aCTLA4 and *Srf*I-linearized pAdEasy-1.5/3-Δ24 (Kanerva A et al., Clin Cancer Res 2002;8:275-80), a rescue plasmid containing the serotype 3 knob and a 24 bp deletion in E1A. The genome of Ad5/3-Δ24aCTLA4 was released by *Pac*I digestion and subsequent transfection to A549 cells. The virus was propagated on A549 cells and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm, and standard TCID50 (median tissue culture infective dose) assay on 293 cells was done to determine infectious particle titer.

To generate Ad5/3-hTERT-Δ24aCTLA4, Ad5/3-hTERT-Δ24aCTLA4-CpG, Ad5/3-E2F-Δ24aCTLA4 and Ad5/3-E2F-Δ24aCTLA4-CpG the anti-CTLA amplification product was first subcloned into pTHSN or pTHSN-CpG and

subsequently recombined with an pAd5/3-hTERT-E1A or pAd5/3-E2F-E1A (Bauerschmitz GJ, et al., Cancer Res 2008;68:5533-9. Hakkarainen T, et al. Clin Cancer Res. 2009;15(17):5396-403.) The obtained plasmid was linearized with PacI and transfected into A549 cells for amplification and rescue. The viruses were propagated on A549 cells and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm, and standard TCID50 (median tissue culture infective dose) assay on 293 cells was done to determine infectious particle titer.

All phases of the cloning were confirmed with PCR and multiple restriction digestions. The shuttle plasmid pTHSN- aCTLA4 was sequenced. The absence of wild type E1 was confirmed by PCR. The E1 region, transgene and fiber were checked in the final virus with sequencing and PCR. All phases of the virus production, including transfection, were done on A549 cells to avoid risk of wild type recombination, as described before (Kanerva A et al. 2003, Mol Ther 8, 449-58; Bauerschmitz GJ et al. 2006, Mol Ther 14, 164-74). aCTLA4 is under the E3 promoter (specifically under endogenous viral E3A gene expression control elements), which results in replication associated transgene expression, which starts about 8h after infection. E3 is intact except for deletion of *6.7K/gp19K*.

To construct the non-replicating E1-deleted control virus Ad5/3-aCTLA4, both chains of the anti-CTLA4 mAb cDNA were ligated into pShuttle-CMV. Homologous recombination was performed between pAdEasy-1.5/3 plasmid (Krasnykh VN, et al., J Virol 1996;70:6839-46), which carries the whole adenovirus genome, and PmeI-linearized pShuttle-CMV-aCTLA4 to construct pAdEasy-1.5/3-aCTLA4. The genome of Ad5/3-aCTLA4 was released by *Pac*I and transfected into 293 cells. The virus was propagated on 293 cells and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm, and standard plaque assay on 293 cells was done to determine infectious particles.

Example 2. Expression and functionality of the constructed adenoviruses *in vitro*

Western blot analysis was used to confirm that the constructed adenoviruses express anti-CTLA4 mAb. A549 or PC3-MM2 tumor cells were infected with the constructed Ad5/3-Δ24aCTLA4 or Ad5/3-aCTLA4 at 10 Virus Particles (VP) per cell. After 48h, the supernatants of virus infected cells were filtrated with 0.02 µm filters (Anotop, Whatman, England), 15 µL were run on a

7.5% SDS–polyacrylamide gel electrophoresis (PAGE) gel under reducing or native conditions and transferred onto a nitrocellulose membrane. The membrane was incubated with goat anti-human IgG (heavy and light chains) (AbD serotec, MorphoSys, Germany), washed and incubated with a secondary antibody coupled to horseradish peroxidase (Dako, Denmark). Signal detection was done by enhanced chemiluminescence (GE Healthcare, Amersham, UK).

In Western blot, Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4 expressed the expected approximately 150 kDa anti-CTLA4 mAb in native conditions and the approximately 50 kDa heavy chains and the approximately 25 kDa light chains in denaturizing conditions (Fig. 2A) in supernatants at 48h after infection.

To compare the anti-CTLA4 mAb expression by the oncolytic virus Ad5/3-Δ24aCTLA4 or by the replicative deficient Ad5/3-aCTLA4, PC3-MM2 cells were seeded at 20000 cells per well and infected at 10VP per cell with the respective viruses. 24h, 48h and 72h post-infection the supernatants were collect and analyzed by ELISA for the amount of human IgG (Fig.10).

