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Nucleic acids encoding CD40/CD40L chimeric polypeptides, methods for their production and uses thereof

The invention relates to nucleic acids encoding CD40/CD40L chimeric polypeptides, to methods for their production, pharmaceutical compositions containing said nucleic acids, and to uses thereof.

CD40 receptor (hereinafter also referred to as CD40), a cell surface receptor from the family of TNF receptors, was first identified and functionally characterized on B lymphocytes, where it is involved in the regulation of growth and differentiation. It may also be found, however, on other cell types, for example on T cells (activation → proliferation stimulation, cytokine secretion), dendritic cells, and monocytes (activation → expression of costimulatory molecules, secretion of inflammatory cytokines, e.g., TNF-α, IL-8, IL-12) as well as on carcinomas (activation leads to inhibition of growth) (van Kooten et al., Int. Arch. Allergy Immunol. 113 (1997) 393-399). It is a member of the group of type I membrane proteins, which implies an extracellular N terminus, a transmembrane domain, and an intracellular C terminus (Laman et al., Critical Reviews in Immunology 16 (1996) 59-108).

CD40L (= CD154 or gp39) is the ligand of CD40 (WO 93/08207). It tends to be expressed on activated CD4+ T cells, but is also found on activated B cells and dendritic cells. Interaction between CD40L and CD40 plays a central role in the development of both humoral and cellular immunity. Ligation of CD40L to CD40 initiates both a CD40-mediated signal (APC (Antigen Presenting Cell) activation) and a T-cell-stimulating signal via CD40L itself (T cell activation). CD40L belongs to the group of type II membrane proteins, which implies an intracellular N terminus, a transmembrane region and an extracellular C terminus (Laman et al., Critical Reviews in Immunology 16 (1996) 59-108). There have also been described soluble forms of CD40L, which means that they only consist of the C terminal extracellular domain and are capable of activating CD40. Consequently, the extracellular domain is sufficient for the formation of trimers, which, in turn, bind to the extracellular domain of the receptor CD40 and thereby trimerize and activate this receptor. Yet, qualitative differences have been discussed in the literature with respect to the mediation of signals by soluble CD40L and by membrane-bound CD40L (Laman et al., Critical Reviews in Immunology 16 (1996) 59-108).

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Gires et al., EMBO J. 16 (1997) 6131-6140, have shown, that fusion constructs of EBV-virus protein LMP1 and CD40 are functionally active in constitutive CD40 signalling.

Hatzivassiliou et al., J. Immunol. 160 (1998) 1116-1121, describe a fusion of LMP-1 and the cytoplasmic domain of CD40 which activates epidermal growth factor receptor, NF-κB and stress-activated protein kinase like LMP-1.

WO 98/26061 describes a method of treating a human neoplasia comprising inserting into a human neoplastic cell a cDNA which encodes a chimeric protein which contains at least a portion of the murine CD40L gene together with portions of the same or other CD40L ligand genes from either mouse, human or other species. Especially preferred is a chimeric gene consisting of the murine CD40L gene transmembrane and cytoplasmatic domains having been attached to the extracellular domains of human CD40L gene.

It is the object of the invention to provide new nucleic acids which are especially useful in gene therapy and are particularly useful for ex vivo treatment of tumor cells and local treatment of solid tumors.

Summary of the Invention

According to the invention it is possible to produce a constitutively active CD40 which needs no foreign components, or only needs a strongly reduced level of foreign components, in order to be active. The invention provides a chimera consisting of the „soluble", that is, the non-membrane-bound, extracellular domain of CD40L covalently bound to the CD40. This is achieved by means of a fusion gene which contains the following components:

- signal peptide sequence, preferably of CD40 or another mammalian signal sequence;
- extracellular domain of CD40L or a part thereof causing binding to CD40 and trimerization;
- spacer sequence;
- extracellular domain of CD40 or a part thereof binding to CD40L (optional);
- transmembrane domain of CD40;

– cytoplasmic domain of CD40 which causes signal transduction.

The invention therefore comprises a nucleic acid encoding a chimeric polypeptide comprising nucleic acid fragments encoding

i) a mammalian signal peptide and downstream thereof;
ii) the binding and trimerization domain of CD40L and downstream thereof;
iii) a spacer of 50 to 100 amino acids;
iv) the transmembrane and signal transduction domains of CD40.

Fragments or modifications of these domains having substantially the same activity in respect of binding, trimerization and signal transduction are also useful according to the invention. Such modifications are, e.g., exchanges of amino acids by other amino acids which do not affect the protein conformation. In addition, such nucleic acids can be used which due to the degeneracy of the genetic code encode a polypeptide encoded by any of the above-mentioned nucleic acids. Particularly preferred are nucleic acids which use the human codon usage. The signal peptide is preferably the signal peptide of CD40 or a fragment thereof.

In a preferred embodiment of the invention (Version A, see Figs. 1 and 2), the encoded polypeptide consists of the signal peptide, the CD40L binding and trimerization domain, no linker or a short linker with about 1 to 30 amino acids, the ligand binding subdomain of CD40, a spacer of 50 to 100 amino acids, the transmembrane domain and the signal transduction domain of CD40. Version A is able to trimerize without the aid of a second cell. The design of the construct of Version A must be such that it is able to trimerize without steric hindrance. This means that the CD40L binding region must be far enough outside of the cell surface. For this, it is preferred that a spacer, preferably the stalk subdomain of CD40 or CD40L, a chimeric stalk domain consisting of a part of the stalk domain and other amino acids or another polypeptide acting as a spacer, having the above-mentioned length, is inserted between the CD40L binding subdomain and the transmembrane domain of CD40. Further preferred is a spacer consisting of at least 80% of the amino acids Glu and/or Ala (linear and flexible spacer, Glu/Ala spacer). The CD40 part of this construct is only necessary for CD40L binding and signal transduction. Therefore, other parts of the extracellular domain of CD40 can be deleted. The CD40L part of this construct is necessary for binding and

trimerization. However, it must be ensured that the binding domains of CD40 and CD40L are able to bind and trimerize. For this, the linker between both binding domains must be sufficient in length. A length of about 8 to 30 amino acids, preferably 15 to 30 amino acids, is therefore preferred.

In a second preferred embodiment of the invention (Version B, see Figs. 1 and 3), after the signal peptide the soluble, that is, the CD40L binding and trimerization domain is covalently bound to the transmembrane region of the CD40 receptor, that is, the extracellular region of the CD40 receptor (or at least the part of the extracellular region causing binding to CD40L), which is required for the binding of CD40L, is deleted. It is necessary to introduce between the extracellular domain of CD40L and the transmembrane domain of CD40 a spacer of a length of approximately 50 to100 amino acids, for example the stalk domain of CD40 and an additional flexible polypeptide like 15 – 30 amino acids from the hinge region of IgD. Such a spacer can be, for example, as in Version A, the stalk domain of CD40 or CD40L (about 80 aa), a chimeric stalk domain consisting of a part of the stalk domain and other amino acids or another polypeptide acting as a spacer, having the above-mentioned length securing trimerization without steric hindrance. Here the above-mentioned Glu/Ala spacer is also preferred. This embodiment offers the advantages that the covalently bound, extracellular CD40L domain trimerizes spontaneously and, as a result, trimerizes the covalently bound cytoplasmic CD40 receptor and activates the same. Another advantage of this embodiment is that the CD40 ligand trimer is still present in an unbound form and can therefore interact *in trans* with other natural CD40 receptors on different cells, trimerize same and, as a result, activate the same as well, that is to say, this embodiment not only acts directly on the cell expressing the chimeric receptor but can also have an activating effect *in trans* on another cell that expresses natural CD40 receptors.

According to the invention it is possible to induce the presentation of tumor-specific antigens on immunocompetent cells in an improved manner and to induce differentiation and proliferation of such immunocompetent cells like T cells, macrophages and dendritic cells. The chimeric CD40/CD40L polypeptide shows a „trans" activity by activation of professional APCs in the same way as CD40L ligated to CD40, that is, it translocates the signal via the JNK/AP-1, JAK/STAT and I-KB/NFkB pathways, which leads to upregulation of genes coding for MHC-I and/or MHC-II, for costimulatory molecules such as B-7.1, B-7.2,

immunostimulatory cytokines such as IL-12, IL-8, and proinflammatory cytokines such as IL-1α, IL-1ß and/or IL-6. It additionally acts in „cis", that means it acts directly on tumor cells by induction of growth inhibition (Eliopoulos, A.G., Oncogene 13 (1996) 2243-2254; van Kooten et al., Int. Arch. Allergy Immunol. 113 (1997) 393-399; Young, L.S., et al., Immunology Today 19 (1998) 502-506).

Several proteolytic cleavage sites are described and actually found in human and murine CD40L (Gauchat, J.-F. et al., FEBS 315 (1993) 259-266; Armitage, R.J. et al., Nature 357 (1992) 80-82; Hsu, Y.-M. et al., The J. of Biol. Chem. 272 (1997) 911-915; Kato, K. et al., J. Clin. Invest. 104 (1999) 947-955).

