Composition of tumour-associated peptides and related anti-cancer vaccine

DESCRIPTION

The present invention relates to immunotherapeutic peptides and their use in immunotherapy, in particular the immunotherapy of cancer. The present invention discloses tumour-associated T-helper cell peptide epitopes, alone or in combination with other tumour-associated peptides that serve as active pharmaceutical ingredients of vaccine compositions which stimulate anti-tumour immune responses. In particular, the composition of the peptides of the present invention can be used in vaccine compositions for eliciting anti-tumour immune responses against colorectal cancer.

For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Background of the invention

Colorectal Carcinoma

According to the American Cancer Society, colorectal cancer (CRC) is the third most common cancer in the US, afflicting more than 175,000 new patients each year. In the US, Japan, France, Germany, Italy Spain and the UK, it affects more than 480,000 patients. It is one of the most common causes of cancer mortality in developed countries.

Research suggests that the onset of colorectal cancer is the result of interactions between inherited and environmental factors. In most cases adenomatous polyps appear to be precursors to colorectal tumours; however the transition may take many years. The primary risk factor for colorectal cancer is age, with 90% of cases diagnosed over the age of 50 years. Other risk factors for colorectal cancer according to the American Cancer Society include alcohol consumption, a diet high in fat and/or red meat and an inadequate intake of fruits and vegetables. Incidence continues to rise, especially in areas such as Japan, where the adoption of westernised diets with excess fat and meat intake and a decrease in fiber intake may be to blame. However, incidence rates are rising not as fast as previously which may be due to increasing screening and polyp removal, thus preventing progression of polyps to cancer.

As in most solid tumours, first line treatment is surgery, however, its benefits remain confined to early-stage patients, yet a significant proportion of patients is diagnosed in advanced stages of the disease. For advanced colorectal cancer chemotherapy regimens based on fluorouracil-based regimens are standard of care. The majority of these regimens are the so-called FOLFOX (infusional 5-FU/leucovorin plus oxaliplatin) and FOLFIRI (irinotecan, leucovorin, bolus and continuous-infusion 5-FU) protocols.

The introduction of third-generation cytotoxics such as irinotecan and oxaliplatin has raised the hope of significantly improving efficacy, but prognosis is still relatively poor, and the survival rate generally remains at approximately 20 months in metastatic disease and, as a result, the unmet needs in the disease remain high.

Recently a novel generation of drugs, molecular-targeted agents, such as Avastin (bevacizumab) and Erbitux (cetuximab), became available and about 40 compounds are in late-stage clinical development for different stages of colorectal cancer. Combinations of several of these compounds increase the number of potential treatment options to be expected for the future. The vast majority of substances is in phase 2, with EGFR addressed by these compounds more often than by any other drug in development for colorectal cancer, which is due to the fact that in ~80% of patients with colorectal cancer EGFR expression is upregulated.

Clinical trials with stage II patients combining chemotherapy with the recently approved monoclonal antibodies (mAbs) (cetuximab + irinotecan or FOLFOX4; bevacizumab as a single-agent or together with FOLFOX4) are currently conducted. Three to four year observation periods are expected for statistically significant results from these trials.

Monoclonal antibodies (mAbs) presently used in oncology in general have an excellent chance of not interfering with active immunotherapy. In fact, there is preclinical evidence suggesting that depletion of VEGF (by bevacizumab) contributes positively to DC-mediated activation of T-cells.

Currently there are about 16 trials testing the safety and potential of novel immunotherapeutic approaches for the treatment of CRC.

Immunotherapeutic approaches for treatment

Stimulation of an immune response is dependent upon the presence of antigens recognised as foreign by the host immune system. The discovery of the existence of tumour associated antigens has now raised the possibility of using a host's immune system to intervene in tumour growth. Various mechanisms of harnessing both the humoral and cellular arms of the immune system are currently explored for cancer immunotherapy.

Specific elements of the cellular immune response are capable of specifically recognising and destroying tumour cells. The isolation of cytotoxic T-cells (CTL) from tumour-infiltrating cell populations or from peripheral blood suggests that such cells play an important role in natural immune defences against cancer (Cheever et al., Annals N.Y. Acad. Sci. 1993 690:101-112; Zeh HJ, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC; J Immunol. 1999, 162(2):989-94; High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy.). CD8-positive T-cells ($TCD8^+$) in particular, which recognise Class I molecules of the major histocompatibility complex (MHC)-bearing peptides of usually 8 to 10 residues derived from proteins or defect ribosomal products (DRIPS) (Schubert U, Antón LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR.;Rapid degradation of a large fraction of newly synthesized proteins by proteasomes; Nature 2000; 404(6779):770-774) located in the cytosols, play an important role in this response. The MHC-molecules of the human are also designated as human leukocyte-antigens (HLA).

There are two classes of MHC-molecules: MHC class I molecules that can be found on most cells having a nucleus which present peptides that result from proteolytic cleavage of endogenous proteins DRIPS, and larger peptides. MHC class II molecules can be found predominantly on professional antigen presenting cells (APCs), and present peptides of exogenous proteins that are taken up by APCs during the course of endocytosis, and are subsequently processed. Complexes of peptide and MHC class I molecule are recognised by CD8-positive cytotoxic T-lymphocytes bearing the appropriate TCR, complexes of peptide and MHC class II molecule are recognised by CD4-positive-helper-T-cells bearing the appropriate TCR.

CD4-positive helper T-cells play an important role in orchestrating the effector functions of anti-tumour T-cell responses and for this reason the identification of CD4-positive T-cell

epitopes derived from tumour associated antigens (TAA) may be of great importance for the development of pharmaceutical products for triggering anti-tumour immune responses (Kobayashi,H., R. Omiya, M. Ruiz, E. Huarte, P. Sarobe, J. J. Lasarte, M. Herraiz, B. Sangro, J. Prieto, F. Borras-Cuesta, and E. Celis. Identification of an antigenic epitope for helper T lymphocytes from carcinoembryonic antigen. Clin. Cancer Res. 2002, 8:3219-3225., Gnjatic, S., D. Atanackovic, E. Jäger, M. Matsuo, A. Selvakumar, N.K. Altorki, R.G. Maki, B. Dupont, G. Ritter, Y.T. Chen, A. Knuth, and L.J. Old. Survey of naturally occurring CD4+ T-cell responses against NY-ESO-1 in cancer patients: Correlation with antibody responses. Proc. Natl. Acad. Sci. U.S.A. 2003, 100 (15): 8862-7) CD4+ T cells can lead to locally increased levels of IFNγ (Qin Z, Schwartzkopff J, Pradera F, Kammertoens T, Seliger B, Pircher H, Blankenstein T; A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells; J Cancer Res; 2003, 63(14): 4095-4100).

It was shown in mammalian animal models, e.g., mice, that even in the absence of cytotoxic T lymphocyte (CTL) effector cells (i.e., CD8-positive T lymphocytes), CD4 positive T-cells are sufficient for inhibiting manifestation of tumours via inhibition of angiogenesis by secretion of interferon-gamma (IFNγ) (Qin, Z. and T. Blankenstein. CD4+ T-cell--mediated tumour rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity. 2000, 12:677-686). Additionally, it was shown that CD4 positive T-cells recognizing peptides from tumour-associated antigens presented by HLA class II molecules can counteract tumour progression via the induction of an antibody (Ab) responses (Kennedy, R.C., M.H. Shearer, A.M. Watts, and R.K. Bright. CD4+ T lymphocytes play a critical role in antibody production and tumour immunity against simian virus 40 large tumour antigen. Cancer Res. 2003, 63:1040-1045). In contrast to tumour-associated peptides binding to HLA class I molecules, only a small number of class II ligands of TAA have been described so far (www.cancerimmunity.org, www.syfpeithi.de).

Since the constitutive expression of HLA class II molecules is usually limited to cells of the immune system (Mach, B., V. Steimle, E. Martinez-Soria, and W. Reith. Regulation of MHC class II genes: lessons from a disease. Annu. Rev. Immunol. 1996, 14: 301-331), the possibility of isolating class II peptides directly from primary tumours was not considered possible. However, the inventors were recently successful in identifying a number of MHC Class II epitopes directly from tumours (EP 1642905, EP 1760088; Dengjel J, Nastke MD, Gouttefangeas C, Gitsioudis G, Schoor O, Altenberend F, Müller M, Krämer B, Missiou A,

Sauter M, Hennenlotter J, Wernet D, Stenzl A, Rammensee HG, Klingel K, Stevanović S.; Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas; Clin Cancer Res. 2006; 12:4163-4170).

In the absence of inflammation, expression of MHC class II molecules is mainly restricted to cells of the immune system, especially professional antigen-presenting cells (APC), e.g., monocytes, monocyte-derived cells, macrophages, dendritic cells. In tumour patients, cells of the tumour have surprisingly been found to express MHC class II molecules (Dengjel J, Nastke MD, Gouttefangeas C, Gitsioudis G, Schoor O, Altenberend F, Müller M, Krämer B, Missiou A, Sauter M, Hennenlotter J, Wernet D, Stenzl A, Rammensee HG, Klingel K, Stevanović S.; Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas; Clin Cancer Res. 2006; 12:4163-4170)

For a peptide to trigger (elicit) a cellular immune response, it must bind to an MHC-molecule. This process is dependent on the allele of the MHC-molecule and specific polymorphisms of the amino acid sequence of the peptide. MHC-class-I-binding peptides are usually 8-10 amino acid residues in length and usually contain two conserved residues ("anchor") in their sequence that interacts with the corresponding binding groove of the MHC-molecule. In this way each MHC allele has a "binding motif" determining which peptides can bind specifically to the binding groove (Rammensee HG, Bachmann J, Stevanovic S. MHC ligands and peptide motifs, Landes Bioscience, USA, 1997).

In the MHC class I dependent immune reaction, peptides not only have to be able to bind to certain MHC class I molecules expressed by tumour cells, they also have to be recognised by T-cells bearing specific T-cell receptors (TCR).

The antigens that are recognised by the tumour specific T-lymphocytes, that is, their epitopes, can be molecules derived from all protein classes, such as enzymes, receptors, transcription factors, etc. Furthermore, tumour associated antigens, for example, can also be present in tumour cells only, for example as products of mutated genes. Another important class of tumour associated antigens are tissue-specific antigens, such as CT ("cancer testis")-antigens that are expressed in different kinds of tumours and in healthy tissue of the testis.