For confirmation of the expression of functional anti-CTLA4 mAb by Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4, increased IL-2 production of stimulated Jurkat cells was examined as described previously (Lee KM et al., Science 1998;282:2263-68).

Jurkat cells (clone 6.1) were stimulated with 0.3 µg/ml of ionomycin (Sigma-Aldrich Co.), 0.03 µg/ml of phorbol myristyl acetate (PMA) (Sigma-Aldrich Co.) and 1 µg/ml of Recombinant Human B7 Fc Chimera (R&D systems) and treated with 0.02 µm filtrated (Anotop, Whatman, England) supernatants of virus infected PC3-MM2 cells. Forty eight hours after Jurkat cells' stimulation, interleukin-2 (IL-2) levels in the growth media were analyzed by BD Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson) according to the instructions of the manufacturer. Ten viral particles (VP) per cell were used and 48h later the supernatants collected. Mouse anti-human CTLA-4 (=CD152) mAb (BD Pharmingen™, Europe) was used has positive control. FCAP Array v.1.0.2 (Soft Flow) software was used for analysis. Figure 7 shows the schematic of the functionality assays.

The anti-CTLA4 mAb binds cell surface CTLA-4 blocking the immunosuppresive interaction with recombinant B7 (rB7). This analysis indicates that mAb anti-CTLA4 activity was found in the supernatants of cells infected with Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4 compared to the

respective isogenic controls Ad5/3-Δ24 or Ad5/3Luc1-infected cells (Fig. 2B). Recombinant anti-CTLA4 mAb was used as a positive control and was more potent than the supernatant collected from Jurkat cells.

In order to further confirm the ability of virus expressed anti-CTLA4 mAb to block signaling through CTLA-4, the loss of function assay was performed. In a loss-of-function assay, Jurkat cells were activated as above, but 0.1 µg/ml of recombinant human CTLA-4/Fc Chimera (R&D Systems) (rCTLA-4) was added to ionomycine, PMA and recombinant B7. rCTLA-4 binds to the anti-CTLA4 mAb in the growth media releasing CTLA-4 on the cell surface to interact with rB7 and repress the T-cell activation which is seen as a decrease in IL-2 production. Loss of anti-CTLA4 mAb function was observed with supernatant from cells infected with Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4 in comparison to the respective isogenic unarmed controls Ad5/3-Δ24 or Ad5/3Luc1 (Figure 2C). Further, the highest loss of function was observed with the supernatant of Ad5/3-Δ24aCTLA4 tumor infected cells, even higher than the positive control. Taken together, this data indicate that infection of cancer cells with Ad5/3-Δ24aCTLA4 leads to high expression of functional fully human mAb against human CTLA-4 which results in reduction of T-cell activity as measured by IL-2.

When the deletions in the adenoviral E1 and E3 regions, i.e. the 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and the *6.7K/gp19K* deletion in E3, respectively, are taken into account, the insertion of the anti-CTLA4 expression cassette equals a genome size gain just below the proposed 105% threshold of compatibility with adenovirus packaging and functionality (Kennedy & Parks, Mol Ther 2009;17:1664-6). To study whether the increase in the genome size or the expression of anti-CTLA4 affect the viral replication, A549 cells were infected with Ad5/3-Δ24aCTLA4, Ad5/3-Δ24 or PBS and virus genomes were measured by qPCR at different time points from the supernatant and from the cells (forward primer, 5′-TCCGGTTTCTATGCCAAACCT-3′, SEQ ID NO:7; reverse primer, 5′-TCCTCCGGTGATAATGACAAGA-3′; SEQ ID NO:8; and probe 5′FAM-TGATCGATCCACCCAGTGA-3′MGBNFQ, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO: 11) (Cerullo et al., 2010; Cancer Res;70:4297-309). No significant differences were seen between the virus groups suggesting intact replicativity.

Example 3. CTLA-4 expression of tumor cell lines and low-passage tumor explants

Since it has been reported that almost 90% of the tumor cell lines express CTLA-4 and that anti-CTLA-4 mAb might have direct anti-tumor activity (13), it was investigated whether that was true also in the tumor cell lines including the HNSCC low-passage tumor explants used.