However fusion proteins of CD40L and CD40 according to the invention are free of typical protease cleavage sites. The cleavage sites described for CD40L exist in a region of the CD40L-molecule which is deleted in the fusion constructs. Therefore the CD40/CD40L chimeric molecules are less sensitive to proteolytic cleavage and thereby less sensitive to downmodulation of their signalling capacity by proteolytic cleavage.

In a further preferred embodiment of the invention, the nucleic acid contains a further gene fragment encoding a cytokine such as IL-2, IL-12, lymphotactin (Dilloo et al., Nature Medicine 2 (1996) 1090, and Entage, Hum. Gene Therapy 10 (1999) 697-709) and/or Interferon-alpha.

The invention further comprises a recombinant vector for the expression of said nucleic acid, wherein the expression of said nucleic acid is under the control of a mammalian promoter, preferably of a CMV promoter or a cytokine-inducible (inflammatory regulated) promoter, more preferably, under the control of an acute phase protein gene promoter, and particularly preferably, under the control of the human acute phase serum amyloid A gene promoter SAA1 or SAA2 (hereafter referred to as „SAA promoter").

The invention further comprises, in a preferred embodiment, a combination of the vector according to the invention with one or more vectors, which expresses, one or more additional genes selected from the group consisting of the genes encoding a cytokine such as IL-2, IL-12 and Interferon-alpha.

If one or more additional genes are used, then said genes can be under the control of the same promoter on the same vector, under the control of two identical or different promoters on the same vector, or on different expression vectors.

The invention further comprises compositions, preferably pharmaceutical compositions, containing at least one expression vector according to the invention as an essential component. The compositions comprise nucleic acids/expression vectors according to the invention together with a pharmaceutically acceptable excipient and/or preservative.

Such compositions are produced by the use of the nucleic acids/expression vectors according to the invention as the essential constituents of such compositions. The compositions are useful for activating antigen presenting cells and T cells.

In a preferred embodiment of the invention, the composition contains at least two genes on one or more vectors. Preferred examples of genes encoded by such vectors are listed below in Tables 1 and 2.

Table 1

Vector 1	Vector 2
chimeric CD40/CD40L	–
chimeric CD40/CD40L	IL-12, IL-2 or interferon-α
chimeric CD40/CD40L	IL-2 and IL-12
chimeric CD40/CD40L	IL-2 and interferon-α
chimeric CD40/CD40L	IL-12 and interferon-α

Genes from Vector 1 and Vector 2 can also be co-expressed on one vector.

The following combinations are also preferred:

Table 2

Vector 1	Vector 2	Vector 3
chimeric CD40/CD40L	IL-2	IL-12
chimeric CD40/CD40L	IL-2	interferon-α
chimeric CD40/CD40L	IL-12	interferon-α

Genes fromVectors 1 to 3 can also be combined on one or two vectors.

Particularly preferred is the combination of the chimeric CD40/CD40L vector with IL-12 (IL-12 as gene or as protein).

The invention further comprises methods for the production of such expression vectors and of compositions, preferably pharmaceutical compositions, containing such vectors. The pharmaceutical compositions are used for ex vivo and in vivo treatment, preferably for in vivo treatment of tumor cells of a patient (gene therapy treatment). According to the invention it was found that vectors containing the chimeric CD40/CD40L gene under the control of the SAA promoter are preferred and improved therapeutic agents for the treatment of tumor diseases.

In a further preferred embodiment of the invention, the expression vectors or host cells according to the invention are combined, for the treatment of tumor disease, with the proteins of Interleukin-2 (IL-2), Interleukin-12 (IL-12) and/or Interferon-alpha (preferably interferon-α2A) and/or with 5-fluorouracil, preferably for application in vivo.

The invention further comprises a mammalian host cell transfected with an expression vector according to the invention and a process for the production of a chimeric CD40/CD40L polypeptide according to the invention by culturing a host cell of the invention under conditions promoting the expression of the CD40/CD40L chimeric gene and presenting said polypeptide on the surface of the host cell. The transfected host cell can also be used as a pharmaceutical agent.

The invention further comprises a process for the production of a modified human tumor cell containing an expression vector encoding a chimeric CD40/CD40L

polypeptide under conditions in which said CD40/CD40L polypeptide is produced in the tumor cell and presented on the surface of said cell.

A further object of the invention is a chimeric polypeptide encoded by a nucleic acid according to the invention, wherein the extracellular domain of CD40L consists of amino acids 47-261 of human CD40L or 47-260 of murine CD40L and/or the intracellular domain of CD40 consists of amino acids 216-277 of human CD40 or 216-289 of murine CD40.

A further object of the invention is a method for the production of a composition, preferably a pharmaceutical composition for activating antigen presenting cells and T cells comprising a nucleic acid according to the invention, characterized by the use of a nucleic acid according to the invention as an essential constituent of said composition.

Detailed Description of the Invention

A promoter (also designated as an expression control region) according to the invention is understood as a nucleic acid region which causes the expression of DNA and hence transcription into mRNA and which usually has a length of 0.5-5 kb. Such expression control regions usually contain enhancer regions and promoter regions to which transcription factors or repressors can bind. Expression control regions can be regulated via binding of activating or repressing factors. A regulatory region according to the invention is understood as a region which influences expression due to induction by cytokines. Based on this, the expression is stimulated.

In a preferred embodiment of the invention, a minimal promoter combined with enhancing elements is used. Minimal promoters and methods for their construction are described, for example, in Luckow, B., et al., Nucleic Acids Res. 10 (1987) 5490 and Spear, B.T., et al., DNA Cell Biol. 14 (1995) 635-642. A minimal promoter useful in the expression vectors according to the invention contains at least a TATA-box, one ore more cytokine-responsive elements (CRE) and one or more binding sites for transactivators such as NFkB, CEBP/NF-IL6 and also others such as YY1, SAF and AP1. Such binding sites usually consist of 4 to 12 nucleotides in length. CREs are described, for example, in Dendorfer, U., Artif. Organs 20

(1996) 437-444; Birt, D.F., et al., J. Nutr. 129 (1999) 25 Suppl., 5715-5745; Kang, D.C., et al., Int. J. Oncol. 13 (1998) 1117-1126; Weber-Nordt, R.M., et al., Leuk. Lymphoma 28 (1998) 459-467; Sen, C.K., FASEB J. 10 (1996) 709-720.

Useful promoters according to the invention are exogenous viral promoters such as Simian virus 40, Rous sarcoma virus and cytomegalovirus (CMV). Such promoters are constitutive promoters and they require no specific inducing signals.

Preferred cytokine-inducible promoters useful in the invention are highly sensitive to cytokine induction during local or systemic inflammation due to the action of pro-inflammatory proteins like IL-1ß, IL-6 and TNFα. Also due to the fact that expression of these cytokines has been found in several tumors (e.g., squamous cell carcinomas) but not in normal uninflamed tissue, a specific expression of such promoters in these types of tumors can be found. Several tumors have been described to express high amounts of pro-inflammatory cytokines, e.g. Knerer et al., Acta Oto-Laryngologica 116 (1996) 132-136 described high level expression of IL-1 and TNF-α in squamous cell carcinoma of the head and neck. But also other tumors have been described to express pro-inflammatory cytokines, e.g. Levy et al., Neurosurgery 39 (1996) 823-823 found IL-6 and IL-1ß expression in meningonomas. Cytokine-inducible promoters such as the preferred SAA2-promoter or the $sPLA_2$-promoter will be activated in tumor tissues such as melanomas, prostate tumors, bladder carcinomas, breast tumors and colon tumors. They are therefore particularly suitable for incorporation into constructs designed to drive the synthesis of desired polypeptides. They are especially advantageous for the treatment of squamous cell carcinomas like head and neck cancers.

Of course other acute phase protein gene promoters could be used, including promoters of C-reactive protein, fibrinogen, serum amyloid protein, complement factor 3, orosomucoid, alpha.sup.1 -antiprotease (antitrypsin) and other isoforms of SAA. Promoters of genes encoding the major APRs (in humans the acute phase serum amyloid As [A-SAAs] and C-reactive protein (CRP)) are extremely responsive to such signals causing them to be massively induced during the acute phase response (Varley, A.W., Proc. Natl. Acad. Sci. USA 92 (1995) 5346-5356; U.S. Patent No. 5,744,304; U.S. Patent No. 5,851,822; Kushner, I., Ann. NY Acad. Sci. USA 389 (1982) 39; Kushner, I., and Mackiewicz, A., Disease Markers 5 (1987) 1; Fey, G.H., and Gauldie, J., In: H. Popper and F. Schaffner (Eds.), Progress in Liver

Diseases. Vol.9. (1989) WB Saunders, Philadelphia, p.89). Consequently major APR promoters have the potential to be used as indicators of the ability of naturally occurring and synthetic molecules to act as pro- and anti-inflammatory reagents.