Various tumour associated antigens have been identified. Further, much research effort is expended to identify additional tumour associated antigens. Some groups of tumour associated antigens, also referred to in the art as tumour specific antigens, are tissue specific. Examples include, but are not limited to, tyrosinase for melanoma, PSA and PSMA for prostate cancer and chromosomal cross-overs (translocations) such as bcr/abl in lymphoma. However, many tumour associated antigens identified occur in multiple tumour types, and some, such as oncogenic proteins and/or tumour suppressor genes (tumour suppressor genes are, for example reviewed for renal cancer in Linehan WM, Walther MM, Zbar B. The genetic basis of cancer of the kidney. J Urol. 2003 Dec; 170 (6Pt1):2163-72) which actually cause the transformation event, occur in nearly all tumour types. For example, normal cellular proteins that control cell growth and differentiation, such as p53 (which is an example for a tumour suppressor gene), ras, c-met, myc, pRB, VHL, and HER-2/neu, can accumulate mutations resulting in upregulation of expression of these gene products thereby making them oncogenic (McCartey et al. Cancer Research, 1998, 15:58 2601-5; Disis et al. Ciba Found. Symp. 1994, 187:198-211). These mutant proteins can also be a target of a tumour specific immune response in multiple types of cancer.

Immunotherapy in cancer patients aims at activating cells of the immune system specifically, especially the so-called cytotoxic T-cells (CTL, also known as "killer cells", also known as CD8-positive T-cells), against tumour cells but not against healthy tissue. Tumour cells differ from healthy cells by the expression of tumour-associated proteins. HLA molecules on the cell surface present the cellular content to the outside, thus enabling a cytotoxic T cell to differentiate between a healthy and a tumour cell. This is realized by breaking down all proteins inside the cell into short peptides, which are then attached to HLA molecules and presented on the cell surface (Rammensee, HG, Falk, K, and Rotzschke, O; Peptides naturally presented by MHC class I molecules, Annu. Rev. Immunol., 1993, 11, 213-244). Peptides that are presented on tumour cells, but not or to a far lesser extent on healthy cells of the body, are called tumour-associated peptides (TUMAPs).

For proteins to be recognised by cytotoxic T-lymphocytes as tumour-specific or –associated antigens, and to be used in a therapy, particular prerequisites must be fulfilled. The antigen should be expressed mainly by tumour cells and not by normal healthy tissues or in comparably small amounts. It is furthermore desirable, that the respective antigen is not only present in a type of tumour, but also in high concentrations (i.e. copy numbers of the

respective peptide per cell). Tumour-specific and tumour-associated antigens are often derived from proteins directly involved in transformation of a normal cell to a tumour cell due to a function e.g. in cell cycle control or apoptosis. Additionally, also downstream targets of the proteins directly causative for a transformation may be upregulated und thus be indirectly tumour-associtated. Such indirectly tumour-associated antigens may also be targets of a vaccination approach. Essential is in both cases the presence of epitopes in the amino acid sequence of the antigen, since such peptide ("immunogenic peptide") that is derived from a tumour associated antigen should lead to an in vitro or in vivo T-cell-response.

Basically, any peptide able to bind a MHC molecule may function as a T-cell epitope. A prerequisition for the induction of an in vitro or in vivo T-cell-response is the presence of a T-cell with a corresponding TCR and the absence of tolerance for this particular epitope. T-helper cells play an important role in orchestrating the effector function of CTLs in anti-tumour immunity. T-helper cell epitopes that trigger a T-helper cell response of the TH1 type support effector functions of CD8-positive Killer T-cells, which include cytotoxic functions directed against tumour cells displaying tumour-associated peptide/MHC complexes on their cell surfaces. In this way tumour-associated T-helper cell peptide epitopes, alone or in combination with other tumour-associated peptides, can serve as active pharmaceutical ingredients of vaccine compositions which stimulate anti-tumour immune responses.

Since both types of response, CD8 and CD4 dependent, contribute jointly and synergistically to the anti-tumour effect, the identification and characterization of tumour-associated antigens recognised by either CD8+ CTLs (MHC class I molecule) or by CD4-positive CTLs (MHC class II molecule) is important in the development of tumour vaccines. It is therefore an object of the present invention, to provide compositions of peptides that contain peptides binding to MHC complexes of either class.

First clinical trials using tumour-associated peptides have started in the mid-1990s by Boon and colleagues mainly for the indication melanoma. Clinical responses in the best trials have ranged from 10% to 30%. Severe side effects or severe autoimmunity have not been reported in any clinical trial using peptide-based vaccine monotherapy. Mild forms of vitiligo have been reported for some patients who had been treated with melanoma-associated peptides.

However, priming of one kind of CTL is usually insufficient to eliminate all tumour cells. Tumours are very mutagenic and thus able to respond rapidly to CTL attacks by changing their protein pattern to evade recognition by CTLs. To counter-attack the tumour evasion mechanisms a variety of specific peptides is used for vaccination. In this way a broad simultaneous attack can be mounted against the tumour by several CTL clones simultaneously. This may decrease the chances of the tumour to evade the immune response. This hypothesis has been recently confirmed in a clinical study treating late-stage melanoma patients. With only few exceptions, patients that had at least three distinct T-cell responses, showed objective clinical responses or stable disease (Banchereau, J, Palucka, AK, Dhodapkar, M, Burkeholder, S, Taquet, N, Rolland, A, Taquet, S, Coquery, S, Wittkowski, KM, Bhardwaj, N, Pineiro, L, Steinman, R, and Fay, J; Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine, Cancer Res., 2001, 61, 6451-6458) as well as increased survival (personal communication with J. Banchereau), while the vast majority of patients with less than three T-cell responses were diagnosed with progressive disease.

A study of the applicants showed a similar effect when patients suffering from renal cell carcinoma were treated with a vaccine composed of 13 different peptides (H. Singh-Jasuja, S. Walter, T. Weinschenk, A. Mayer, P. Y. Dietrich, M. Staehler, A. Stenzl, S. Stevanovic, H. Rammensee, J. Frisch; Correlation of T-cell response, clinical activity and regulatory T-cell levels in renal cell carcinoma patients treated with IMA901, a novel multi-peptide vaccine; ASCO Meeting 2007 Poster # 3017; M. Staehler, A. Stenzl, P. Y. Dietrich, T. Eisen, A. Haferkamp, J. Beck, A. Mayer, S. Walter, H. Singh, J. Frisch, C. G. Stief; An open label study to evaluate the safety and immunogenicity of the peptide based cancer vaccine IMA901, ASCO meeting 2007; Poster # 3017).

The major task in the development of a tumour vaccine is therefore the not only the identification and characterisation of novel tumour associated antigens and immunogenic T-helper epitopes derived thereof, but also the combination of different epitopes to increase the likelihood of a response to more than one epitope for each patient. It is therefore an object of the present invention to provide combinations of amino acid sequences of such peptides that have the ability to bind to a molecule of the human major histocompatibility complex (MHC) class-I (HLA class I) or II (HLA class II). It is a further object of the present invention, to provide an effective anti-cancer vaccine that is based on a combination of the peptides.

In the present invention, the inventors did isolate and characterise peptides binding to HLA class I or II molecules directly from mammalian tumours, i.e. colorectal carcinomas.

The present invention provides peptides that stem from antigens associated with tumourigenesis, and have the ability to bind sufficiently to MHC (HLA) class II molecules for triggering an immune response of human leukocytes, especially lymphocytes, especially T lymphocytes, especially CD4-positive T lymphocytes, especially CD4-positive T lymphocytes mediating T_{H1}-type immune responses.

The present invention also provides peptides that stem from antigens associated with tumourigenesis, and have the ability to bind sufficiently to MHC (HLA) class I molecules for triggering an immune response of human leukocytes, especially lymphocytes, especially T lymphocytes, especially CD8-positive cytotoxic T-lymphocytes as well as combinations of the two that are particularly useful for vaccination of patients that suffer from cancer.

According to the present invention, the object is solved by providing a pharmaceutical composition comprising at least two peptides containing an amino acid sequence selected from the group consisting of SEQ ID NO 1 to SEQ ID NO 7, and/or containing a variant amino acid sequence that is at least 80 % homologous to that of SEQ ID NO 1 to SEQ ID NO 7, and/or a polynucleotide containing a nucleic acid encoding SEQ ID NO 1 to SEQ ID NO 7 or the variant amino acid sequence, and a pharmaceutically acceptable carrier. Pharmaceutical compositions of the present invention may also further comprise at least one additional peptide containing an amino acid sequence selected from the group consisting of SEQ ID NO: 8 to SEQ ID NO: 15, or containing a variant amino acid sequence that is at least 80 % identical to that of SEQ ID NO: 8 to SEQ ID NO: 15, or polynucleotide containing a nucleic acid encoding SEQ ID NO: 8 to SEQ ID NO: 15 or the variant amino acid sequence. The peptides may have an overall length of between 8 and 100, preferably between 8 and 30, and most preferably between 8 and 16 amino acids. The peptides may also have non-peptide bonds.

As described herein below, the peptides that form the basis of the present invention have all been identified as presented by MHC class I or II bearing cells. Thus, these particular peptides as well as other peptides containing the sequence (i.e. derived peptides) all elicit a specific T-

cell response, although the extent to which such response will be induced might vary from individual peptide to peptide and from individual patient to patient. Differences, for example, could be caused due to mutations in the peptides. The person of skill in the present art is well aware of methods that can be applied to determine the extent to which a response is induced by an individual peptide, in particular with reference to the examples herein and the respective literature.

Preferably the variants of the invention will induce T-cells cross-reacting with the respective peptide of the invention.

The percentage of homology between the amino acid sequence of a peptide or a nucleic acid sequence encoding the peptide and a variant can be calculated using algorithms well known in the art. In the present invention, the term "homologous" refers to the degree of identity between sequences of two amino acid sequences, i.e. peptide or polypeptide sequences. The aforementioned "homology" is determined by comparing two sequences aligned under optimal conditions over the sequences to be compared. The amino acid or nucleic acid sequences to be compared herein may have an addition or deletion (for example, gap and the like) in the optimum alignment of the two sequences. Such a sequence homology can be calculated by creating an alignment using, for example, ClustalW algorithm (Nucleic Acid Res., 22(22): 4673 4680 (1994). Commonly available sequence analysis software, more specifically, Vector NTI, GENETYX or analysis tools provided by public databases, such as e.g. http://restools.sdsc.edu/biotools/biotools16.html may also be used.