Indirect immunofluorescence was performed in low passage tumor cell culture UT-SCC8 or in tumor cell lines A549, SKOV3-ip1 and PC3-MM2 for analyzing the surface CTLA-4. Briefly, the cell pellet was incubated for 30 min at 4°C with mouse anti-human CTLA-4 mAb (BD Pharmingen™, Europe) as primary antibody followed by incubation for a further 30 min at 4°C with an Alexa Fluor® 488 donkey anti-mouse IgG (Invitrogen) as secondary antibody. The fluorescence intensity was measured on a LSR flow cytometer (BD Pharmingen™, Europe). At least 40 000 cells/sample were counted. A Clontech Discovery Labware immunocytometry systems (BD Pharmingen™, Europe) and the FlowJo 7.6.1 software were used for analysis.

A549, SKOV3-ip1 and PC3-MM2 tumor cell lines presented 99.5%, 96.6% and 96.6% CTLA-4 expression, respectively, while UT-SCC8 low-passage tumor explants presented a 90.3% CTLA-4 expression (Figure 2D).

Example 4. Assessment of oncolytic potency of the constructed adenoviruses *in vitro* and *in vivo*

The oncolytic efficacy (or cell killing) of Ad5/3-Δ24aCTLA4 on different tumor cell lines and on HNSCC low passage tumor cell culture was evaluated.

HNSCC low passage tumor cell culture or tumor cell lines PC3-MM2, SKOV3-ip1 or A549 were seeded at 1.5×10^4 or 1.0×10^4 cells/well on 96-well plates. On the next day, the viruses were diluted in DMEM with 2% FCS at different concentrations (1, 10, 100, 1000 VP/cell), cells were infected for 1 hour at 37°C, washed and incubated in 5% FCS in DMEM. The cell viability was determined according to the manufacturer's protocol (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Promega). The results are shown in Figure 3.

Ad5/3-Δ24aCTLA4 had oncolytic potency similar to the positive control virus Ad5/3-Δ24 in all cell lines. Also replication deficient Ad5/3-aCTLA4 had anti-tumor activity because tumor cells express CTLA4.

The oncolytic effect of Ad5/3-Δ24aCTLA4 resulted in 97.6%, 78.2%, 69.1% and 57.3% cell killing, on PC3-MM2, SKOV3-ip1, A549 and UT-SCC8, respectively (Figure 3). Ad5/3-Δ24aCTLA4 and its counterpart with no transgene in E3 (Ad5/3-Δ24) showed no statistical difference in cytotoxicity in any of the analyzed tumor cell lines.

With non-replicating Ad5/3-aCTLA4, no cytotoxicity was observed with lower virus doses (Fig.3). However, cytotoxicity was observed in the highest Ad5/3-aCTLA4 doses with a maximum cell killing rates of 96.2%, 74.3%, 49.1% and 56.15% on PC3-MM2, SKOV3-ip1, A549 and UT-SCC8, respectively (Fig.3). This result is in accord with previously demonstration of direct binding of anti-CTLA-4 with CTLA expressed on the surface of cancer cells inducing cell death (Contardi E et al., Int J Cancer 2005;117:538-50).

Example 5. Assessment of antitumor activity of Ad5/3-Δ24aCTLA4

Tumor growth suppression and apoptosis of the adenoviruses were assessed in immune deficient nude mice with human prostate cancer xenografts by treating the mice with anti-CTLA-4 monoclonal antibody expressing oncolytic adenovirus.

Human prostate explant xenografts were established by injecting $5x10^6$ PC3-MM2 cells into the flanks of 5- to 6-week-old female NMRI/nude mice (Taconic, Ejby, Denmark). After 7 days tumors (n=8/group, 5-8 mm in diameter) were injected with a volume of 50 µL for 3 times every other day with $1x10^8$ VP (days 0, 2 and 4) and control tumors were injected with DMEM only. The formula (length x $width^2$ x 0.5) was used to calculate tumor volume.