The human acute-phase serum amyloid A promoter (SAA2-promoter) have been cloned by Uhlar et al., J. Immunol. Methods 203 (1997) 123-130. The SAA2-promoter is described to be active in inflamed tissue and can be highly activated (by factor 70) in vitro by monocyte conditioned medium as well as by IL-1ß IL-6 and TNF-α IL-6. IL-1ß mediated stimulation can be blocked by IL-1 receptor antagonist (Uhlar et al., J. Immunol. Methods 203 (1997) 123-130 and Steel et al., Biochem. Journal 291 (1993) 701-707).

It is further preferred to use within the vectors according to the invention a translation control element within the SAA2 5'-UTR that plays a crucial role in modulating A-SAA production. This element is a cell- and/or tissue-specific translational enhancer. Its efficiency could be mediated by an intracellular factor that is activated or synthesized de novo after cytokine treatment. The sequence of this enhancer element is shown in SEQ ID NO:4 of WO 98/40506. Preferably, the enhancer is used in conjunction with the A-SAA promotor (i.e., downstream of the promotor and upstream of the gene encoding the product of interest).

The SAA2 promoter which is preferred according to the invention is a promoter which is very silent in healthy noninflammated tissues but which is specifically active in inflammated but also in tumor tissue. A murine squamous carcinoma cell line like SCC-VII is a model system to show the functionality and tumor specificity of the SAA2 promoter in tumors expressing preinflammatory cytokines as it is already described for human squamous carcinomas.

"SAA promoter" according to the invention therefore means a promoter which has the function of an A-SAA promoter and which is therefore inducible by a cytokine in substantially the same manner as the SAA promoter described in WO 98/40506 and which is essentially identical to the sequence of the SAA1 and/or SAA2 promoter. Also preferred are promoters which are coded by DNA sequences which hybridize with SEQ ID NO:1 shown in WO 98/40506 under stringent conditions and have the ability to act as an expression control sequence inducible by cytokines.

The phrase "hybridize under stringent conditions" means that two nucleic acid fragments are capable of hybridization to one another under standard hybridization conditions described in Sambrook et al., "Expression of cloned genes in E.coli" in Molecular cloning: a laboratory manual (1989), Cold Spring Harbor Laboratory Press, New York, USA, 9.47-9.63 and 11.45-11.61. More specifically, "stringent conditions" as used herein refers to hybridization in 6.0 x SSC at about 45°C followed by a wash of 2.0 x SSC at 50°C. For a selection of the stringency the salt concentration in the wash step can be selected, for example, from about 2.0 x SSC at 50°C for low stringency to about 0.2 x SSC at 50°C for high stringency. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions, about 65°C.

The term "under the control" means that the promoter is located in a position relative to that of the DNA encoding the desired polypeptide that allows the promoter to efficiently direct transcription of the structural gene or the genes for the desired polypeptide(s).

The reading direction of DNA- and RNA-polymerases is always from the 5' to the 3' end of a DNA strand. The term "downstream" means everything located in 3' direction on the transcribed DNA-strand and the term "upstream" means everything located in 5' direction on the DNA-strand.

Signal peptides (signal sequences) are essential parts of membrane-bound or secreted polypeptides which are needed for membrane translocation of the polypeptide and which are processed after or during membrane translocation. A signal sequence has a length of about 13 and 36 amino acids and contains at least one positive residue at the aminoterminal end. The center of the signal sequence is a strongly hydrophobic part of 10 to 15 residues and is described, for example, by Nunnari, J., et al., Curr. Opin. Cell Biol. 4 (1992) 573-580 and by Gilmore, R., et al., Ann. N.Y. Acad. Sci. 674 (1992) 27-37. Preferred signal peptides are the signal peptides of CD40, CD40L or TNF-R.The signal peptide is cleaved off on integration into the membrane of the target cell.

To determine whether a signal peptide is useful for the chimeric polypeptide according to the invention or whether a fragment or modification of the signal

peptide of CD40 or TNF-R has the activity of the signal peptide of, respectively, CD40 or TNF-R, the correct cell surface expression of epitope tagged CD40/CD40L fusion proteins can be tested by FACS-analysis using antibodies against said epitope. Only signal peptides with proper function will allow detectable cell surface expression of the tagged CD40/CD40L fusion proteins. This method is reviewed by Jarvik, J.W., et al., Ann. Rev. Genet. 32 (1998) 601-618.

The CD40L domains of the fusion genes trimerize and, therefore, trimerize the intramolecularly bound CD40 receptor which causes activation of receptor-mediated signal transduction in the same manner as a transactivating CD40 ligand trimer in a natural system.

To determine whether a fragment or modification of the binding and trimerization domain of human or murine CD40L has the activity of the binding and trimerization domain of human or murine CD40L, the functionality of the CD154 binding and trimerization domain (CD154 is the ligand for CD40) or modifications thereof can be tested. This can be done by determination of NF-κB (a transcription factor which is one of the principal outcomes of CD40 signaling) activation. Coincubation of cells expressing CD154, the CD154 binding and trimerization domain or modifications thereof with cells expressing CD40 plus a NF-κB reporter gene plasmid (e.g. pNF-κB-CAT, where the CAT-gene is controlled by a repeat of four NF-kB binding sites: 5'-GGGGAATTTCC (SEQ ID NO:1) (Garceau, N., J. Exp. Med. 191 (2000) 381-385; Urban, M.B., et al., Gene Dev. 4 (1990) 1975-1984; and Mihm, S., AIDS 5 (1991) 497-503 wherein pNF-kB-CAT is described) and determination of the reporter gene expression (e.g. by CAT-ELISA) allows the meassurement of correct CD154-mediated transactivation of CD40 signaling.

To determine whether a fragment or modification of the transmembrane and signal transduction domain of human or murine CD40 polypeptide having the activity of the transmembrane and signal transduction domain of human or murine CD40, the functionality of CD40, or CD40 with modifications in the transmembrane and signal transduction domain can be tested. This can also be done by determination of NF-κB activation as described above. Coincubation of cells expressing CD154 with cells expressing CD40 or CD40 with modifications of the transmembrane and/or signal transduction domains plus a NF-κB reporter gene plasmid and

determination of the reporter gene expression (e.g. by CAT-ELISA) allows the meassurement of correct CD40 signal transduction.

To determine whether a fragment or modification of the ligand binding subdomain of CD40 has the activity of the ligand binding subdomain of CD40, the functionality of CD40 or CD40 with a fragment or modification of the ligand binding subdomain can be tested. This can also be done by determination of NF-κB activation as described above. Coincubation of cells expressing CD154 with cells expressing CD40 or CD40 with a fragment of or with modifications in the ligand binding domain plus a NF-κB reporter gene plasmid and determination of the reporter gene expression (e.g. by CAT-ELISA) allows the meassurement of correct CD40L binding and therethrough a correct CD40 signal transduction.

CD40 according to the invention is understood as a polypeptide with the activity and biochemical characteristics of CD40 receptor. Such polypeptides are, for example, human or murine CD40 or modifications thereof such as mutations, deletions or substitutions. Preferred modified CD40 polypeptides are at least 80% homologous to human CD40 preferably within the intracellular domain. Homology according to the invention can be determined with the aid of the computer programs Gap or BestFit (University of Wisconsin; Needleman and Wunsch, J. Mol. Biol. 48 (1970) 443-453; Smith and Waterman, Adv. Appl. Math. 2 (1981) 482-489).

The human CD40 molecule is 277 amino acids in length (MW = 30,619 Da) and starts with a hydrophobic signal sequence of a length of 19 amino acids (Pos. 1-19), followed by an extracellular domain of a length of 174 amino acids (Pos. 20-193), a hydrophobic transmembrane domain of a length of 22 amino acids (Pos. 194-215), and a cytoplasmic domain of a length of 62 amino acids (Pos. 216-277) (Swiss-Prot.: P25942 and Stamenkovic et al., The EMBO J. 8 (1989) 1403-1410). The extracellular domain is responsible for the ligand binding and for the thereby mediated trimerization of the active complex. It consists of two subdomains (ligand binding subdomain and stalk subdomain). According to the invention, the extracellular domain is to be understood as the full length domain, or at least as part thereof, which causes ligand (CD40L) binding. The ligand binding subdomain consists of about 100 amino acids (Pos. 20-120), and the stalk subdomain consists of about 73 amino acids (Pos. 121-193) according to Bajorath et al., Proteins 27

(1997) 59-70. The stalk subdomain is a domain which is necessary for steric reasons. This subdomain ensures an optimal distance between the binding domain and the cell surface which is necessary for trimerization.

The cytoplasmic domain mediates the signal transduction of the complex that is activated by trimerization. Signal transduction is mediated by the socalled TRAF proteins (TNF-R Associated Factor), whose binding sites are located in the cytoplasmic domain of CD40, and ultimately leads to the activation of various transcription factors, NF-κB being the most prominent of these transcription factors. The analogous murine CD40 molecule is 289 amino acids in length (MW = 21,111 Da) (Grimaldi et al., The J. of Immunol. 149 (1992) 3921-3926 as well as Swiss-Prot.: P27512) and has a signal sequence of a length of 19 amino acids (Pos. 1-19), followed by an extracellular domain of a length of 174 amino acids (Pos. 20-193), a hydrophobic transmembrane domain of a length of 22 amino acids (Pos. 194-215), and a cytoplasmic domain of a length of 74 amino acids (Pos. 216-289).