Pharmaceutically acceptable carriers are well known and are usually liquids, in which an active therapeutic agent is formulated. The carrier generally does not provide any pharmacological activity to the formulation, though it may provide chemical and/or biological stability, release characteristcs, and the like. Exemplary formulations can be found, for example, in Alfonso R. Gennaro. Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, MD: Lippincott Williams & Wilkins, 2000 and include, but are not limited to, saline, water, buffered water, 0.3% glycine, hyaluronic acid, dextrose and the like. Recently, it was found that certain fat emulsions, which have been in use for many years for intravenous nutrition of human patients, can also act as a vehicle for peptides. Two examples of such emulsions are the available commercial fat emulsions known as Intralipid and Lipofundin. "Intralipid" is a registered trademark of Kabi Pharmacia, Sweden, for a fat

emulsion for intravenous nutrition, described in U.S. Pat. No. 3,169,094. "Lipofundin" is a registered trademark of B. Braun Melsungen, Germany. Both contain soybean oil as fat (100 or 200 g in 1,000 ml distilled water: 10% or 20%, respectively). Egg-yolk phospholipids are used as emulsifiers in Intralipid (12 g/l distilled water) and egg-yolk lecithin in Lipofundin (12 g/l distilled water). Isotonicity results from the addition of glycerol (25 g/l) both in Intralipid and Lipofundin.

The peptides stem from tumour-associated antigens, especially tumour-associated antigens with functions in, e.g., proteolysis, angiogenesis, cell growth, cell cycle regulation, cell division, regulation of transcription, regulation of translation, tissue invasion, etc. Table 1 provides the peptides and the function of the protein the peptides are derived from.

Table 1: Peptides of the present invention and function of the parent protein

SEQ ID NO	Peptide ID	Sequence	Gene Symbol	Function	binds to MHC
1	C20-001	ALSNLEVTL	C20orf42	implicated in linking actin cytoskeleton to ECM	HLA-A*02
2	NOX-001	ILAPVILYI	NOX1	NADPH oxidase	HLA-A*02
3	ODC-001	ILDQKINEV	ODC1	Ornithine decarboxylase	HLA-A*02
4	PCN-001	KLMDLDVEQL	PCNA	DNA polymerase delta auxiliary protein	HLA-A*02
5	TGFBI-001	ALFVRLLALA	TGFBI	transforming growth factor, beta-induced	HLA-A*02
6	TOP-001	KIFDEILVNA	TOP2A/TOP2B	Topoisomerase	HLA-A*02
7	TGFBI-004	TPPIDAHTRNLLRNH	TGFBI	transforming growth factor, beta-induced	HLA-DR

Chromosome 20 open reading frame 42

C20orf42 is a focal adhesion protein involved in attachment of the actin cytoskeleton to the plasma membrane and in integrin-mediated cellular processes. Deficiency of C20orf42 as a result of loss-of-function mutations causes Kindler syndrome, an autosomal recessive genodermatosis characterized by skin blistering, progressive skin atrophy, photosensitivity and, occasionally, carcinogenesis (Herz, C, Aumailley, M, Schulte, C, Schlotzer-Schrehardt, U, Bruckner-Tuderman, L, and Has, C; Kindlin-1 is a phosphoprotein involved in regulation of polarity, proliferation, and motility of epidermal keratinocytes, J Biol Chem., 2006, 281, 36082-36090). Recently, a severe gastrointestinal tract involvement with hemorrhagic colitis

has been reported in a patient with a loss-of-function mutation (Sadler, E, Klausegger, A, Muss, W, Deinsberger, U, Pohla-Gubo, G, Laimer, M, Lanschuetzer, C, Bauer, JW, and Hintner, H; Novel KIND1 gene mutation in Kindler syndrome with severe gastrointestinal tract involvement, Arch. Dermatol., 2006, 142, 1619-1624).

In the context of cancer, C20orf42 has been described within studies investigating gene expression in cancer-relevant settings. It was found to be overexpressed in 70% of colon carcinomas and 60% of lung carcinomas tested (n = 10). Normal tissue expression by Northern Blot was restricted to neuromuscular tissues (Weinstein, EJ, Bourner, M, Head, R, Zakeri, H, Bauer, C, and Mazzarella, R; URP1: a member of a novel family of PH and FERM domain-containing membrane-associated proteins is significantly over-expressed in lung and colon carcinomas, Biochim. Biophys. Acta, 2003, 1637, 207-216). Furthermore, C20orf42 has been identified as a gene involved in TGF-β-mediated cell migration and tumour invasion (Kloeker, S, Major, MB, Calderwood, DA, Ginsberg, MH, Jones, DA, and Beckerle, MC; The Kindler syndrome protein is regulated by transforming growth factor-beta and involved in integrin-mediated adhesion, J. Biol. Chem., 2004, 279, 6824-6833).

NADPH oxidase homolog-1 (NOX1)

NOX1, is a growth factor-responsive enzyme that catalyzes formation of the reactive oxygen species superoxide (O_2-) and hydrogen peroxide (H_2O_2). Its expression was originally identified in colon, prostate, uterus, and proliferating vascular smooth muscle cells (Suh, Y. A. et al. Cell transformation by the superoxide-generating oxidase Mox1. Nature 1999, 401, 79-82). Its expression is linked to a number of biological responses including cellular proliferation, angiogenesis, and activation of cellular signaling pathways (Harper, R. W., Xu, C., Soucek, K., Setiadi, H. and Eiserich, J. P. A reappraisal of the genomic organization of human Nox1 and its splice variants. Arch. Biochem. Biophys. 2005, 435, 323-330).

NOX1 is highly expressed in the colon but its function in colonic physiology or pathology is still poorly understood. In normal tissues, NOX1 expression was low in the ileum, intermediate in the right colon, and high in the left colon. There was no statistical difference in NOX1 expression between samples derived from adenomas, well differentiated or poorly differentiated colon adenocarcinomas. NOX1 was highly expressed in colon epithelial cells, both within the crypts and on the luminal surface. In conclusion, NOX1 is an enzyme that is constitutively expressed in colon epithelium and is not directly associated with

tumourigenesis (Szanto, I. et al. Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease. J Pathol. 2005, 207, 164-176).

Immunohistochemistry showed that NOX1 was constitutively expressed in surface mucous cells. Adenomas and well differentiated adenocarcinomas up-regulated NOX1 expression. Nuclear factor (NF)-kappaB was predominantly activated in adenoma and adenocarcinoma cells expressing abundant NOX1, suggesting that NOX1 may stimulate NF-kappaB-dependent antiapoptotic pathways in colon tumours (Fukuyama, M. et al. Overexpression of a novel superoxide-producing enzyme, NADPH oxidase 1, in adenoma and well differentiated adenocarcinoma of the human colon. Cancer Lett. 2005, 221, 97-104).

Wnt3a/beta-Catenin signaling has been described to induce NOX1 expression (Petropoulos, H. & Skerjanc, I. S. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. J Biol Chem. 2002, 277, 15393-15399).

Recently, reactive oxygen species have been suggested to induce endothelial apoptosis that subsequently induces the expression of various adhesion molecules for tumour cells. This indicates that by tackling the production of ROS preventing tumour recurrence at distant sites might be feasible (Ten, KM, van der Wal, JB, Sluiter, W, Hofland, LJ, Jeekel, J, Sonneveld, P, and van Eijck, CH; The role of superoxide anions in the development of distant tumour recurrence, 2006, Br.J Cancer,).

Ornithine decarboxylase 1 (ODC1)

ODC1 is the rate-limiting enzyme of the polyamine biosynthesis pathway which catalyses ornithine to putrescine. The activity level for the enzyme varies in response to growth-promoting stimuli and exhibits a high turnover rate in comparison to other mammalian proteins.

Polyamine metabolism is an integral component of the mechanism of carcinogenesis in epithelial tissues. Increases in ODC1 are often associated with initiation of normal cell growth and with sustained neoplastic cell growth. Inhibitors of ODC1 suppress tumour formation in experimental models of bladder, breast, colon and skin carcinogenesis. Over-expression of ODC1 activity is a well-recognized feature of many cancers1 and ODC1 has been considered

as a proto-oncogene (Auvinen, M., Paasinen, A., Andersson, L. C. and Holtta, E. Ornithine decarboxylase activity is critical for cell transformation. Nature 1992, 360, 355-358).

Germline mutations in the adenomatous polyposis coli (APC) gene are one of the most clearly defined inherited predispositions for colon cancer. APC mutations cause a substantial increase in free β-catenin levels, which moves into the nucleus, where it forms a complex with members of the lymphoid-enhancing factor (LEF)/T-cell factor (Tcf) family of sequence-specific transcription factors. The c-myc oncogene is one of the Tcf target genes He, T. C. et al. Identification of c-MYC as a target of the APC pathway (Science 281, 1509-1512 (1998). c-Myc RNA and protein are overexpressed in both early and late stages of colorectal tumorigenesis). ODC is a c-Myc target gene.

Loss of APC function causes an upregulation of ODC1 (Gerner, EW and Meyskens, FL, Jr., Polyamines and cancer: old molecules, new understanding, Nat. Rev. Cancer, 2004, 4, 781-792) and overexpression has been frequently observed in colorectal carcinoma (Hu, H. Y. et al. Ornithine decarboxylase gene is overexpressed in colorectal carcinoma, World J. Gastroenterol. 2005, 11, 2244-2248; Kitahara, O. et al. Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia; Cancer Res. 2001, 61, 3544-3549; Nemoto, T., Kubota, S., Ishida, H., Murata, N. and Hashimoto, D. Ornithine decarboxylase, mitogen-activated protein kinase and matrix metalloproteinase-2 expressions in human colon tumors. World J. Gastroenterol. 2005, 11, 3065-3069).

ODC1 has pro-angiogenic properties by acting as an endostatin suppressor (Nemoto, T., Hori, H., Yoshimoto, M., Seyama, Y. & Kubota, S. Overexpression of ornithine decarboxylase enhances endothelial proliferation by suppressing endostatin expression. Blood 2002, 99, 1478-1481).

Infection of the CRC cell line HT-29 with an adenovirus encoding antisense RNA for ODC1 and S-adenosylmethionine decarboxylase (another important enzyme of the polyamine biosynthesis pathway) leads to a downregulation of CCND1 and cell cycle arrest. Moreover, nuclear translocation of beta-catenin was also inhibited (Gong, L, Jiang, C, Zhang, B, Hu, H, Wang, W, and Liu, X; Adenovirus-mediated Expression of Both Antisense Ornithine Decarboxylase and S-adenosylmethionine Decarboxylase Induces G(1) Arrest in HT-29 Cells,

J Biochem. Mol. Biol, 2006, 39, 730-736). The adenovirus also induced tumor regression in established tumors in nude mice (Zhang, B, Liu, XX, Zhang, Y, Jiang, CY, Hu, HY, Gong, L, Liu, M, and Teng, QS; Polyamine depletion by ODC-AdoMetDC antisense adenovirus impairs human colorectal cancer growth and invasion in vitro and in vivo, 2006, J Gene Med, 8, 980-989).