On day 5, tumor cryosections were stained for expression of anti-CTLA4 (Human IgG) or apoptosis (active caspase-3) by immunohisto-chemistry. 4-5 µm cryosections of frozen tumors embedded in Tissue Tek OCT (Sakura, Torrance, CA, USA) were prepared and fixed in acetone for 10 min at -20°C. As primary antibodies a goat anti-human IgG (heavy & light chains) (AbD serotec, MorphoSys) and a rabbit monoclonal antibody against active caspase-3 at dilution 1:200 for 1hour at room temperature (BD Pharmingen Tm, AB559565) were used. Further, sections were incubated according to manufacturer instructions with LSAB2 System-HRP kit (K0673, DakoCytomation, Carpinteria, CA, USA). Bound antibodies were visualized using 3,3'-diaminobenzidine (DAB, Sigma, St Louis, MO, USA). Lastly, sections were counterstained with hematoxyline and dehydrated in ethanol, clarified in xylene and sealed with Canada balsam. Representative pictures

were captured at 40 x magnification using a Leica DM LB microscope equipped with Olympus DP50 color camera.

On day 7, human IgG levels in tumors and plasma of mice treated with Ad5/3-Δ24aCTLA4 or Ad5/3-aCTLA4 were measured (Figure 4C). In Ad5/3-Δ24aCTLA4 treated tumors 81-fold more anti-CTLA4 mAb was found in comparison to Ad5/3-aCTLA4 treated tumors ($p<0.05$). In addition, 43.3-fold more anti-CTLA4 mAb was found in tumors treated with Ad5/3-Δ24aCTLA4 than in the plasma of the same animals ($p<0.05$). The average plasma concentration was 392.6 µg/g (SE 312.0), which is below concentrations reported tolerated in humans treated with ipilimumab (561 µg/mL) and tremelimumab 450 µg/mL, respectively (Weber JS, et al. J Clin Oncol 2008;26:5950-6. Ribas A, et al. J Clin Oncol 2005;23:8968-77. Tarhini AA, Iqbal F, Oncol Targets Ther 2010;3:15-25.) (1 mL of plasma weighs circa 1g). In Ad5/3-Δ24aCTLA4 tumors, mAb concentration was 16 977 µg/g and therefore much higher than in plasma.

Human anti-CTLA-4 mAb does not bind to mouse CTLA-4, and xenograft experiments require T-cell deficient nude mice. Thus, this model only assays oncolytic and pro-apoptotic effects. Nevertheless, Ad5/3-Δ24aCTLA4 showed a significant antitumor effect compared to mock ($p < 0.01$; Figure 4A) in this aggressive subcutaneous prostate cancer xenograft model. No other treated group presented significant effect compared to mock. No statistical difference was observed between Ad5/3-Δ24aCTLA4 and Ad5/3-Δ24 ($p=0.43$), confirming the *in vitro* data that the anti-CTLA4 mAb expression does not reduce the antitumor potency of the virus.

Given that CTLA-4–blocking Abs may induce direct killing of tumor cells by triggering apoptosis, it was assessed if anti-tumor efficacy was associated with human anti-CTLA4 mAb production and subsequently increased apoptosis. Human mAb staining in Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4 treated tumors was observed but not with Ad5/3-Δ24 or Ad5/3-Luc1 (Fig. 4B). The expression of human mAb seems to correlate with increased apoptosis (Fig. 4B). Thus Ad5/3-Δ24aCTLA4 treated tumors express anti-CTLA-4 mAb resulting in enhanced apoptosis.

To further assess the oncolytic and pro-apoptotic effects of Ad5/3-Δ24aCTLA4, a human lung cancer xenograft model was used. This model does not take into account the immunomodulatory functions of the transgene. Human lung cancer tumors were established by injecting 5 x 10^6 A549 cells

into the flanks of 5-6-week-old female NMRI nude mice. Tumors were treated with Ad5/3-Δ24aCTLA4, recombinant anti-CTLA4 protein, a non-replicating control virus Ad5/3lucI or an oncolytic Ad5/3-Δ24 and measured as described above for PC3-MM2 tumors. Mice were killed when a tumor reached an average diameter of 15 mm. No statistical difference was observed between the Ad5/3-Δ24aCTLA4 and Ad5/3-Δ24, suggesting that the replication dependent oncolytic potency of the viruses of the present invention is similar to the parent virus (Figure 4D).