CD40L (CD154) according to the invention is understood as a polypeptide with the activity and biochemical characteristics of CD40L. Such polypeptides are, for example, human or murine CD40L or modifications thereof such as mutations, deletions or substitutions. Preferred modified CD40L polypeptides are at least 80% homologous to human CD40L preferably within the extracellular domain.

The human CD40L protein is 261 amino acids in length (Swiss-Prot.: P29965, MW = 29,273 Da) and starts with a cytoplasmic region of a length of 22 amino acids (Pos. 1-22), followed by a signal anchor of a length of 24 amino acids (type II membrane protein; without a typical signal peptide) (Pos. 23-46), and an extracellular, signal-mediating domain of a length of 215 amino acids (Pos. 47-261). The extracellular domain of CD40L consists of two subdomains: the stalk domain (Pos. 47-119) and the trimerization domain (Pos. 120-261). The mature glycoprotein has a molecular weight of 35 kDa.

The murine CD40L (Swiss-Prot.: P27548) is 260 amino acids in length (MW = 29,396 Da) and starts with a cytoplasmic region of a length of 22 amino acids (Pos. 1-22), followed by a signal anchor of a length of 24 amino acids (Pos. 23-46) and an extracellular, signal-mediating domain of a length of 214 amino acids (Pos. 47-260). The mature murine glycoprotein has a molecular weight of 33 kDa.

The three-dimensional structure of CD40L has been elucidated: The structure of the CD40 / CD40L complex has been derived from the known homologous structure of TNF-R / TNF-ß and from a crystal-structure determination of the soluble extracellular domain of the CD40L trimer (Karpusas, M., et al., Structure 3 (1995) 1031-1039). This has led to the model according to which CD40L trimerizes and therefore mediates trimerization of CD40. Human CD40 and murine CD40 exhibit 76% homology. Human CD40L and murine CD40L exhibit 86% homology.

One may introduce the nucleic acid segments according to the invention ex vivo or in vivo into a mammalian cell or a mammalian host by any of several means, including vector transfection. Viral vectors may be used to infect human and animal cells with the recombinant DNA; certain adenoviral vectors have proved particularly useful. Adenoviral vectors are preferred. Of course DNA segments need not be introduced into cells by a viral vector: Direct transfection may be performed by electroporation, gene gun techniques, or DNA-liposome complexes, for example. DNA/liposome complexes have been used to introduce DNA encoding prostaglandin synthase into rabbits, with subsequent production of prostaglandin E2 and prostacyclin (Conary et al., J. Clin. Invest. 93 (1994) 1834-1840). An appropriate vector includes the gene encoding the selected protein or proteins such that the nucleic acid or acids according to the invention is or are under the transcriptional control of a promoter which is active in mammalian, preferably in human cells such as the CMV promoter, the SV40 promoter or the SAA promoter. The vector is introduced into a host cell by any of a number of procedures known to those skilled in the art, such as direct introduction of DNA by gene gun techniques, liposomal transfection or direct local injection. Direct infusion is preferred in the case of bladder carcinomas and infusion with an endoscopic probe is preferred in the case of colon carcinomas.

In a preferred embodiment, the chimeric nucleic acid and the genes encoding IL-2, IL-12 and/or Interferon-alpha are being co-expressed on the same or on different vectors. This can be effected by means of cotransfection or cotransduction of two viral or plasmid vectors which carry both genes or of a single vector on which both genes are present in coded form. In the latter embodiment, both genes can be expressed by separate promoters (the promoters may be identical or different from one another), they may be present coupled via an IRES sequence (internal ribosomal entry site) in the expression, or coupled via a splice-donor sequence

before the first gene and a splice-acceptor sequence after the first gene, that is, before the second gene. In the latter embodiment, in the case of unprocessed mRNA, the first gene would be read, and in the case of processed mRNA, the first gene would be spliced out and the second gene would be read.

Genes encoding chimeric nucleic acids according to the invention represent „immunological master genes", that is to say, after ligand binding through CD40L, CD40 induces a great number of co-stimulatory factors, inflammation-inducing or maintaining factors, and thus, a strong immune reaction (Stout et al., Immunology Today 17 (1996) 487-492). CD40 also induces the NFkB transduction pathway, at the end of which there are a great number of inflammation mediators such as, for instance, IL-12, IL1-ß, IL-6 or TNF-α. The preferred embodiment of the invention therefore consists of a chimeric nucleic acid under the expression control of the cytokine-inducible promoter. It is an advantage of this embodiment that, after being inserted into a tumor, which even expresses pro-inflammatory cytokines only to a little extent, it induces an autocrine activation cascade, i.e., the inflammatory cytokines will induce the promoter on the inserted vector according to the invention and the chimeric polypeptide will, in turn, activate the NFkB transduction pathway, at the end of which there is the activation of the promoter. This effect can be further enhanced by inserting into the cytokine-inducible promoter the binding sites for pro-inflammatory transactivators, such as NFkB or NF-IL6 (e.g., the region from -190 to -78 of the SAA2 promoter or only the individual binding sites), preferably in multimeric form.

A gene therapy agent according to the invention is understood to mean a pharmaceutical composition which contains one or more expression vectors according to the invention as essential components, in an amount needed by the tumor patient to ensure an effective treatment. Such a composition preferably contains at least one vector together with a non-viral delivery system, as an adenoviral vector or as a retroviral vector. In such cases, the delivery system or the viral vector per se or the expressed CD40/CD40L gene will cause a local or systemic inflammatory response in the tumor and/or in the tissue surrounding the tumor. In such cases, cytokine mobilization caused by the administration of the delivery/targeting vehicle would lead to the promoter-driven production of the therapeutic agent at up to at least a hundred times over its uninduced basal level.

Gene therapy of somatic cells can be accomplished by using, e.g., retroviral vectors, other viral vectors or by non-viral gene transfer (cf. Friedman, T., Science 244 (1989) 1275; Morgan 1993, RAC Data Management Report, June 1993).

Vector systems suitable for gene therapy are, for instance, retroviruses (Mulligan, R.C., (1991) in Nobel Symposium 8: Etiology of human disease at the DNA level (Lindsten, J., and Pattersun, eds.) 143-189, Raven Press), adeno-associated virus (McLughlin, J. Virol. 92 (1988) 1963), adenoviruses (Zhang, W.W. et al., Cancer Gene Therapy 6 (1999) 113-138), Vaccinia virus (Moss et al., Ann. Rev. Immunol. 5 (1987) 305), bovine papilloma virus (Rasmussen et al., Methods Enzymol. 139 (1986) 642) or viruses from the group of the Herpes viruses, such as Epstein-Barr virus (Margolskee et al., Mol. Cell. Biol. 8 (1988) 2937) or Herpes simplex virus. However, adenoviruses are preferred.

The adenoviral vectors could also be applied as formulations of cationic lipids (e.g., DOSPER) with ADV as described by Fasbender, A., et al., J. Biol. Chem. 272 (1997) 6479-6489 and Dodds, E., et al., J. Neurochem. 72 (1999) 2105-2112.

There are also known non-viral delivery systems. For this, usually "nude" nucleic acid, preferably DNA, is used, or nucleic acid together with an auxiliary agent, such as, e.g., transfer reagents (liposomes, dendromers, polylysine transferrin conjugates (Felgner et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7413).

It was found that it is possible according to the invention to create and activate tumor-specific T cells which are cytolytically active for a very long period of time. The combination of CD40 and CD40L expression, preferably in combination with the expression of IL-2, IL-12 and/or Interferon-alpha or in combination with a therapeutically active amount of IL-2, IL-12 and/or Interferon-alpha polypeptides or 5-fluorouracil further lead to a synergistic enhancement of the level and duration of the activation phase of said tumor-specific T cells.

The compositions may be administered parenterally, using, for example, injectable solutions, preferably for intratumoral injection and preferably into head and neck cancer (a squamous cell carcinoma). For the preparation of such injectable solutions, the vectors according to the invention are admixed with pharmaceutical inert, inorganic or organic excipients, buffers and/or preservatives. Such excipients

are, for example, water, alcohols, polyols, glycerol, preferably having a neutral pH value (pH 6-8). Pharmaceutically acceptable buffers are, for example, phosphate, lactate, phosphate buffered saline, Tris. The pharmaceutical compositions may also contain preserving agents, toxicity agents, stabilizing agents, wetting agents, clarification agents, viscosity agents, salts for the variation of osmotic pressure, buffers or antioxidants. They may also contain other therapeutically valuable agents.