A specific and irreversible inhibitor of ODC1 is 2-difluoromethylornithine (DMFO, Eflornithine (Sanofi-Aventis)). It is marketed for the treatment of sleeping sickness (caused by trypanosomes) and is the active ingredient of the hair removal cream Vaniqa.

With respect to cancer, DMFO has been widely used in pre-clinical models and shown promising anti-tumor effects by decreasing polyamine levels (Gerner, EW and Meyskens, FL, Jr.; Polyamines and cancer: old molecules, new understanding, Nat. Rev. Cancer, 2004, 4, 781-792). Clinical trials have been performed for several cancers and some are currently underway for CRC. However, these studies are mostly combination approaches performed in preventive settings with patients especially susceptible to CRC (adenomatous polyps). The immunogenic ODC peptide ODC-001 has been identified previously (M. Diehl, PhD Thesis, University of Tübingen, 1998)

Proliferating Cell Nuclear Antigen (PCNA)

PCNA is found in the nucleus and is a cofactor of DNA polymerase delta. The encoded protein acts as a homotrimer and helps increase the processivity of leading strand synthesis during DNA replication. Therefore, it is expressed in all proliferating cells, especially tumour cells, and is used as a marker to detect proliferation.

Proliferation indexes in neoplastic and adjacent normal mucosa, as defined by PCNA immunohistochemical analysis, have long been known as independent predictors of recurrence and poor survival in patients with colorectal cancer (al-Sheneber, IF, Shibata, HR, Sampalis, J, and Jothy, S; Prognostic significance of proliferating cell nuclear antigen expression in colorectal cancer, Cancer, 1993, 71, 1954-1959; Mayer, A, Takimoto, M, Fritz, E, Schellander, G, Kofler, K, and Ludwig, H; The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer, Cancer, 1993, 71, 2454-2460; Nakamura, T, Tabuchi, Y, Nakae, S, Ohno, M, and Saitoh, Y; Serum carcinoembryonic antigen levels and proliferating cell nuclear antigen

labeling index for patients with colorectal carcinoma. Correlation with tumor progression and survival, Cancer, 1996, 77, 1741-1746)

DNA topoisomerase II (TOP2)

TOP2A and TOP2B encode isoforms of a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription. This nuclear enzyme is involved in processes such as chromosome condensation, chromatid separation, and the relief of torsional stress that occurs during DNA transcription and replication. It catalyses the transient breaking and rejoining of two strands of duplex DNA, which allows the strands to pass through one another, thus altering the topology of DNA. The two isoforms of this enzyme exist as likely products of a gene duplication event. The gene encoding the alpha form is localised to chromosome 17 and the beta gene is localised to chromosome 3.

TOP2A is the target for several anticancer agents and a variety of mutations in this gene have been associated with the development of drug resistance.

The TOP2A gene is located adjacent to the HER-2 oncogene, the most frequently amplified oncogene in breast cancer, at the chromosome location 17q12-q21 and is either amplified or deleted, with equal frequency, in almost 90% of HER-2 amplified primary breast tumours (Jarvinen, TA and Liu, ET; Topoisomerase IIalpha gene (TOP2A) amplification and deletion in cancer--more common than anticipated, Cytopathology, 14, 309-313). Furthermore, TOP2A amplifications have been reported for other cancers. Recent experimental as well as numerous, large, multi-center trials suggest that amplification (and/or deletion) of TOP2A may account for both sensitivity or resistance to commonly used cytotoxic drugs, i.e. topoisomerase II inhibitors (anthracyclines etc. Kellner, U, Sehested, M, Jensen, PB, Gieseler, F, and Rudolph, P; Culprit and victim -- DNA topoisomerase II, Lancet Oncol., 2002, 3, 235-243), depending on the specific genetic defect at the TOP2A locus (Jarvinen, TA and Liu, ET; Simultaneous amplification of HER-2 (ERBB2) and topoisomerase IIalpha (TOP2A) genes--molecular basis for combination chemotherapy in cancer, Curr.Cancer Drug Targets., 2006, 6, 579-602).

Without TOP2A DNA replication and cell division are impossible. It has therefore become the main target of many antitumour therapy regimens, even though the exact mechanism of cell killing remains elusive (Kellner, U, Sehested, M, Jensen, PB, Gieseler, F, and Rudolph,

P; Culprit and victim -- DNA topoisomerase II, Lancet Oncol., 2002, 3, 235-243). The success of this approach is limited by the development of spontaneous resistance, and drug-induced DNA damage can increase malignancy.

TOP2B, the second potential source protein for TOP-001, has not been in the focus of cancer research so much because it is located in a chromosomal region (3p24) that is not known for frequent amplification in tumors. However, TOP2B is similar in primary structure to TOP2A and has almost identical catalytic properties (Leontiou, C, Lightowlers, R, Lakey, JH, and Austin, CA; Kinetic analysis of human topoisomerase IIalpha and beta DNA binding by surface plasmon resonance, FEBS Lett., 2003, 554, 206-210). In another study it has also been shown that both isoforms can substitute for each other (Sakaguchi, A and Kikuchi, A; Functional compatibility between isoform alpha and beta of type II DNA topoisomerase, J Cell Sci., 2004, 117, 1047-1054).

In the present invention the inventors provide conclusive evidence that tumour-associated peptides sufficiently binding to HLA-class I molecules are able to elicit immune responses mediated by human CD8-positive cytotoxic T-lymphocytes, also demonstrating that the claimed peptides are suitable for triggering responses of the human immune system against selected peptides of the tumour cell peptidome.

Similarly, it was found that tumour-associated peptides sufficiently binding to HLA-class II molecules, especially those HLA class II alleles genetically encoded by HLA DR loci of the human genome, are able to elicit immune responses mediated by human CD4–positive T-cells. CD4-positive T-cells were isolated from human peripheral blood, demonstrating that the claimed peptides are suitable for triggering T-cell responses of the human immune system against selected peptides of the tumour cell peptidome. As exemplified below with a peptide TGFBI-004 this HLA-DR-binding, tumour-associated peptide was found to be recognized by CD4-positive T-cells.

As peptides can be synthesized chemically and can be used as active pharmaceutical ingredients of pharmaceutical preparations, the peptides provided by the present invention can be used for immunotherapy, preferentially cancer immunotherapy.

In another aspect the pharmaceutical composition further comprises at least one additional peptide containing an amino acid sequence selected from the group consisting of SEQ ID NO 8 to SEQ ID NO 15, or containing a variant amino acid sequence that is at least 80 % homologous to that of SEQ ID NO 8 to SEQ ID NO 15, or a polynucleotide containing a nucleic acid encoding SEQ ID NO 8 to SEQ ID NO 15 or the variant amino acid sequence. The peptides of SEQ ID NO 8 to SEQ ID NO 13 and 15 are immunogenic peptides previously identified and bind to MHC class I and MHC class II molecules (see Table 2).

These peptides were shown to elicit T-cell responses in vivo in patients suffering from renal cell carcinoma (RCC) (H. Singh-Jasuja, S. Walter, T. Weinschenk, A. Mayer, P. Y. Dietrich, M. Staehler, A. Stenzl, S. Stevanovic, H. Rammensee, J. Frisch; Correlation of T-cell response, clinical activity and regulatory T-cell levels in renal cell carcinoma patients treated with IMA901, a novel multi-peptide vaccine; ASCO Meeting 2007 Poster # 3017; M. Staehler, A. Stenzl, P. Y. Dietrich, T. Eisen, A. Haferkamp, J. Beck, A. Mayer, S. Walter, H. Singh, J. Frisch, C. G. Stief; An open label study to evaluate the safety and immunogenicity of the peptide based cancer vaccine IMA901, ASCO meeting 2007; Poster # 3017). Since the parent proteins are not only overexpressed in RCC but also CRC and other types of cancer these peptides are also useful in vaccines for the treatment of other tumour types, in particular antiCRC vaccines.

Table 2: Additional immunogenic peptides useful in a composition of the invention

SEQ ID NO	Peptide ID	Sequence	Gene Symbol	Function	binds to MHC
8	CEA-006	SPQYSWRINGIPQQHT	CEACAM5	Carcinoembryonic antigen	HLA-DR
9	CCN-001	LLGATCMFV	CCND1	Cyclin D1	HLA-A*02
10	MUC-001	STAPPVHNV	MUC1	Mucin 1	HLA-A*02
11	MMP-001	SQDDIKGIQKLYGKRS	MMP7	Metalloproteinase 7	HLA-DR
12	CEA-005	YLSGADLNL	*CEACAM5*	variant of CEA peptide	HLA-A*02
13	MET-001	YVDPVITSI	MET	met proto-oncogene	HLA-A*02
14	(HBV-001)	FLPSDFFPSV		control peptide	
15	CEA-004	YLSGANLNL	CEACAM5	CEA peptide	HLA-A*02

Carcinoembryonic antigen-related cell adhesion molecule 5

Carcinoembryonic antigen (CEA = CEACAM5) is a 180 kDa heavily glycosylated membrane protein composed of three C2 Ig-like repeating units flanked by a N-terminal Ig V-like region and a C-terminal region, which includes glycophosphatidylinositol linkage region (Hegde, P, Qi, R, Gaspard, R, Abernathy, K, Dharap, S, Earle-Hughes, J, Gay, C, Nwokekeh, NU, Chen, T, Saeed, AI, Sharov, V, Lee, NH, Yeatman, TJ, and Quackenbush, J; Identification of tumour markers in models of human colorectal cancer using a 19,200-element complementary DNA microarray, Cancer Res., 2001, 61, 7792-7797).

As an oncofetal antigen, CEA is expressed during foetal development, but also, at low levels, in the gastrointestinal epithelium of adults. However, CEA is overexpressed in a high percentage of human tumours, including 90% of gastrointestinal, colorectal and pancreatic cancer, 70% of non-small cell lung cancer cells and 50% of breast cancers (Thompson, JA, Grunert, F, and Zimmermann, W; Carcinoembryonic antigen gene family: molecular biology and clinical perspectives, J Clin Lab Anal., 5, 344-366 2005). Due to its high expression by tumour cells and its secretion to the serum, CEA has been broadly used as a tumour marker (Sikorska, H, Shuster, J, and Gold, P; Clinical applications of carcinoembryonic antigen, Cancer Detect.Prev., 12, 321-355 1988) and is the standard serum marker for colorectal cancer monitoring (Locker, GY, Hamilton, S, Harris, J, Jessup, JM, Kemeny, N, Macdonald, JS, Somerfield, MR, Hayes, DF, and Bast, RC, Jr.; ASCO 2006 update of recommendations for the use of tumour markers in gastrointestinal cancer, J Clin Oncol, 24, 5313-5327, 2006).