Example 6. Immunomodulation of cancer patients' T-cells by anti-CTLA4 mAb expressing oncolytic adenoviruses

To extend the preclinical findings into humans, PBMCs of patients with advanced solid tumors refractory to chemotherapy were studied. PBMCs of cancer patients (patient I244 suffering from chondroideal melanoma, patient C261 suffering from colon cancer, patient M158 suffering from mesothelioma or patient X258 suffering from cervical cancer) were stimulated with 0.3 µg/ml of ionomycin (Sigma-Aldrich Co.), 0.03 µg/ml of phorbol myristyl acetate (PMA) (Sigma-Aldrich Co.) and 1µg/ml of Recombinant Human B7 Fc Chimera (R&D systems) to mimic tumor induced immune suppression. After stimulation PBMCs were treated with 0.02 µm filtrated (Anotop, Whatman, England) supernatants of PC3-MM2 cells infected Ad5/3-Δ24aCTLA4, Ad5/3-Δ24, Ad5/3-aCTLA4 or Ad5/3-Luc1.

In the loss of function assay 0.1 µg/ml of recombinant human CTLA-4/Fc Chimera (R&D Systems) was added to ionomycine, PMA and recombinant B7. Twenty four hours after PBMC stimulation interleukin-2 (IL-2) or interferon-γ (IFN-γ) levels in the growth media were analyzed by BD Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson) according to the instructions of the manufacturer. Ten viral particles (VP) per cell were used and 48h later the supernatants collected. Mouse anti-human CTLA-4 (=CD152) mAb (BD Pharmingen™, Europe) was used has positive control. FCAP Array v.1.0.2 (Soft Flow) software was used for analysis.

In all four patients' samples, the supernatant from anti-CTLA4 mAb expressing oncolytic adenoviruses was able to increase T-cell activity, as measured by IL-2 and interferon gamma (Figure 5). The rationale for the experiment is shown in Figure 7. Interestingly, while with Jurkat cells, which is an immortalized T-cell line, the recombinant mAb was more effective, with

patient samples the viral supernatants were often more potent. Similar data were obtained in a loss function assay (Figure 8), rationale in Figure 7E-F.

Example 7. Effect of anti-CTLA4 mAb on PBMCs from healthy individuals

The experiments described in Example 6 were also performed using PBMCs of healthy individuals. In contrast to cancer patients, supernatant from Ad5/3-Δ24aCTLA4 infected cells did not increase IL-2 or interferon gamma production. Significant changes were not seen in the loss of function assay either. However, since the positive control recombinant mAb was effective in both cases (increased IL-2 and interferon gamma in Figure 6 and decreased them in Figure 8 (increased IL-2 $p<0.05$ and $p=0.206$, increased INF-γ $p<0.05$ and $p=0.120$, respectively in donor 1 and donor 2 in Figure 6; decreased IL-2 $p<0.001$ and $p<0.05$; and decreased INF-γ $p<0.001$ and $p<0.05$ respectively in donor 1 and donor 2 in Figure 8; comparing to rB7, rCTLA4. ionomycin and PMA only treated cells), it was assumed that this is a dose effect. This is supported by the non-significant trend seen for Ad5/3-Δ24aCTLA4 in the loss-of-function assay (Figure 8) and several cases of significant effect by supernatant from Ad5/3-aCTLA4 infected cells (Figures 6 and 8). Nevertheless, the effect of anti-CTLA-4 mAb was more pronounced in cancer patients, perhaps because they have a higher degree of immunosuppressive processes ongoing due to the advanced tumor present.

Example 8: Utility of the replication competent platform in increasing anti-CTLA4 mAb expression

The utility of the replication competent platform in increasing anti-CTLA4 mAb expression in comparison to the replication deficient virus was analyzed. PC3-MM2 cells were seeded at 20000 cells per well and infected at 10VP per cell with Ad5/3-Δ24aCTLA4 and Ad5/3-aCTLA4, respectively. 24h, 48h and 72h post-infection the supernatants were collected and analyzed by ELISA for the amount of human IgG. A 3-fold increase was observed with the oncolytic virus Ad5/3-Δ24aCTLA4 in comparison to the replicative deficient Ad5/3-aCTLA4. Ad5/3-aCTLA4 (Figure 10).

One utility of the oncolytic platform is the higher level of transgene expression obtained. After expression of early genes, the viral genome amplifies and up to 10 000 copies of viral DNA is produced. This results in many more copies that can produce the transgene (Figure 10).