Suitable preservatives for use in such preparations include benzalkonium chloride, benzethonium chloride, chlorobutanol, thimerosal, and the like. Suitable buffers include boric acid, sodium and potassium bicarbonate, sodium and potassium borates, sodium and potassium carbonate, sodium acetate, sodium biphosphate and the like, in amounts sufficient to maintain the pH between about pH 6 and pH 8, preferably between about pH 7 and pH 7.5. Suitable tonicity agents are dextran 40, dextran 70, dextrose, glycerine, potassium chloride, propylene glycol, sodium chloride, and the like. Suitable antioxidant and stabilizers include sodium bisulfite, sodium metabisulfite, sodium thiosulfate, thiourea and the like. Suitable wetting and clarifying agents include polysorbate 80, polysorbate 20, poloxamer 282, and tyloxapol. Suitable viscosity increasing agents include dextran 40, gelatin, glycerin, hydroxyethyl cellulose, hydroymethylpropyl cellulose, lanolin, methylcellulose, petrolatum, polyethylene glycol, polyvinyl alcohol, polyvinyl polyvinylpyrrolidone, carboxymethyl cellulose and the like.

The interaction between CD40 on antigen-presenting cells (APC) and its ligand CD40L on T cells exerts a prominent role in the upregulation of APC functions. These include the activation of B7 surface expression and IL-12 synthesis, two proteins which cooperate in the induction of an effective anti-tumor response. It was found that tumor cells from basal and squamous cell carcinoma exhibit a down-regulation or a dramatic loss of CD40 which may account for a tumor escape mechanism, by which activated T cells expressing CD154 are not able to kill the tumor cells any longer. The potential of the substances according to the invention in inducing an anti-tumor response can be shown by a stable transfection of a non-immunogenic $CD40^-$ tumor cell (e.g., a squamous cell carcinoma cell line like SCCVII) with such constructs. Wild-type SCCVII, the neo control and $CD40^+$ cells were indistinguishable in their proliferation rate and morphology in vitro. Syngeneic C3H/HeN mice injected s.c. with $CD40^+$ SCCVII had a significant

reduction in tumor growth compared to the $CD40^-$ wild-type SCCVII or the neo control. In contrast, CD40-transfected cells injected into nude mice exerted the same tumor growth kinetic as the two controls indicating that immunocompetent T cells are necessary for anti-tumor activity. Histological analysis of tumor explants from $CD40^+$ tumors revealed predominant infiltration of $CD4^+$ T cells and expression of the CD25 T cell activation marker. These changes were not detectable in control group tumors. Chimeras according to the invention induce apoptosis and inhibition of the proliferation rate in tumor cells

In accordance with the invention, the vectors according to the invention can be used in the control or treatment of tumor diseases, preferably in treatment of vascularized tumors. The dosage is a biologically effective amount of the vector and can vary within wide limits and is, of course, fitted to the individual requirements in each particular case. The preferable dose for viral vectors is 10^8-10^{11} pFU/injection and 50-1000 µg DNA/injection (see also Kikuchi et al., Human Gene Therapy 10 (1999) 1375-1387), preferably 50-200 µg DNA/injection for nonviral vectors. The preferable volume per injection is between 1 and 10 ml.

Viral vectors are preferably formulated in a pharmaceutical composition containing phosphate buffered saline, pH 7.4 or other buffers of pH 6 to 8 (Caruso, M., et al., Proc. Natl. Acad. Sci. USA 93 (1995) 11302-11306). Non-viral vectors are preferably formulated in a pharmaceutical composition with liposomes also at a pH value of 6 to 8 (cf. Yanagihara, I., et al., Mol. Cell Biol. Hum. Dis. Ser. 5 (1995) 64-82; Thierry, A.R., et al., Gene Ther. 4 (1997) 226-237; Gao, X., et al., Gene Ther. 2 (1995) 710-722; Abdallah, B., et al., Biol. Cell 85 (1995) 1-7; Treco, D.A., et al., Mol. Med. Today 1 (1995) 314-321).

The patient is preferably treated in such a way that 1-2 injections per week are administered directly into the tumor over a period of 3-10 weeks, which induces a cytotoxic immune response against the tumor cells. After that period, the extent of change of the tumor is examined and, if necessary, as well as if possible, the tumor is removed. A therapy regimen of this kind is of particular importance with respect to improving the results of therapy, because after post-operative treatment, a sufficient number of tumor cells will still be present. The presurgical immunization results, however, in a cytotoxic immune response also after the removal of the

tumor and therefore cytotoxic T cells are formed which are capable of destroying metastasizing cells and cells of minimal residual disease.

It is further preferred to administer the individual injections into a plurality of sites in the tumor and/or in the vicinity of the tumor, which allows the pharmaceutical agent according to the invention to reach not only the actual tumor cells but also other non-tumoral cells of the tumor tissue, for example fibroblasts, macrophages, T cells or dendritic cells.

It is further preferred that prior to, during or directly after administration of the pharmaceutical agent according to the invention, the inflammatory reaction on the tumor should be intensified locally. This can be accomplished by means of, for example, local thermal treatment (microwaves), pressure, or by injection of the pharmaceutical agent into a plurality of sites in and in the vicinity of the tumor.

The vectors according to the invention may be injected as formulations with transfer reagents directly into tumors, post-operatively into tumor caves, or systemically.

In a preferred embodiment of the invention, the vector-containing pharmaceutical agent according to the invention (preferably containing no expression vector for IL-2, IL-12 and/or Interferon-alpha) is administered as an adjuvant and in combination with a polypeptide having the activity of IL-2, IL-12 and/or Interferon-alpha. In this case, the vector-containing agent is administered as described above, whereas the polypeptide is preferably administered systemically. In this connection, it is preferred to administer the polypeptide before and after (immediately before or after or up to 12 hours before or after) the injection of the vector-containing agent.

Table 3 shows the preferred administration scheme for the adjuvant administration of polypeptides.

Table 3

Polypeptide	Injections per day	Amount per injection	Amount per day
IL-2	1-2	0.6-2.5 10^6 units	0.6-5x10^6 units
IL-12	1-2	1-2x10^4 units	1-4x10^4 units
Interferon-alpha	1-2	0.5-1x10^6 units	0.5-2x10^6 units

5-fluorouracil is administered with 12 mg/kg body weight per day for the first four days of the tumor therapy and with 6 mg/kg body weight on days 6, 8, 10 and 12.

According to the invention, it is important to use endotoxin-free DNA and to avoid any inflammatory reactions of the tissue. This would induce preinflammatoy cytokine expression which on the other hand would lead to a stimulation of the inducible promoter in other tissues but squamous cell carcinomas. Such endotoxin-free DNA can be produced according to WO 97/29113.

For this treatment long lasting and high expression of the immune stimulatory gene is favourable as well as tumor cell specificity which would reduce side effects due to draining of the plasmids or adenoviral vectors from the injected tumors into neighbouring tissues or other organs like liver or lung. Additionally tumor specific treatment could result in systemic injection of the tumor-targeted immune stimulatory plasmid-formulation. Tumor specificity can be achieved by either targeting of the transfection reagents to tumors or tumor-specific gene expression or combinations of both. Targeting of the transfection reagents is not yet feasible with sufficient specificity and efficiency but tumor specific promoters are available.

According to the description, this invention will have great impact on the treatment of tumors, metastases and minimal residual disease by vectors which code for immune stimulatory genes. The use of the promoters according to the invention allows high-level, long-lasting, potentially inducible/repressable and tumor specific expression. Therefore in cancer treatment regimens using vector DNA injection it could optimize the results due to longer lasting and higher amounts of the immune stimulatory proteins in the tumor, the possibility of systemic treatment which would reach not only the primary tumors but also metastases, and a wider

therapeutic window (higher doses and multiple dosing) due to reduced toxic side effects.

A further preferred embodiment of the invention is the treatment of non-solid tumors of hematologic origin like chronic and acute lymphocytic leukemia, multiple myeloma, chronic and acute myelomic leukemia. In the case of these non-solid tumors the treatment will be preferentially ex vivo. This includes removing of tumor cells from the circulation by leukapheresis, transduction of these tumor cells ex vivo by vectors according to this invention and reinfusion of the transduced tumor cells into the patient. Such a method, using gene transfer of CD40L, is described by Kato et al., J. Clin. Invest. 101 (1998) 1133 and by Kikuchi, T., et al., Human Gene Therapy 20 (1999) 1375-1382. A treatment using the vectors according to the invention can be performed in the same manner.

The following examples, references and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Fig. 1 shows a schematic comparison of Version A and Version B.

Fig. 2 describes a CD40 - CD40L fusion construct, Version A.

Fig. 3 describes a CD40 - CD40L fusion construct, Version B.