Despite the overexpression of CEA in tumour cells, cancer patients do not normally show an immune response against this antigen (Orefice, S, Fossati, G, Pietrojusti, E, and Bonfanti, G; Delayed cutaneous hypersensitivity reaction to carcinoembryonic antigen in cancer patients, Tumouri, 1982, 68, 473-475,). The immune system commonly becomes tolerant to CEA, because it is normally expressed at low levels in the body. However, in a series of clinical vaccine trials, the immunogenicity of CEA has been demonstrated (Sarobe, P, Huarte, E, Lasarte, JJ, and Borras-Cuesta, F; Carcinoembryonic antigen as a target to induce anti-tumour immune responses, Curr. Cancer Drug Targets., 2004, 4, 443-454), especially in colorectal carcinoma (CRC) (Mosolits, S, Ullenhag, G, and Mellstedt, H; Therapeutic vaccination in patients with gastrointestinal malignancies. A review of immunological and clinical results, Ann.Oncol., 2005, 16, 847-862), and CEA is the tumour associated antigen (TAA) with the

greatest number of vaccine platforms tested in this tumour type (von Mehren, M; Colorectal cancer vaccines: what we know and what we don't yet know, Semin. Oncol., 2005, 32, 76-84).

Several cytotoxic and helper T-cell epitopes have been described for CEA (Crosti, M, Longhi, R, Consogno, G, Melloni, G, Zannini, P, and Protti, MP; Identification of novel subdominant epitopes on the carcinoembryonic antigen recognized by CD4+ T-cells of lung cancer patients, J Immunol., 2006, 176, 5093-5099; Novellino, L, Castelli, C, and Parmiani, G; A listing of human tumour antigens recognized by T-cells: March 2004 update, Cancer Immunol.Immunother., 2004, 54, 187-207; Ruiz, M, Kobayashi, H, Lasarte, JJ, Prieto, J, Borras-Cuesta, F, Celis, E, and Sarobe, P; Identification and characterization of a T-helper peptide from carcinoembryonic antigen, Clin Cancer Res., 2004, 10, 2860-2867), enabling a variety of peptide-based vaccination trials in CRC (Babatz, J, Rollig, C, Lobel, B, Folprecht, G, Haack, M, Gunther, H, Kohne, CH, Ehninger, G, Schmitz, M, and Bornhauser, M; Induction of cellular immune responses against carcinoembryonic antigen in patients with metastatic tumours after vaccination with altered peptide ligand-loaded dendritic cells, Cancer Immunol. Immunother., 2006, 55, 268-276; Fong, L, Hou, Y, Rivas, A, Benike, C, Yuen, A, Fisher, GA, Davis, MM, and Engleman, EG; Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumour immunotherapy, Proc. Natl. Acad. Sci. U.S.A, 2001, 98, 8809-8814; Liu, KJ, Wang, CC, Chen, LT, Cheng, AL, Lin, DT, Wu, YC, Yu, WL, Hung, YM, Yang, HY, Juang, SH, and Whang-Peng, J; Generation of carcinoembryonic antigen (CEA)-specific T-cell responses in HLA-A*0201 and HLA-A*2402 late-stage colorectal cancer patients after vaccination with dendritic cells loaded with CEA peptides, Clin Cancer Res., 2004, 10, 2645-2651; Matsuda, K, Tsunoda, T, Tanaka, H, Umano, Y, Tanimura, H, Nukaya, I, Takesako, K, and Yamaue, H; Enhancement of cytotoxic T-lymphocyte responses in patients with gastrointestinal malignancies following vaccination with CEA peptide-pulsed dendritic cells, Cancer Immunol. Immunother., 2004, 53, 609-616; Ueda, Y, Itoh, T, Nukaya, I, Kawashima, I, Okugawa, K, Yano, Y, Yamamoto, Y, Naitoh, K, Shimizu, K, Imura, K, Fuji, N, Fujiwara, H, Ochiai, T, Itoi, H, Sonoyama, T, Hagiwara, A, Takesako, K, and Yamagishi, H; Dendritic cell-based immunotherapy of cancer with carcinoembryonic antigen-derived, HLA-A24-restricted CTL epitope: Clinical outcomes of 18 patients with metastatic gastrointestinal or lung adenocarcinomas, Int. J Oncol., 2004, 24, 909-917; Weihrauch, MR, Ansen, S, Jurkiewicz, E, Geisen, C, Xia, Z, Anderson, KS, Gracien, E, Schmidt, M, Wittig, B, Diehl, V, Wolf, J, Bohlen, H, and Nadler, LM; Phase I/II combined chemoimmunotherapy with carcinoembryonic antigen-derived HLA-A2-restricted

CAP-1 peptide and irinotecan, 5-fluorouracil, and leucovorin in patients with primary metastatic colorectal cancer, Clin Cancer Res., 2005, 11, 5993-6001). These and other clinical trials to date have demonstrated safety of CEA vaccinations and evidence for the induction of immune response against this antigen (von Mehren, M; Colorectal cancer vaccines: what we know and what we don't yet know, Semin.Oncol., 2005, 32, 76-84).

A variant of CEA-006 was published previously (Ruiz, M, Kobayashi, H, Lasarte, JJ, Prieto, J, Borras-Cuesta, F, Celis, E, and Sarobe, P; Identification and characterization of a T-helper peptide from carcinoembryonic antigen, Clin Cancer Res., 2004, 10, 2860-2867). CEA-005 is a mutant with a single amino acid exchange and has been reported to be overcome the central immune tolerance (Zaremba, S, Barzaga, E, Zhu, M, Soares, N, Tsang, KY, and Schlom, J; Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen, Cancer Res., 1997, 57, 4570-4577).

Transforming Growth Factor, Beta-Induced (TGFBI)

TGFBI was first identified as a TGF-beta-inducible gene in a human lung adenocarcinoma cell line. It encodes for a secreted extracellular matrix protein, which is thought to act on cell attachment and extracellular matrix composition.

TGFBI was shown to be among the most significantly elevated genes in colorectal cancers and it is expressed at high levels in adenomas as well. Quantitative PCR results demonstrated strong elevation in both unpurified tumours and purified tumour epithelial cells. Accordingly, in situ hybridization experiments revealed TGFBI to be expressed in many cell types, in both the stromal and epithelial compartments (Buckhaults, P, Rago, C, St, CB, Romans, KE, Saha, S, Zhang, L, Vogelstein, B, and Kinzler, KW; Secreted and cell surface genes expressed in benign and malignant colorectal tumours, Cancer Res., 2001, 61, 6996-7001).

In a meta-analysis of studies investigating gene expression in colorectal carcinoma, TGFBI was identified as one of only nine genes described as upregulated repeatedly (4 studies for TGFBI) (Shih, W, Chetty, R, and Tsao, MS; Expression profiling by microarrays in colorectal cancer, Oncol.Rep., 2005, 13, 517-524).

In human pancreatic tissues, there was a 32.4-fold increase in TGFBI mRNA levels in pancreatic cancers in comparison to normal control tissues. In situ hybridization analysis

revealed that TGFBI mRNA was expressed mainly in the cancer cells within the pancreatic tumour mass (Schneider, D, Kleeff, J, Berberat, PO, Zhu, Z, Korc, M, Friess, H, and Buchler, MW; Induction and expression of betaig-h3 in pancreatic cancer cells, Biochim.Biophys.Acta, 2002, 1588, 1-6).

TGFBI was identified as a gene promoting angiogenesis in an in vitro model. Additionally, dramatically enhanced expression of TGFBI was detected in several tumours. Antisense oligonucleotides to TGFBI blocked both gene expression and endothelial tube formation in vitro, suggesting that TGFBI may play a critical role in endothelial cell-matrix interactions (Aitkenhead, M, Wang, SJ, Nakatsu, MN, Mestas, J, Heard, C, and Hughes, CC; Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: induction of ESM-1, (beta)ig-h3, and NrCAM, Microvasc. Res., 2002, 63, 159-171).

Mucin-1 (MUC1)

Mucins are high-molecular weight epithelial glycoproteins with a high content of clustered oligosaccharides O-glycosidically linked to tandem repeat peptides rich in threonine, serine, and proline. There are two structurally and functionally distinct classes of mucins: transmembrane mucins, to which MUC1 belongs, and secreted gel-forming mucins. Colon cancer mucins have differences in carbohydrate structures that are investigated as diagnostic and prognostic markers, and also as targets for cancer vaccines.

The extracellular domain of the MUC1 protein is made up of highly conserved repeats of 20 amino acids, the actual number varying between 25 and 100 depending on the allele. Each tandem repeat contains five potential glycosylation sites, and between doublets of threonines and serines lies an immunodominant region containing epitopes recognized by various anti-MUC1 antibodies (Taylor-Papadimitriou, J, Burchell, J, Miles, DW, and Dalziel, M; MUC1 and cancer, Biochim. Biophys. Acta, 1999, 1455, 301-313).

Compared to most other epithelia, the MUC1 of colon is more heavily glycosylated thereby masking the MUC1 protein for immunohistochemical staining by MUC1-specific antibodies. In colorectal adenocarcinomas, MUC1 is less glycosylated, allowing immunodetection. The aberrantly glycosylated MUC1 confers new binding properties and can simultaneously mediate and block binding to adhesion molecules with some molecular specificity, thereby playing a dual role in the metastatic spread of tumor cells (McDermott, KM, Crocker, PR,

Harris, A, Burdick, MD, Hinoda, Y, Hayashi, T, Imai, K, and Hollingsworth, MA; Overexpression of MUC1 reconfigures the binding properties of tumor cells, Int. J Cancer, 2001, 94, 783-791).