Example 9: Oncolytic adenovirus vectors with increased immune response

To increase immune response further, oncolytic adenoviruses featuring toll-like receptor 9 (TLR-9) stimulating CpG molecules in the backbone of the virus (Figure 1) were studied using a xenograft mouse model of lung cancer. NMRI nude mice (5 mice per group, two tumors per mouse) were implanted with A549 cells and treated $1x10^8$ VP of a CpG-rich oncolytic adenovirus Ad5-Δ24CpG, an oncolytic adenovirus containing Δ24 deletion, oncolytic adenovirus + CpG containing recombinant oligos (ODN 2395, InvivoGen, USA). Tumor growth was measured at two days' intervals for 12 days as described earlier. The CpG rich virus Ad5-Δ24CpG was most effective in mediating antitumor immunity (Figure 11).

Splenocytes harvested from the same mice were stimulated with a UV inactivated virus and co-cultured at a ratio of 1:1 and 10:1 with A549 cells. A MTS cell killing assay was performed at 72 hours. The percentage of A549 cells still alive at the indicated time point is given. The data shown in Figure 12A demonstrates that the CpG modified virus was able to stimulate antigen presenting cells and this resulted in an enhanced anti-tumor immune response. The response was so potent that it could be seen even in nude mice which lack T-cells. Therefore, even better data is expected in immune competent animals and humans. The utility of TLR-9 stimulation (which induces anti-tumor immunity) is likely to be most prominent with simultaneous down-regulation of suppressive signals with aCTLA-4.

The expression of anti-CTLA4 produced by a virus may enhance the anti-virus immunity and thereby counteract virotherapy. To this end, the effect of mouse anti-CTLA4 together with an adenovirus on splenocytes of immunocompetent mice was assessed. Immunocompetent C57Bl/6 mice (n=5) were treated three times with PBS, Ad5/3-Δ24 alone, Ad5/3-Δ24 and mouse aCTLA4 antibody in combination or with mouse aCTLA4 antibody only (Figure 12B). Two weeks later spleens were collected and splenocytes analyzed by interferon gamma ELISPOT (ELISPOT^{PRO} for human IFN-γ, 3420-2APT-10, MABTECH AB, Sweden) Stimulation was done by a UV-inactivated Ad5/3 chimeric virus or Ad5 peptide mix. No significant difference was observed between the groups suggesting that mouse anti-CTLA4 antibody does not affect the immunity caused by the virus.

Example 10. Safety and efficacy of oncolytic adenovirus vectors featuring monoclonal antiCTLA-4 antibodies in human cancer patients

I. Patients

Patients with advanced and treatment refractory solid tumors are enrolled in a Finnish Medicines Association (FIMEA) approved Advanced Therapy Access Program (ATAP).

Patients are chosen among cancer patients with advanced solid tumors refractory to standard therapies. Inclusion criteria were solid tumors refractory to conventional therapies, WHO performance score 3 or less and no major organ function deficiencies. Exclusion criteria were organ transplant, HIV, severe cardiovascular, metabolic or pulmonary disease or other symptoms, findings or diseases preventing oncolytic virus treatment. Written informed consent was obtained and treatments were administered according to Good Clinical Practice and the Declaration of Helsinki. Treatments are given intratumorally, intravenously or intraperitoneally as appropriate

II. Treatments with an adenoviral vector encoding aCTLA-4 MAb

Oncolytic adenoviruses are produced according to clinical grade and the treatment of patients is initiated.

The treatment is given by intratumoral injections. The total number of treatments is three every 3 weeks. The viral doses are chosen based on previous data of the inventors with other oncolytic viruses. However, different administration schemas may be used as appropriate. For instance, for the first round of serial treatment, patients receive a portion of the dose, *e.g.*, four fifths or two fifths of the dose, intratumorally or intraperitoneally and the rest of the dose intravenously.

The virus is diluted in sterile saline solution at the time of administration under appropriate conditions. Following virus administration all patients are monitored overnight at the hospital and subsequently for the following 4 weeks as outpatients. Physical assessment and medical history were done at each visit and clinically relevant laboratory values were followed.

Side effects of treatment are recorded and scored according to Common Terminology for Adverse Events v3.0 (CTCAE). Since many cancer patients have symptoms due to disease, pre-existing symptoms are not scored if they do not become worse. However, if the symptom became more severe, e.g. pre-treatment grade 1 changed to grade 2 after treatment, it is scored as grade 2.