Fig. 4 shows a Western Blot with pcDNA3-CD40-CD40L chimeric constructs A and B transfected into HEK293 cells and stained with a monoclonal anti<CD40> antibody directed against the intracellular region of CD40. Control shows pcDNA3-CD40 transfected into HEK293 cells. Version A bands at about 40 kD; version B bands at about 47 kD.
1 = CD40 (0.1 µg); 2 = CD40 (0.4 µg); 3 = CD40 (1.6 µg); 4 = control; 5 = CD40 (0.1 µg); 6 = version A (0.1 µg); 7 = version B (0.1 µg); 8 = CD40 (0.4 µg); 9 = version A (0.4 µg); 10 = version B

(0.4 µg); 11 = CD40 (1.6 µg); 12 = version A (1.6 µg); 13 = version B (1.6 µg).

Fig. 5 shows an in vitro function analysis of CD40L/CD40 fusion constructs. Test for cis-activity: pcDNA3-CD40-CD40L and pNF-KB-CAT reporter plasmid were cotransfected into human 293 cells and tested for CAT expression (CAT-ELISA) after 48 h.

Fig. 6a shows an in vitro function analysis of CD40L/CD40 fusion construct version B. Test for trans-signaling: pcDNA3-CD40-CD40L chimeric alone and pcDNA3-CD40 + pNF-KB-CAT were each transfected into 293 cells. 24 h after transfection both cell pools were coincubated for 24 h and then tested for CAT expression.

Fig. 6b shows an in vitro function analysis of CD40L/CD40 fusion construct version B. Test for trans-activity: pcDNA3-CD40-CD40L alone and pcDNA3-CD40 + pNF-KB-CAT were each transfected into 293 cells. 24 h after transfection both cell pools were coincubated for 24 h and then tested for CAT expression.

Materials and Methods

Animals:

Six to eight week old female C57BL/6 mice are delivered from Charles River. All animals were IVC's with daily cycles of 12 h light/12 h darkness according to international guidelines. C57BL/6 mice are syngeneic to B16F10 mouse melanoma cells and used for implantation experiments with this cell line.

Female syngeneic C3H/HeN (H-2K^k) mice were purchased from Charles River, Germany. The mice were between 8-12 weeks old. Female Balb/c nu/nu mice (20 weeks old) were obtained from Bomholtgard Breeding, Denmark.

B16 F10 melanoma cell line was grown in syngeneic C57BL/6 mice(H-2b).

Cell lines:

B16F10 mouse melanoma cells were obtained from ATCC (CRL 6475) and cultured in DMEM (Biochrom Ltd., UK) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria), 4 mM L-glutamine (Gibco - Life Technologies, MD, USA), 1 mM sodium pyruvate and 4,5 g/l glucose. The culture conditions are 37°C in a water saturated atmosphere containing 5% CO_2. Culture passage is performed with trypsin/EDTA 1x (Roche Diagnostics GmbH, DE) splitting twice a week.

HEK293 cells were obtained from ATCC (CRL-1573) and cultured in DMEM (Biochrom Ltd., UK) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria), 2 mM L-glutamine (Gibco – Life Technologies).

A murine squamous carcinoma cell line (e.g., SCCVII/SF; O'Malley, B., et al., J. Cancer Res. 56 (1996) 1737-1741 and Snit, H.D., et al., Radiation Research 104 (1985) 47-65) and a murine colon carcinoma cell line (e.g., CT26; Xiang, R., et al., Cancer Res. 58 (1998) 3918-3925), both being $CD40^-$ and $CD40L^-$, were cultured in DMEM high glutamax® medium (GIBCO Life Technologies) supplemented with 10% heat-inactivated FCS (GIBCO Life Technologies) penicillin and streptomycin (Roche Diagnostics GmbH, DE). This cell line can also be used used instead of B16F10 in the examples.

DNA constructs and transfection:

The CD40/CD40L fusion protein:

Two versions of the fusion protein were expressed:

Version A:

a) This version comprises the CD40 signal peptide (aa Pos. 1-21) fused to the extracellular part of murine CD40L (aa Pos. 47-260). A linker derived from the hinge region of immunoglobulin D (IgD) (Accession 709448.1, Locus MUSIGCD15.EM_ROD → ht 64-123), see Tucker, Science 209 (1980) 1353-1360), connects this part with the residual murine CD40 protein (aa Pos. 22-277 (human) or 289 (murine)).

b) In comparison with a), the extracellular part of CD40L is truncated leaving the aa position 120-260, thus omitting the stalk region of CD40L.

Version B:

This version comprises the CD40 signal peptide (aa Pos. 1-21) fused to the extracellular part of CD40L (aa Pos. 120-260 (or 121 – 261 of human CD40L) → compare to Version A b) which is directly fused (without a linker or with a short linker (1-30 aa/ e.g. a 20 aa linker derived from IgD-hinge to a truncated form of the CD40 (aa Pos. 122-277 (human) or 289 (murine)), therefore deleting the CD40-CD40L binding domain of CD40.

The final fusion constructs are human/human, murine/human or murine/murine CD40L/CD40. The construction of the murine/murine chimeric constructs is described in example 1.

The fusion of the different parts of the cDNA constructs was performed by standard PCR techniques. DNA sequence was verified by sequencing the resulting PCR products.

The chimeric cDNA was cloned into the pcDNA3 (Invitrogen Corp., San Diego, US) mammalian expression vector. Transfection of HEK293 cells was carried out using transfection reagents FUGENE™ (Roche Diagnostics GmbH, DE) consisting essentially of a blend of lipids (non-liposomal formulation).

Flow cytometry and antibodies:

Cells were characterized for the expression of relevant cell surface markers by direct immunofluorescence using either PE and/or FITC conjugated monoclonal antibodies against CD40, MHC-I (H-2K^d ≙ BALB/c), CD40L, ICAM-I (CD54), B7.1 (CD80), B7.2 (CD86), Fas receptor (Fas, APD-1/CD95), Fas ligand (FasL, CD95L) or tags (e.g. HA-tag, Flag-tag) fused to the N-termini of the CD40-CD40L fusion proteins (→ CD40-CD40L^{tag}).The corresponding isotype controls were used for determination of unspecific binding. Antibody binding was carried out for 20 min. at room temperature, followed by washing and analyzing using a FACScan® (Becton Dickinson, DE).

In vivo experiments:

Tumor cells were inoculated in a concentration of 5 x 10^5/200 µl subcutaneously (s.c.) in the right hind flank of syngenic or athymic animals. Tumor growth was quantitated five days post-inoculation three times a week using a calipper. Tumor

volumes were calculated by the approximately ellipsoid formula: $4/3 \times \pi$ / 8 x (length x width x depth).

For the experiments on subcutaneous implantation of in vitro Ad-CD40-CD40L transduced B16F10 melanoma cells into C57BL6 mice, the B16F10 cells were seeded into 12-well plates (Falcon), 4×10^5 cells per well. When cells were adherent vectors in appropriate dilutions made in transduction medium (DMEM without supplements) were added to the wells. After determination of surface expression of parallel transduced tagged CD40-CD40L^{tag} fusion constructs by FACS-analysis, 5×10^5 tumor cells in a volume of 200 µl were implanted subcutaneous into lateral abdominal wall. For this procedure mice were anesthetized with ether. Tumor growth was quantitated five days post-inoculation three times a week using a caliper. Tumor volumes were calculated by the approximately ellipsoid formula: $4/3 \times \pi$ / 8 x (length x width x depth).

Example 1

Construction of expression vectors

Material and Methods:

pcDNA3-CD40-CD40L (version A and B) murine / murine fusion constructs:
(murine/human and human/human chimeric constructs were constructed and tested in an analogous manner)

The signal peptide sequence of murine CD40 comprising amino acids 1 – 21 was amplified by PCR using two overlapping oligonucleotides with a KpnI-restriction site immediatedly upstream of the ATG start codon and a BsmBI and a HindIII restriction site at the 3'-end of the resulting fragment. Two different versions of the mCD40 receptor part of the fusion gene were amplified by PCR using pcDNA3-mCD40 (pcDNA3 with cloned mCD40 cDNA) as template. The first fragment (corresponding to fusion gene version B) codes for aa 122 – 289 of mCD40 and the second fragment (corresponding to fusion gene version A) codes for aa 21 – 289 of mCD40. In the first case a HindIII-site followed by a BsmBI-site followed by 5 nucleotides corresponding to the 3'-end of the coding sequence of mCD40L were introduced at the 5'-end of the PCR-fragment. In both cases a HindIII-site followed by a BsmBI-site followed by 5 nucleotides corresponding to the 3'-end of

the 20 aa IgD-hinge region were introduced at the 5'-end of the PCR-fragment. In both cases the downstream primer introduced a XhoI-site at the 3'-end of the PCR-fragments with a stop-codon directly upstream.

The IISK+ Bluescript vector (Stratagene) was cut with KpnI/XhoI. The PCR fragment of the signal peptide was cut with KpnI/HindIII and the PCR fragments with the two different versions of the mCD40 gene were cut with HindIII/XhoI. In 3-fragment ligations the intermediate constructs pBluescript-Signalpeptide-CD40 (version B; aa 122 - 289) and pBluescript – Signalpeptide – CD40 (version A; aa 21 – 289) were obtained.