MUC1 as detected immunologically is increased in expression in colon cancers, which correlates with a worse prognosis (Byrd, JC and Bresalier, RS; Mucins and mucin binding proteins in colorectal cancer, Cancer Metastasis Rev., 2004, 23, 77-99), indicating that upregulation of MUC1 may be involved in the progression of CRC. Colon cancers with metastasis express MUC1 more strongly than those without metastasis (Nakamori, S, Ota, DM, Cleary, KR, Shirotani, K, and Irimura, T; MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma, Gastroenterology, 1994, 106, 353-361), and MUC1 staining was positive in all colorectal cancers with hepatic involvement in one study (Matsuda, K, Masaki, T, Watanabe, T, Kitayama, J, Nagawa, H, Muto, T, and Ajioka, Y; Clinical significance of MUC1 and MUC2 mucin and p53 protein expression in colorectal carcinoma, Jpn. J Clin Oncol., 2000, 30, 89-94). A recent study in 462 colorectal cancer patients found MUC1 expression to be an independent prognostic marker of poor prognosis (Duncan, TJ, Watson, NF, Al-Attar, AH, Scholefield, JH, and Durrant, LG; The role of MUC1 and MUC3 in the biology and prognosis of colorectal cancer, World J Surg. Oncol, 2007, 5, 31).

There is a pathophysiological significance of circulating anti-MUC1 antibodies in CRC: anti-MUC1 antibodies were detected in 5 of 31 (16.1%) healthy subjects and in 27 of 56 (48.2%) patients with colorectal cancer (Nakamura, H, Hinoda, Y, Nakagawa, N, Makiguchi, Y, Itoh, F, Endo, T, and Imai, K; Detection of circulating anti-MUC1 mucin core protein antibodies in patients with colorectal cancer, J Gastroenterol., 1998, 33, 354-361).

Apart from its role as an antibody target, MUC1 is also a well-established target for cytotoxic T cells. Several reports demonstrated that cytotoxic MHC-unrestricted T cells from ovarian, breast, pancreatic, and multiple myeloma tumors can recognize epitopes of the MUC1 protein core localised in the tandem repeat (Apostolopoulos, V and McKenzie, IF; Cellular mucins: targets for immunotherapy, Crit Rev. Immunol., 1994, 14, 293-309; Finn, OJ, Jerome, KR, Henderson, RA, Pecher, G, Domenech, N, Magarian-Blander, J, and Barratt-Boyes, SM; MUC-1 epithelial tumor mucin-based immunity and cancer vaccines, Immunol. Rev., 1995, 145, 61-89; Barnd, DL, Lan, MS, Metzgar, RS, and Finn, OJ; Specific, major

histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells, Proc. Natl. Acad. Sci. U.S.A, 1989, 86, 7159-7163; Takahashi, T, Makiguchi, Y, Hinoda, Y, Kakiuchi, H, Nakagawa, N, Imai, K, and Yachi, A; Expression of MUC1 on myeloma cells and induction of HLA-unrestricted CTL against MUC1 from a multiple myeloma patient, J. Immunol., 1994, 153, 2102-2109; Noto, H, Takahashi, T, Makiguchi, Y, Hayashi, T, Hinoda, Y, and Imai, K; Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin, Int. Immunol., 1997, 9, 791-798). However, HLA-A*02 restricted T cell epitopes derived from the MUC1 protein have also been identified (Apostolopoulos, V, Karanikas, V, Haurum, JS, and McKenzie, IF; Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen, J Immunol., 1997, 159, 5211-5218; Brossart, P, Heinrich, KS, Stuhler, G, Behnke, L, Reichardt, VL, Stevanovic, S, Muhm, A, Rammensee, HG, Kanz, L, and Brugger, W; Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies, Blood, 1999, 93, 4309-4317). One of those peptides is MUC-001. It is derived from the tandem repeat region of the MUC1 protein. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells in patients with advanced breast or ovarian cancer using these MUC1 peptides has been successful (Brossart, P, Wirths, S, Stuhler, G, Reichardt, VL, Kanz, L, and Brugger, W; Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells, Blood, 2000, 96, 3102-3108; Wierecky, J, Mueller, M, and Brossart, P; Dendritic cell-based cancer immunotherapy targeting MUC-1, Cancer Immunol. Immunother., 2005 Apr. 28: 288-94). Moreover, such vaccinations have successfully induced clinical responses in renal cell carcinoma patients (Wierecky, J, Muller, MR, Wirths, S, Halder-Oehler, E, Dorfel, D, Schmidt, SM, Hantschel, M, Brugger, W, Schroder, S, Horger, MS, Kanz, L, and Brossart, P; Immunologic and clinical responses after vaccinations with peptide-pulsed dendritic cells in metastatic renal cancer patients, Cancer Res., 2006, 66, 5910-5918).

Upregulation of immunoreactive MUC1 in colorectal cancer is mostly not based on mRNA overexpression but rather caused by its decreased glycosylation which unmasks epitopes for antibody recognition, especially in the tandem repeat region of MUC1. This deglycosylation provides at the same time an opportunity for the generation of T cell epitopes by altered antigen processing in tumor cells, which is prevented in normal cells by glycosylation. This mechanism may explain the striking features of MUC-001 as a tumor associated T cell

epitope despite the absence of strong mRNA overexpression. Some evidence that glycosylation changes may indeed affect antigen processing comes from a recent observation that altered glycosylation of MUC1 in colorectal cancer can be actually detected by antigen presenting cells via a receptor specifically recognizing tumor glycoforms (Saeland, E, van Vliet, SJ, Backstrom, M, van, dB, V, Geijtenbeek, TB, Meijer, GA, and van, KY; 2006, The C-type lectin MGL expressed by dendritic cells detects glycan changes on MUC1 in colon carcinoma, Cancer Immunol.Immunother., 2007, 56(8): 1225-36). A specific uptake and processing of tumor glycoforms by antigen presenting cells may also explain the fact that MUC-001 specific T cells have been observed naturally (without immunisation) in breast (Rentzsch, C, Kayser, S, Stumm, S, Watermann, I, Walter, S, Stevanovic, S, Wallwiener, D, and Guckel, B; Evaluation of pre-existent immunity in patients with primary breast cancer: molecular and cellular assays to quantify antigen-specific T lymphocytes in peripheral blood mononuclear cells, Clin Cancer Res., 2003, 9, 4376-4386) and colorectal cancer patients (Dittmann, J, Keller-Matschke, K, Weinschenk, T, Kratt, T, Heck, T, Becker, HD, Stevanovic, S, Rammensee, HG, and Gouttefangeas, C; CD8+ T-cell response against MUC1-derived peptides in gastrointestinal cancer survivors, Cancer Immunol. Immunother., 2005, 54, 750-758). In these patients no autoimmune effects were reported. This demonstrates the natural role of MUC-001 as a tumor associated peptide inducing specific T cells and suggests that the administration of MUC-001 can be considered safe although no overexpression can be detected for the MUC1 antigen at the mRNA level.

Met proto-oncogene (hepatocyte growth factor receptor) (c-Met)

The MET proto-oncogene protein product is the hepatocyte growth factor receptor. It contains a tyrosine kinase domain that activates signaling pathways involved in cell proliferation, motility, adhesion, and invasion (Trusolino, L and Comoglio, PM; Scatter-factor and semaphorin receptors: cell signalling for invasive growth, Nat. Rev. Cancer, 2002, 2, 289-300).

Studies in various tumor types have demonstrated several mechanisms for c-Met activation, including HGF/c-Met autocrine loop, activating point mutations, TPR-Met fusion protein, and failure to cleave c-Met into the α and β chains (Di Renzo, MF, Olivero, M, Martone, T, Maffe, A, Maggiora, P, Stefani, AD, Valente, G, Giordano, S, Cortesina, G, and Comoglio, PM; Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas, Oncogene, 2000, 19, 1547-1555; Ebert, M, Yokoyama, M, Friess, H,

Buchler, MW, and Korc, M; Coexpression of the c-met proto-oncogene and hepatocyte growth factor in human pancreatic cancer, Cancer Res., 1994, 54, 5775-5778; Mondino, A, Giordano, S, and Comoglio, PM; Defective posttranslational processing activates the tyrosine kinase encoded by the MET proto-oncogene (hepatocyte growth factor receptor), Mol. Cell Biol., 1991, 11, 6084-6092; Olivero, M, Valente, G, Bardelli, A, Longati, P, Ferrero, N, Cracco, C, Terrone, C, Rocca-Rossetti, S, Comoglio, PM, and Di Renzo, MF; Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family, Int. J Cancer, 1999, 82, 640-643; Park, M, Dean, M, Cooper, CS, Schmidt, M, O'Brien, SJ, Blair, DG, and Vande Woude, GF; Mechanism of met oncogene activation, Cell, 1986, 45, 895-904; Park, WS, Dong, SM, Kim, SY, Na, EY, Shin, MS, Pi, JH, Kim, BJ, Bae, JH, Hong, YK, Lee, KS, Lee, SH, Yoo, NJ, Jang, JJ, Pack, S, Zhuang, Z, Schmidt, L, Zbar, B, and Lee, JY; 1999, Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas, Cancer Res., 59, 307-310; Rahimi, N, Tremblay, E, McAdam, L, Park, M, Schwall, R, and Elliott, B; 1996, Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma, Cell Growth Differ., 7, 263-270;Schmidt, L, Duh, FM, Chen, F, Kishida, T, Glenn, G, Choyke, P, Scherer, SW, Zhuang, Z, Lubensky, I, Dean, M, Allikmets, R, Chidambaram, A, Bergerheim, UR, Feltis, JT, Casadevall, C, Zamarron, A, Bernues, M, Richard, S, Lips, CJ, Walther, MM, Tsui, LC, Geil, L, Orcutt, ML, Stackhouse, T, Lipan, J, Slife, L, Brauch, H, Decker, J, Niehans, G, Hughson, MD, Moch, H, Storkel, S, Lerman, MI, Linehan, WM, and Zbar, B; 1997, Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas, Nat.Genet., 16, 68-73; Schmidt, L, Junker, K, Weirich, G, Glenn, G, Choyke, P, Lubensky, I, Zhuang, Z, Jeffers, M, Vande, WG, Neumann, H, Walther, M, Linehan, WM, and Zbar, B; 1998, Two North American families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene, Cancer Res., 58, 1719-1722). Mechanistically, c-Met overexpression cooperates with oncogenic Ki-ras mutation to enhance tumorigenicity of colon cancer cells in vivo (Long, IS, Han, K, Li, M, Shirasawa, S, Sasazuki, T, Johnston, M, and Tsao, MS; Met receptor overexpression and oncogenic Ki-ras mutation cooperate to enhance tumorigenicity of colon cancer cells in vivo, Mol.Cancer Res., 2003, 1, 393-401).