Tumor size is assessed by contrast-enhanced computer tomography (CT) scanning. Maximum tumor diameters are obtained. Response Evaluation Criteria in Solid Tumors (RECIST1.1) criteria are applied to overall disease, including injected and non-injected lesions. These criteria are: partial response PR (>30% reduction in the sum of tumor diameters), stable disease SD (no reduction/increase), progressive disease PD (>20% increase). Clear tumor decreases not fulfilling PR are scored as minor responses (MR). Serum tumor markers are also evaluated when elevated at baseline, and the same percentages are used.

Patient serum samples are analyzed for immunological and virological parameters, including virus copy number in blood over time, induction of anti-viral neutralizing antibodies, changes in anti-viral and anti-tumor T-cells, other immunological cell types and changes in anti-tumor antibodies. Additionally adverse events are graded according to Common Terminology for Adverse Events v3.0 (CTCAE), neutralizing antibody titers are measured and the efficacy is evaluated according to RECIST criteria for computer tomography (CT) (Therasse P et al. 2000, J Natl Cancer Inst 92, 205-16) or PERCIST criteria (Wahl et al 2009 J Nucl Med 50 Suppl 1:122S-50S) for positron emission tomography computer tomography (PET-CT). All patients have had progressing tumors prior to treatment and are at different stages of disease.

Claims

1. An oncolytic adenoviral vector comprising

1) an adenovirus serotype 5 (Ad5) nucleic acid backbone comprising a capsid modification,

2) a 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and

3) a nucleic acid sequence encoding a fully human monoclonal antibody specific for CTLA-4 (aCTLA MAb) in the place of the deleted adenoviral genes *gp19k/6.7K* in the E3 region.

2. An oncolytic adenoviral vector according to claim 1 further comprising one or more regions selected from a group consisting of E2, E4, and late regions.

3. An oncolytic adenoviral vector according to claim 1 or 2 comprising the following regions: a left ITR, partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5, E4, and a right ITR.

4. An oncolytic adenoviral vector according to claim 3, wherein the regions are in a sequential order in the 5' to 3' direction.

5. An oncolytic adenoviral vector according to any one of the previous claims, wherein a wild type region is located upstream of the E1 region.

6. An oncolytic adenoviral vector according to any one of the previous claims, wherein the E1 region comprises a viral packaging signal.

7. An oncolytic adenoviral vector according to any one of the previous claims, wherein a nucleic acid sequence encoding aCTLA MAb is under the control of the viral E3 promoter.

8. An oncolytic adenoviral vector according to any one of the previous claims, which additionally comprises a nucleic acid sequence encoding a tumor specific human telomerase reverse transcriptase (hTERT) promoter or an E2F promoter upstream of the E1 region.

9. An oncolytic adenoviral vector according to any one of the previous claims, which additionally comprises a CpG site in the viral backbone.

10. An oncolytic adenoviral vector according claim, wherein the CpG site is in the E3 region.

11. An oncolytic adenoviral vector according to any one of the previous claims, wherein the E4 region is of a wild type.

12. An oncolytic adenoviral vector according to any one of the previous claims, wherein the capsid modification is Ad5/3 chimerism, insertion of an integrin binding (RGD) region and/or heparin sulphate binding polylysine modification into the fiber.

13. An oncolytic adenoviral vector according to claim 12, wherein the capsid modification is Ad5/3 chimerism.

14. An oncolytic adenoviral vector according to claim 12, wherein the capsid modification is a RGD-4C modification.

15. An oncolytic adenoviral vector according to any one of the previous claims comprising at least one expression cassette.

16. An oncolytic adenoviral vector according to any one of the previous claims comprising only one expression cassette.

17. An oncolytic adenoviral vector according to any one of the previous claims, wherein the vector is capable of selectively replicating in cells in cells having defects in the Rb/p16-pathway.

18. An oncolytic adenoviral vector according to any one of the previous claims, wherein the vector is capable of selectively replicating in cells expressing telomerase.

19. A cell comprising the adenoviral vector according to any one of claims 1-18.

20. A pharmaceutical composition comprising the adenoviral vector according to any one of claims 1-18.

21. An oncolytic adenoviral vector or pharmaceutical composition according to any one of the previous claims, which acts as an *in situ* cancer vaccine.

22. Adenoviral vector according to any one of claims 1-18 for treating cancer in a subject.

23. A method of treating cancer in a subject, wherein the method comprises administration of the vector or pharmaceutical composition according to any one of claims 1-18 or 20 to a subject.