The mCD40L binding and trimerization domain (aa 120 – 260) and the mCD40-L stalk plus binding and trimerization domain (aa 47-260) were also obtained by PCR amplification using pcDNA3-mCD40L (pcDNA3 with cloned mCD40L cDNA) as template. In each case the upstream primer introduced an EcoRI-restriction site followed by a BsmBI-restriction site followed by 5 nucleotides corresponding to the 3'-end of the signal sequence to the 5'-end of the PCR product. The downstream primer introduced a XbaI restriction site to the 3'-end of the PCR-fragment with a BsmBI restriction site immediately upstream. The resulting PCR fragments were cloned into the EcoRI/XbaI sites of the IISK+ Bluescript vector (Stratagene).

A linker derived from the hinge region of immuneglobulin D (IgD-hinge; see above) comprising 20 amino acids was obtained by PCR amplification using 2 overlapping oligonucleotides. A flanking EcoRI site at the 5'-end was inserted followed by a BsmBI site followed by 5 nucleotides corresponding to the 3'-end of the coding mCD40L sequence. At the 3'-end of the IgD-hinge fragment a XbaI-site was introduced with a BsmBI-site immediately upstream. The resulting fragment was cloned into the EcoRI/XhoI sites of the polylinker of the IISK+ Bluescript vector.

CD40L (aa 47 – 260) and CD40L (aa 120 – 260) as well as the IgD hinge were cut out from pBluescript by a BsmBI digestion and cloned in a 3-fragment ligation into also BsmBI cut pBluescript - signal peptide – CD40 (version A; aa 21 – 289). and into the BsmBI-site of pBluescript – signal peptide – CD40 (version B; aa 122 – 289).

The resulting vectors were:

- pBluescript – signal peptide – CD40L (aa 47 – 260) – hinge – CD40 (version A / long)
- pBluescript – signal peptide – CD40L (aa 120 – 260) – hinge – CD40 (version A/ short; in the following the term „version A" means „version A/short")
- pBluescript – signal peptide – CD40L (aa 120 – 260) – hinge- CD40 (version B)

The CD40-CD40L fusion genes were excised from the Bluescript vectors by KpnI / XhoI digestion and subcloned into the polylinker of pcDNA3 (Clontech, CA, USA), in which the fusion genes are transcriptional regulated by a CMV-promoter.

pSAA2-CD40-CD40L (Version A and B) were constructed by removing of the Cat gene from pSAA2-Cat (European Application No. 99107611.8) by NotI-digestion and filling up of the protruding ends with Klenow enzyme. The CD40-CD40L fusion genes were excised from pcDNA3-CD40-CD40L by KpnI / XhoI digestion, the protruding ends were blunted by Klenow-polymerase treatment and the blunt ended fragments were ligated to the pSAA2-CAT vector fragment. In pSAA2-CD40-CD40L constructs the fusion genes are under the control of the SAA2-promoter.

pSAA2-IL2 was constructed by removing the Cat gene in pSAA2-Cat by NotI-digestion, and inserting of the IL2 cDNA which was amplified by PCR using primers with NotI-overhangs.

pcDNA3-IL2 was constructed by cloning the PCR amplified IL2 gene into the mammalian expression vector pcDNA3 (Clontech).

pSAA2-IL12 was constructed by removing the Cat gene in pSAA2-Cat by NotI-digestion. The 2 subunit-genes p35 and p40 of the IL12 were separately amplified by PCR. Their transcription was coupled by the use of an IRES sequence from EMCV (derived from pIRES1neo; Clontech). P35 was amplified by a 5'-primer with a NotI-site in the overhang and a 3'-primer with a SalI-site in the overhang. EMCV IRES was amplified by a 5'-primer with a SalI-site in the overhang and a 3'-primer with a XhoI-site in the overhang. P40 was amplified by a 5'-primer with a XhoI-site in the overhang and a 3'-primer with a NotI-site as well as a XhoI-site in the

overhang. The amplified fragments were then cloned into pBluescript (Stratagene) which was cut by NotI and XhoI. The final plasmid pBluescript-p35-IRES-p40 was then cut with NotI and the fragment containing p35 and p40 transcriptionally coupled by EMCV IRES was cloned into pSAA2-Cat instead of the Cat-gene.

pcDNA3-IL12 was constructed by subcloning the p35-IRES-p40 gene cassette from pSAA2-IL12 into the mammalian expression vector pcDNA3 (Clontech).

AdV-CD40-CD40L:

Adenovirus vectors (AdV) are constructed by cloning the genes to be expressed into an Adenovirus transfer vector which contains the expression cassette and regions homologous to the Adenovirus genome. The AdV transfer vector and the AdV genome are linearised and cotransfected into 293A cells (ATCC CRL 1573.1). There they recombine to form AdV vectors which can be packed into viral particles but cannot replicate due to deletions in their E1 region. This deletions result in the cloning capacity of 7 kb. The AdV vectors can only be propagated and therefore be produced in 293A cells which are stable transfected by sheared AdV DNA and therefore provide the E1 gene products in trans.

The Adenovirus vector AdV-CD40-CD40L was constructed by insertion of the whole expression cassette from pcDNA3-CD40-CD40L into the Adenovirus transfer vector pQBI-AdCMV5 (QUANTUM Biotechnologies Inc.) from which the CMV5 promoter / enhancer and the globin-poly-A site have been removed. Due to the lack of suitable restriction sites the whole expresssion cassette from pcDNA3-CD40-CD40L (CMV promoter, CD40, CD40-L, poly-A site) was amplified by PCR and also the pQBI-AdCMV5 vector without the CMV5 promoter/enhancer and the globin polyA was amplified by PCR. The 2 amplified DNA fragments were restricted with PmeI (the recognition sites were provided by the overhangs of each PCR-primer) and ligated by T4-DNA ligase.

The Adenovirus transfer vector AdV-CD40-CD40L was linearised by ClaI digestion at the linearization site and cotransfected with linear QBI-viral DNA into QBI-293A cells according to manufacturer's protocol (QUANTUM Biotechnologies Inc.).

In the 293A the transfer vector and the cotransfected adenoviral DNA recombine in their overlapping regions to form the Adenovirus vector AdV-CD40-CD40L.

AdV-CD40-CD40L has deletions in the E1 region of human Adenovirus type 5; the CD40-CD40L expression cassette is cloned instead of the deleted E1 region. Due to the E1 deletion the Adenovirus vector is not able to replicate alone. Only in 293A cells which contain the sheared genomic DNA from human Adenovirus type 5, the E1A and E1B genes which are essential for virus replication are provided in trans and therefore the virus could be produced in that cell line. The virus vector was purified via several cycles of plaque purification and isolated in large scale from the infected 293A cells by methods described in the QUANTUM Adeno-Quest Kit protocol or reviewed by Graham et al., Mol. Biotechnol. 3 (1995) 207–220.

The Adenovirus vector AdV-IL2 was constructed analogous AdV-CD40-CD40L by using the expression cassette of pcDNA3-Il2 instead of pcDNA3-CD40-CD40L.

The Adenovirus vector AdV-IL-12 was constructed analogous AdV-CD40-CD40L by using the expression cassette of pcDNA3-IL-12 instead of pcDNA3-CD40-CD40L.

Coexpression Vectors:

Vectors as well as adenoviral vectors coexpressing two genes were constructed by using one of the expression plasmids above, inserting an IRES sequence from EMCV (Encephalomyocarditis virus, derived from pIRES1neo; Clontech) directly downstream of the first gene to be expressed and inserting the second gene to be expressed between the IRES sequence and the poly-(A) sequence. The IRES-sequence (Internal Ribosome Entry Site) allows a reinitiation of the translational machinery and therefore an expression coupling of both genes.

According to this method the following vectors are produced:
pSAA2-CD40/CD40L-IL2
pSAA2-CD40/CD40L-IL12
AdV-CD40/CD40L-IL2

Example 2

Vector expression

The pcDNA3-CD40/CD40-chimera were transiently transfected into HEK293 cells. Two days after transfection, the expression of the fusion proteins at the cell surface was verified by Western-blots (see Figure 4) using anti-<CD40> antibody directed against the CD40 C-terminus as well as with analogous CD40-CD40L chimeric constructs N-terminally tagged with a Flag-tag or a HA-tag (named CD40-CD40L^{tag}), and analyzed by FACS analysis using anti Flag- or HA-tag antibodies.

The constitutive activity of the fusion proteins was examined by cotransfecting an NF-kB (e.g., CAT-gene (CAT-ELISA, Roche Diagnostic GmbH, DE) → pNF-kB-CAT) reporter plasmid to test the activation of the transcription factor NF-kB, one of the principal outcomes of CD40 signaling.

To test for the cis-activity of the CD40L/CD40 chimera, the pCDNA3-CD40L/CD40-chimera plasmids were cotransfected with the reporter plasmid into HEK293 cells and tested for CAT-activity after 48 h hours.

Figure 5. shows the results from the assay for CD40-CD40L cis-activity.