Interestingly, there is some evidence for interactions of MET signalling with the Wnt/beta-catenin pathway frequently upregulated in colon cancer. MET can be activated by Prostaglandin E2 (PGE2) and PGE2-activated c-Met associates with β-catenin and increases

its tyrosine phosphorylation thereby inducing colon cancer cell invasiveness (Pai, R, Nakamura, T, Moon, WS, and Tarnawski, AS; Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors, FASEB J, 2003, 17, 1640-1647). Recently, mutual activation of MET and beta-catenin has been described, resulting in a positive feedback loop between these two key players in colorectal tumorigenesis (Rasola, A, Fassetta, M, De, BF, D'Alessandro, L, Gramaglia, D, Di Renzo, MF, and Comoglio, PM; A positive feedback loop between hepatocyte growth factor receptor and beta-catenin sustains colorectal cancer cell invasive growth, Oncogene, 2007, 26, 1078-1087).

The c-Met mRNA expression level in primary CRC tumors (n = 36) is an important predicitive marker for early-stage invasion and regional disease metastasis, thus correlating directly with colon cancer stage (Takeuchi, H, Bilchik, A, Saha, S, Turner, R, Wiese, D, Tanaka, M, Kuo, C, Wang, HJ, and Hoon, DS; c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases, Clin Cancer Res., 2003, 9, 1480-1488). Another analysis of c-Met expression of 130 CRC samples showed overexpression (T/N > 2.0) of c-Met in 69% primary CRC and significantly higher c-Met levels in CRC with blood vessel invasion (P = 0.04), and in advanced stage (P = 0.04) supporting the role for c-Met in human CRC progression and metastasis (Zeng, Z, Weiser, MR, D'Alessio, M, Grace, A, Shia, J, and Paty, PB; Immunoblot analysis of c-Met expression in human colorectal cancer: overexpression is associated with advanced stage cancer, Clin Exp. Metastasis, 2004, 21, 409-417). In another study 69% and 48% of 60 colon adenocarcinomas showed a greater than 2- and greater than 10-fold elevation in c-Met mRNA, respectively, compared to adjacent normal mucosa (Kammula, US, Kuntz, EJ, Francone, TD, Zeng, Z, Shia, J, Landmann, RG, Paty, PB, and Weiser, MR; Molecular co-expression of the c-Met oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome, Cancer Lett., 2007, 248, 219-228). Thus, increased c-Met signalling is a common occurrence in early stage CRC, but with even greater expression occurring in advanced and metastatic disease.

Cyclin D1 (CCND1)

CCND1 belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation

patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity are required for cell cycle G1/S transition. Mutations, amplification and overexpression of this gene, which alters cell cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis (Fu, M, Wang, C, LI, Z, Sakamaki, T, and Pestell, RG; Minireview: Cyclin D1: normal and abnormal functions, Endocrinology, 2004, 145, 5439-5447).

A common A/G single nucleotide polymorphism (A870G) results in two distinct mRNA isoforms a and b. The alternately spliced isoform b encodes a truncated protein which has been linked to higher incidence of tumor onset including lung cancer, colon cancer, and other cancer types (Fu, M, Wang, C, LI, Z, Sakamaki, T, and Pestell, RG; Minireview: Cyclin D1: normal and abnormal functions, Endocrinology, 2004, 145, 5439-5447).

For colorectal cancer, overexpression of CCND1 at the mRNA and protein levels has been frequently described (Sutter, T, Doi, S, Carnevale, KA, Arber, N, and Weinstein, IB; Expression of cyclins D1 and E in human colon adenocarcinomas, J Med, 1997, 28, 285-309; Mermelshtein, A, Gerson, A, Walfisch, S, Delgado, B, Shechter-Maor, G, Delgado, J, Fich, A, and Gheber, L; Expression of D-type cyclins in colon cancer and in cell lines from colon carcinomas, Br. J Cancer, 2005, 93, 338-345; Balcerczak, E, Pasz-Walczak, G, Kumor, P, Panczyk, M, Kordek, R, Wierzbicki, R, and Mirowski, M; Cyclin D1 protein and CCND1 gene expression in colorectal cancer, Eur. J Surg.Oncol., 2005, 31, 721-726; Bondi, J, Husdal, A, Bukholm, G, Nesland, JM, Bakka, A, and Bukholm, IR; Expression and gene amplification of primary (A, B1, D1, D3, and E) and secondary (C and H) cyclins in colon adenocarcinomas and correlation with patient outcome, J Clin Pathol., 2005, 58, 509-514; Perez, R, Wu, N, Klipfel, AA, and Beart, RW, Jr.; A better cell cycle target for gene therapy of colorectal cancer: cyclin G, J Gastrointest. Surg., 2003, 7, 884-889; Wong, NA, Morris, RG, McCondochie, A, Bader, S, Jodrell, DI, and Harrison, DJ; Cyclin D1 overexpression in colorectal carcinoma in vivo is dependent on beta-catenin protein dysregulation, but not k-ras mutation, J Pathol., 2002, 197, 128-135; McKay, JA, Douglas, JJ, Ross, VG, Curran, S, Murray, GI, Cassidy, J, and McLeod, HL; Cyclin D1 protein expression and gene polymorphism in colorectal cancer. Aberdeen Colorectal Initiative, Int.J Cancer, 2000, 88, 77-81; Bartkova, J, Lukas, J, Strauss, M, and Bartek, J; The PRAD-1/cyclin D1 oncogene

product accumulates aberrantly in a subset of colorectal carcinomas, Int. J Cancer, 1994, 58, 568-573).

This can be explained by the well-established fact that CCND1 is a target gene of the β-Catenin-TCF/LEF pathway which is frequently upregulated in colorectal carcinoma (Shtutman, M, Zhurinsky, J, Simcha, I, Albanese, C, D'Amico, M, Pestell, R, and Ben-Ze'ev, A; The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway, Proc. Natl. Acad. Sci. U.S.A, 1999, 96, 5522-5527; Tetsu, O and McCormick, F; Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells, Nature, 1999, 398, 422-426).

Enhanced CCND1 expression has been linked to higher tumor grades, metastasis, and decreased survival (Balcerczak, E, Pasz-Walczak, G, Kumor, P, Panczyk, M, Kordek, R, Wierzbicki, R, and Mirowski, M; Cyclin D1 protein and CCND1 gene expression in colorectal cancer, Eur. J Surg. Oncol., 2005, 31, 721-726; Bahnassy, AA, Zekri, AR, El-Houssini, S, El-Shehaby, AM, Mahmoud, MR, Abdallah, S, and El-Serafi, M; Cyclin A and cyclin D1 as significant prognostic markers in colorectal cancer patients, BMC. Gastroenterol., 2004, 4, 22; McKay, JA, Douglas, JJ, Ross, VG, Curran, S, Murray, GI, Cassidy, J, and McLeod, HL; Cyclin D1 protein expression and gene polymorphism in colorectal cancer. Aberdeen Colorectal Initiative, Int. J Cancer, 2000, 88, 77-81; Maeda, K, Chung, Y, Kang, S, Ogawa, M, Onoda, N, Nishiguchi, Y, Ikehara, T, Nakata, B, Okuno, M, and Sowa, M; Cyclin D1 overexpression and prognosis in colorectal adenocarcinoma, Oncology, 1998, 55, 145-151).

Matrix metallopeptidase 7 (matrilysin, uterine) (MMP7)

Matrix metalloproteinases (MMPs) are a large family of structurally related zinc-dependent proteinases typically described as capable of degrading components of the extracellular matrix. Individual MMPs have been identified that show increased expression in tumors and most tumors show enhanced MMP activity (Curran, S and Murray, GI; 1999, Matrix metalloproteinases in tumour invasion and metastasis, J Pathol., 189, 300-308; Curran, S and Murray, GI; 2000, Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis, Eur.J Cancer, 36, 1621-1630).

Basal membrane and extracellular matrix represent two physical barriers to malignant invasion, and their degradation by MMPs plays a key role in tumor progression and metastatic

spread (Johnsen, M, Lund, LR, Romer, J, Almholt, K, and Dano, K; 1998, Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation, Curr.Opin.Cell Biol., 10, 667-671; Nelson, AR, Fingleton, B, Rothenberg, ML, and Matrisian, LM; 2000, Matrix metalloproteinases: biologic activity and clinical implications, J Clin Oncol., 18, 1135-1149; Wang, FQ, So, J, Reierstad, S, and Fishman, DA; 2005, Matrilysin (MMP-7) promotes invasion of ovarian cancer cells by activation of progelatinase, Int.J Cancer, 114, 19-31). Apart from this function, MMPs are now discussed for their involvement in tumour development and progression including roles in apoptosis, cell proliferation, and cell differentiation. These functions are linked to MMP-mediated proteolysis of non-matrix proteins and actions distinct from their enzyme activity (Egeblad, M and Werb, Z; 2002, New functions for the matrix metalloproteinases in cancer progression, Nat.Rev.Cancer, 2, 161-174; Leeman, MF, Curran, S, and Murray, GI; 2003, New insights into the roles of matrix metalloproteinases in colorectal cancer development and progression, J.Pathol., 201, 528-534).

Recent studies have shown that several matrix metalloproteinases, especially matrilysin (MMP7), interact with the specific molecular genetic and signalling pathways involved in colorectal cancer development. In particular, matrilysin is activated at an early stage of colorectal tumourigenesis by the beta-catenin signalling pathway (Brabletz, T, Jung, A, Dag, S, Hlubek, F, and Kirchner, T; 1999, beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer, Am.J Pathol., 155, 1033-1038; Leeman, MF, Curran, S, and Murray, GI; 2003, New insights into the roles of matrix metalloproteinases in colorectal cancer development and progression, J.Pathol., 201, 528-534; Zucker, S and Vacirca, J; 2004, Role of matrix metalloproteinases (MMPs) in colorectal cancer, Cancer Metastasis Rev., 23, 101-117).