24. The adenoviral vector or method according to claims 21-23, wherein the cancer is selected from a group consisting of nasopharyngeal cancer, synovial cancer, hepatocellular cancer, renal cancer, cancer of connective tissues, melanoma, lung cancer, bowel cancer, colon cancer, rectal cancer, colorectal cancer, throat cancer, oral cancer, liver cancer, bone cancer, pancreatic cancer, choriocarcinoma, gastrinoma, pheochromocytoma,

prolactinoma, T-cell leukemia/lymphoma, neuroma, von Hippel-Lindau disease, Zollinger-Ellison syndrome, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, ureter cancer, oligodendroglioma, neuroblastoma, meningioma, spinal cord tumor, osteochondroma, chondrosarcoma, Ewing's sarcoma, cancer of unknown primary site, carcinoid, carcinoid of gastro-intestinal tract, fibrosarcoma, breast cancer, Paget's disease, cervical cancer, esophagus cancer, gall bladder cancer, head cancer, eye cancer, neck cancer, kidney cancer, Wilms' tumor, Kaposi's sarcoma, prostate cancer, testicular cancer, Hodgkin's disease, non-Hodgkin's lymphoma, skin cancer, meso-thelioma, multiple myeloma, ovarian cancer, endocrine pancreatic cancer, glucagonoma, pancreatic cancer, parathyroid cancer, penis cancer, pituitary cancer, soft tissue sarcoma, retinoblastoma, small intestine cancer, stomach cancer, thymus cancer, thyroid cancer, trophoblastic cancer, hydatidiform mole, uterine cancer, endometrial cancer, vagina cancer, vulva cancer, acoustic neuroma, mycosis fungoides, insulinoma, carcinoid syndrome, somatostatinoma, gum cancer, heart cancer, lip cancer, meninges cancer, mouth cancer, nerve cancer, palate cancer, parotid gland cancer, peritoneum cancer, pharynx cancer, pleural cancer, salivary gland cancer, tongue cancer, tonsil cancer.

25. The adenoviral vector or method according to claims 21-24, wherein the subject is a human or an animal.

26. The adenoviral vector or method according to any one of claims 21-25, wherein the administration is conducted through an intratumoral, intramuscular, intra-arterial, intravenous, intrapleural, intravesicular, intracavitary or peritoneal injection, or an oral administration.

27. The adenoviral vector or method according to any one of claims 21-26, wherein oncolytic adenoviral vectors or pharmaceutical compositions are administered several times during the treatment period.

28. The adenoviral vector or method according to any one of claims 21-27, wherein the oncolytic adenoviral vector having a different fiber knob of the capsid compared to the vector of the earlier treatment, is administered to a subject.

29. The adenoviral vector or method according to any one of claims 21-28, wherein the method further comprises administration of concurrent radiotherapy or concurrent chemotherapy or other concurrent cancer therapies to a subject.

30. The adenoviral vector or method according to any one of claims 21-29, wherein the method further comprises administration of an auxiliary agent, selected from the group consisting of verapamil or another calcium channel blocker; an autophagy inducing agent; temozolomide; a substance capable to downregulating regulatory T-cells; cyclophosphamide and any combination thereof in a subject to a subject.

31. The adenoviral vector or method according to any one of claims 21-29, wherein the method further comprises administration of chemotherapy or anti-CD20 therapy or other approaches for blocking of neutralizing antibodies.

32. A method of producing a fully human monoclonal antibody specific for CTLA-4 in a cell, wherein the method comprises:

a) carrying a vehicle comprising an oncolytic adenoviral vector according to any one of claims 1-18 to a cell, and

b) expressing a fully human monoclonal antibody specific for CTLA-4 of said vector in the cell.

33. A method of increasing tumor specific immune response in a subject, wherein the method comprises:

a) carrying a vehicle comprising an oncolytic adenoviral vector according to any one of claims 1-15 to a target cell or tissue,

b) expressing a fully human monoclonal antibody specific for CTLA-4 of said vector in the cell, and

c) increasing amount of expressed anti-CTLA4 mAb by using the oncolytic platform.

d) increasing the tumor to plasma ratio of anti-CTLA4 mAb by using the oncolytic platform.

34. A use of the oncolytic adenoviral vector according to any one of claims 1-18 for producing anti-CTLA4 mAb in a cell.

35. Oncolytic adenoviral vector of any one of claims 1-18 for producing a fully human monoclonal antibodies against CTLA4.