To test for the trans-activity of the CD40L/CD40 chimera, the reporter plasmid pNF-kB-CAT was cotransfected with pcDNA3-CD40 (expression of CD40 gene alone) into HEK293 cells and the pcDNA3-CD40/CD40L-chimera plasmid was seperately transfected into HEK293 cells. After 24 h and verification of cell surface expression of the CD40/CD40L chimera both cell clones were coincubated for additional 24 h and than tested for CAT-expression.

Figure 6a/b. show design/results from the assay for CD40-CD40L trans-activity.

Example 3

In vitro growth and surface expression of CD40-CD40L chimera

SCCVII, CT26 and B16-F10 cells were infected with adenoviruses expressing the CD40-CD40L chimera or a reporter gene expressing a cell surface molecule such as a low affinity nerve growth factor receptor (LNGFR) or an indicator gene such as

lacZ as a control. The expression of relevant cell surface markers (CD40-CD40L, MHCI, ICAM-1, ...) in response to viral infection and fusion protein expression was characterized. Further, growth kinetics and apoptotic behavior of the infected cells was examined.

Non- viral transfection:

Cells were seeded in 6-well plates at a density of 1-2x10^5 cells/well 16 h before transfection. Transfection was performed with FuGENE™6 transfection reagent (Roche Diagnostics GmbH, DE). Therefore, 8 µl FuGENE™6 was added to 100 µl serum-free DMEM medium, incubated for 5 min at room temperature and then mixed with 2 µg DNA. This mixture was incubated for 15 min at room temperature and added dropwise to the cells with a volume of 2 ml cell culture medium (DMEM, 10% FCS, 2mM glutamine, 4.5 g/l glucose). For transient expression assays the cells were tested for gene expression after 2 days; for stable transfections the cells were selected by addition of G418 (at a concentration of 0.5–1 mg/ml) which selects for neo^R marker gene expression.

Example 4

In vivo growth of B16F10-derived tumors in syngenic mice (C57BL/6)

To examine the tumor growth kinetics of the murine B16F10 cell line, which does not express CD40 or CD40L or CD40-CD40L chimera, 5 x 10^5 cells / 200µl were injected per mouse. Tumor formation occurred within 10-15 days. Quantification of tumor growth was performed by caliper.

Example 5

Subcutaneous implantation of in vitro Ad-CD40L/CD40 transduced B16F10 melanoma cells into C57BL6 mice

For the experiments on subcutaneous implantation of in vitro Ad-CD40-CD40L transduced B16F10 melanoma cells into C57BL6 mice, the B16F10 cells were seeded into 12-well plates (Falcon), 4 x 10^5 cells per well. When cells were adherent, vector preparations of Ad-CD40-CD40L chimera, Ad-empty (control) and Ad-lacZ (control) in appropriate dilutions made in transduction medium (DMEM without

supplements) as well as no vector (mock-control) were added to the wells. The infection was done on an incubator at 37°C and 5% CO_2. After 2 hours transduction medium was removed, culture medium with its supplements was added and the incubation was contiunued for another 2 days.

Cell surface expression was indirectly verified by FACS-analysis of a tagged AdCD40-CD40L^{tag} construct transduced in parallel under the same conditions.

At day zero 5 x 10^5 tumor cells in a volume of 200 µl were implanted subcutaneous into lateral abdominal wall. For this procedure mice were anesthetized with ether. Tumor growth was quantitated five days post-inoculation three times a week using a caliper. Body weight was monitored three times a week. General disturbance was checked daily. Tumor volumes were calculated by the approximately ellipsoid formula: 4/3 x π / 8 x (length x width x depth).

Example 6

Effect of Ad-CD40-CD40L administration on primary tumor growth

Murine B16F10 cells were injected at a dose of 5 x 10^5 cells / 200µl per mouse. 14 to 16 days after tumor cell inoculation Ad-CD40-CD40L (versions A and B) or AdCMV-LacZ as a control were injected intratumorally and the tumor size was measured every third day for a period of about 21 days. Dose response curves with different concentrations of viral vector (1 x 10^7 to 1 x 10^9 / 50µl) and comparison of single vs. multiple injections were performed. After termination of the experiment, histopathology is performed with explanted tumors and checked for activated T cells (CD4, CD25).

From these studies the optimal regimen can be established.

Example 7

T-cell memory experiment

Example 6 was performed according to Example 5 however in the case of tumor regression, treated mice are challenged with tumor cells i.p. into the contralateral side. Growth of secondary tumors is measured to monitor memory T-cell response.

Example 8

Vaccination experiment

In a further approach ex vivo Ad-CD40-CD40L- or AdCMV-LacZ-transduced SCCVII tumor cells were irradiated (5000 rad) and implanted in syngeneic mice with a preexisting B16F10 -tumor (established according to Example 4) at a distant site. Tumor growth of the primary tumor was monitored to show the antigen-specificity of the anti-tumor effect. As control Ad-CD40-CD40L- or AdCMV-LacZ-transduced different (e.g. CT-26) tumor cells were implanted, which should give no anti- B16F10 reaction.

Example 9

In vivo tumor growth in athymic mice

In order to demonstrate whether tumor-specific CTL (cytotoxic T cell)-activity plays a pivotal role for in vivo tumor growth reduction, the Balb/c nu/nu athymic mice strain which lacks mature T cells was used. B16F10 cells were inoculated at the same time pattern as described for syngeneic C57BL/6 mice. The tumor growth kinetics were measured under the conditions described in Example 5.

Example 10

Pharmaceutical compositions

a) Nonviral vectors

For the production of a pharmaceutical composition 100 µg of a nonviral vector according to the invention are dissolved in 1 ml of phosphate buffered saline (PBS: 8 g sodium chloride, 1.44 g di-sodium-hydrogen-phosphate, 0.24 g potassium-di-hydrogen-phosphate per liter H_2O at a pH of 7.4).

b) Viral vectors

For the production of a pharmaceutical composition 10^{10} pFU adenovirus vector according to the invention in a volume of 1 ml PBS was added to 1 ml of liposomal transfection reagent (Roche Diagnostics GmbH, DE; DOSPER: 1,3-di-oleoyloxy-2-

(6-Carboxy-spermyl)-propylamide) in PBS, incubated for 15 min. at room temperature and stored at 4°C.

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Patent Claims

1. A nucleic acid encoding a chimeric polypeptide comprising nucleic acid fragments encoding

   i) a mammalian signal peptide and downstream thereof,
   ii) the binding and trimerization domain of CD40L and downstream thereof,
   iii) a spacer of 50 to 100 amino acids and downstream thereof
   iv) the transmembrane and signal transduction domains of CD40.

2. A nucleic acid according to claim 1, characterized in that the spacer is the stalk domain of CD40 or CD40L.

3. A nucleic acid according to claim 1 or 2 encoding, between the binding and trimerization domain of CD40L and the spacer, the extracellular region of CD40 or at least the part of said region causing binding to CD40L.

4. A nucleic acid according to claim 3, characterized in that between the binding and trimerization domain of CD40L and said extracellular region of CD40 or said part thereof an additional spacer encoding 1-30 amino acids is inserted.

5. A recombinant expression vector comprising a nucleic acid according to claims 1 to 4.

6. A mammalian host cell transformed or transfected with an expression vector according to claim 5.

7. A process for preparing a chimeric CD40/CD40L polypeptide characterized by culturing a host cell transformed or transfected with an expression vector comprising a nucleic acid according to claims 1 to 4 under conditions promoting the expression of said nucleic acid encoding the chimeric polypeptide and presenting said polypeptide on the surface of said host cell.

8. A process for the production of a modified human tumor cell characterized by cultivating a human tumor cell with an expression vector comprising a

nucleic acid according to claims 1 to 4 under conditions at which CD40/CD40L polypeptide is produced in the tumor cell and presented on the surface of said cell.

9. A chimeric polypeptide encoded by a nucleic acid according to claims 1 to 4, characterized in that the extracellular domain of CD40L is preceded by the signal peptide of CD40 or a fragment thereof.

10. A chimeric polypeptide according to claim 9, characterized in that the intracellular domain of CD40 consists of amino acids 216-277 of human CD40L or 216-289 of murine CD40L and/or the extracellular domain of CD40L consists of amino acids 47-261 of human CD40L or 47-260 of murine CD40L.

11. A chimeric polypeptide according to claim 8 or 9, characterized in that at least 80% of the polypeptidic spacer consist of amino acids Glu and/or Ala.

12. A composition characterized by a nucleic acid as claimed in claims 1 to 4 together with a pharmaceutically acceptable buffer, excipient and/or preservative.

13. A method for the production of a composition for activating antigen presenting cells and T cells comprising a nucleic acid as claimed in claims 1 to 4, characterized by the use, as an essential constituent of said composition, of a nucleic acid as claimed in claims 1 to 4.

14. A composition for the treatment of a patient for tumor therapy consisting as essential components of a vector according to claim 5 and a substance selected from the group consisting of Interleukin-2, Interleukin-12, Interferon-alpha and 5-fluoro-uracil.