MMP7 is overexpressed both in benign and malignant colorectal tumors (Ishikawa, T, Ichikawa, Y, Mitsuhashi, M, Momiyama, N, Chishima, T, Tanaka, K, Yamaoka, H, Miyazakic, K, Nagashima, Y, Akitaya, T, and Shimada, H; 1996, Matrilysin is associated with progression of colorectal tumor, Cancer Lett., 107, 5-10; McDonnell, S, Navre, M, Coffey, RJ, Jr., and Matrisian, LM; 1991, Expression and localization of the matrix metalloproteinase pump-1 (MMP-7) in human gastric and colon carcinomas, Mol.Carcinog., 4, 527-533; Miyazaki, K, Hattori, Y, Umenishi, F, Yasumitsu, H, and Umeda, M; 1990, Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin

(pump-1), secreted from human rectal carcinoma cell line, Cancer Res., 50, 7758-7764; Nagashima, Y, Hasegawa, S, Koshikawa, N, Taki, A, Ichikawa, Y, Kitamura, H, Misugi, K, Kihira, Y, Matuo, Y, Yasumitsu, H, and Miyazaki, K; 1997, Expression of matrilysin in vascular endothelial cells adjacent to matrilysin-producing tumors, Int.J Cancer, 72, 441-445; Newell, KJ, Witty, JP, Rodgers, WH, and Matrisian, LM; 1994, Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis, Mol.Carcinog., 10, 199-206; Yoshimoto, M, Itoh, F, Yamamoto, H, Hinoda, Y, Imai, K, and Yachi, A; 1993, Expression of MMP-7(PUMP-1) mRNA in human colorectal cancers, Int.J Cancer, 54, 614-618). MMP7 is one of only a few MMPs that is actually secreted by tumour cells (Overall, CM and Kleifeld, O; 2006, Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy, Nat.Rev.Cancer, 6, 227-239). Furthermore, the levels of MMP7 mRNA expression correlate with the stage of CRC progression (Ishikawa, T, Ichikawa, Y, Mitsuhashi, M, Momiyama, N, Chishima, T, Tanaka, K, Yamaoka, H, Miyazakic, K, Nagashima, Y, Akitaya, T, and Shimada, H; 1996, Matrilysin is associated with progression of colorectal tumor, Cancer Lett., 107, 5-10; Mori, M, Barnard, GF, Mimori, K, Ueo, H, Akiyoshi, T, and Sugimachi, K; 1995, Overexpression of matrix metalloproteinase-7 mRNA in human colon carcinomas, Cancer, 75, 1516-1519). In CRC metastases, MMP7 plays also a critical role (Adachi, Y, Yamamoto, H, Itoh, F, Hinoda, Y, Okada, Y, and Imai, K; 1999, Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers, Gut, 45, 252-258; Mori, M, Barnard, GF, Mimori, K, Ueo, H, Akiyoshi, T, and Sugimachi, K; 1995, Overexpression of matrix metalloproteinase-7 mRNA in human colon carcinomas, Cancer, 75, 1516-1519).

Elevated MMP7 serum levels are associated with a poor prognosis advanced colorectal cancer patients (Maurel, J, Nadal, C, Garcia-Albeniz, X, Gallego, R, Carcereny, E, Almendro, V, Marmol, M, Gallardo, E, Maria, AJ, Longaron, R, Martinez-Fernandez, A, Molina, R, Castells, A, and Gascon, P; 2007, Serum matrix metalloproteinase 7 levels identifies poor prognosis advanced colorectal cancer patients, Int.J Cancer, Published Online: 8 May 2007) and overexpression in CRC patients, associated again with decreased survival, has been suggested to promote escape from immune surveillance by cleaving Fas on tumor cells (Wang, WS, Chen, PM, Wang, HS, Liang, WY, and Su, Y; 2006, Matrix metalloproteinase-7 increases resistance to Fas-mediated apoptosis and is a poor prognostic factor of patients with colorectal carcinoma, Carcinogenesis, 27, 1113-1120).

The proteins of the invention can be the target of a tumour specific immune response in multiple types of cancer.

The Hepatitis B Virus Core Antigen peptide HBV-001 is not derived from an endogenous human tumour-associated antigen, but is derived from the Hepatitis B virus core antigen. Firstly, it allows quantitative comparisons of the magnitude of T-cell responses induced by TUMAPs and hence allows important conclusions on the capacity to elicit anti-tumour responses. Secondly, it functions as an important positive control in the case of lack of any T-cell responses in the patient. And thirdly, it also allows conclusions on the status of immunocompetence of the patient.

Hepatitiv B virus (HBV) infection is among the leading causes of liver disease, affecting approximately 350 million people world-wide (Rehermann, B and Nascimbeni, M; Immunology of hepatitis B virus and hepatitis C virus infection, Nat.Rev.Immunol., 2005, 5, 215-229). Due to the ease of horizontal and vertical transmission and the potential for chronic disease that may lead to liver cirrhosis and hepatocellular carcinoma, HBV represents a major impact on the public health system for many countries worldwide. The HBV genome (Previsani, N and Lavanchy, D; 2002, Hepatitis B, (Epidemic and Pandemic Alert and Response, World Health Organization, Geneva, 2002)) is comprised of partially double-stranded circular DNA. In HBV virions, it is packed together with the core protein HBc and other proteins to form the nucleocapsid, which is surrounded by an outer envelope containing lipids and the surface protein family HBs (also called envelope protein). The antigenic determinants which are associated with HBc and HBs are noted as HBcAg and HBsAg, respectively. These antigens are associated with serological, i.e. antibody responses found in the patient blood and are among the clinically most useful antigen-antibody systems for the diagnosis of HBV infection. HBc will represent a novel foreign antigen for all individuals without prior history of HBV infection. As immunogenic peptides are well known for this antigen (Bertoletti, A, Chisari, FV, Penna, A, Guilhot, S, Galati, L, Missale, G, Fowler, P, Schlicht, HJ, Vitiello, A, Chesnut, RC, and .; 1993, Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein, J.Virol., 67, 2376-2380;Livingston, BD, Crimi, C, Grey, H, Ishioka, G, Chisari, FV, Fikes, J, Grey, H, Chesnut, RW, and Sette, A; 1997, The hepatitis B virus-specific CTL responses induced in humans by lipopeptide vaccination are comparable to those elicited by acute viral infection, J.Immunol., 159, 1383-1392), one ten-amino acid peptide from HBcAg was selected as a positive control

antigen within IMA. The induction of HBc peptide-specific CTLs will then be used as a marker for patient immunocompetence and successful vaccination.

The pharmaceutical composition can furthermore contain additional peptides and/or excipients to be more effective, as will be further explained below.

By a "variant amino acid sequence" of the given amino acid sequence the inventors mean that the side chains of, for example, one or two of the amino acid residues are altered (for example by replacing them with the side chain of another naturally occurring amino acid residue or some other side chain) such that the peptide is still able to bind to an HLA molecule in substantially the same way as a peptide consisting of the given amino acid sequence. For example, a peptide may be modified so that it at least maintains, if not improves, the ability to interact with and bind a suitable MHC molecule, such as HLA-A or -DR, and so that it at least maintains, if not improves, the ability to generate activated CTL which can recognise and kill cells which express a polypeptide containing an amino acid sequence as defined in the aspects of the invention. As can derived from the database, certain positions of HLA-A binding peptides are typically anchor residues forming a core sequence fitting to the binding motif of the HLA binding groove.

Those amino acid residues that are not essential to interact with the T-cell receptor can be modified by replacement with another amino acid whose incorporation does not substantially affect T-cell reactivity and does not eliminate binding to the relevant MHC. Thus, apart from the proviso given, the peptide of the invention may be any peptide (by which term the inventors include oligopeptide or polypeptide) which includes the amino acid sequences or a portion or variant thereof as given.

It is furthermore known for MHC-class II presented peptides that these peptides are composed of a "core sequence" having a certain HLA-specific amino acid motif and, optionally, N- and/or C-terminal extensions which do not interfere with the function of the core sequence (i.e. are deemed as irrelevant for the interaction of the peptide and the T-cell). The N- and/or C-terminal extensions can, for example, be between 1 to 10 amino acids in length, respectively. These peptide can be used either directly to load MHC class II molecules or the sequence can be cloned into the vectors according to the description herein below. As these peptides form the final product of the processing of larger peptides within the cell, longer

peptides can be used as well. The peptides of the invention may be of any size, but typically they may be less than 100,000 in molecular weight, preferably less than 50,000, more preferably less than 10,000 and typically about 5,000. In terms of the number of amino acid residues, the peptides of the invention may have fewer than 1000 residues, preferably fewer than 500 residues, more preferably fewer than 100 residues. Accordingly the present invention provides also compositions of peptides and variants thereof wherein the peptide or variant has an overall length of between 8 and 100, preferably between 8 and 30, and most preferred between 8 and 16, namely 8, 9, 10, 11, 12, 13, 14, 15 or 16 amino acids.

Correspondingly, variants that induce T-cells cross-reacting with a peptide of the invention are often length variants.

If a peptide is longer than around 12 amino acid residues is used directly to bind to a MHC class II molecule, it is preferred that the residues that flank the core HLA binding region do not substantially affect the ability of the peptide to bind specifically to the binding groove of the MHC class II molecule or to present the peptide to the CTL. However, as already indicated above, it will be appreciated that larger peptides may be used, especially when encoded by a polynucleotide, since these larger peptides may be fragmented by suitable antigen-presenting cells.

It is also possible, that MHC class I epitopes, although usually between 8-10 amino acids long, are generated by peptide processing from longer peptides or proteins that include the actual epitope. Similar to MHC class II epitopes, it is preferred that the residues that flank the binding region do not substantially affect the ability of the peptide to bind specifically to the binding groove of the MHC class I molecule or to present the peptide to the CTL nor mask the sites for proteolytic cleavage necessary to expose the actual epitope during processing.

Accordingly the present invention also provides peptides and variants of MHC class I epitopes having an overall length of between 8 and 100, preferably between 8 and 30, and most preferred between 8 and 16, namely 8, 9, 10, 11, 12, 13, 14, 15 or 16 amino acids.

Of course, the peptide or variant according to the present invention will have the ability to bind to a molecule of the human major histocompatibility complex (MHC) class I or II. Binding of a peptide or a variant to a MHC complex may be tested by methods known in the

art, for example those described in the examples of the present invention below or those described in the literature for different MHC class II alleles (e.g. Vogt AB, Kropshofer H, Kalbacher H, Kalbus M, Rammensee HG, Coligan JE, Martin R; Ligand motifs of HLA-DRB5*0101 and DRB1*1501 molecules delineated from self-peptides; J Immunol. 1994; 153(4):1665-1673; Malcherek G, Gnau V, Stevanovic S, Rammensee HG, Jung G, Melms A; Analysis of allele-specific contact sites of natural HLA-DR17 ligands; J Immunol. 1994; 153(3):1141-1149; Manici S, Sturniolo T, Imro MA, Hammer J, Sinigaglia F, Noppen C, Spagnoli G, Mazzi B, Bellone M, Dellabona P, Protti MP; Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11; J Exp Med. 1999; 189(5): 871-876; Hammer J, Gallazzi F, Bono E, Karr RW, Guenot J, Valsasnini P, Nagy ZA, Sinigaglia F; Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association; .J Exp Med. 1995 181(5):1847-1855; Tompkins SM, Rota PA, Moore JC, Jensen PE; A europium fluoroimmunoassay for measuring binding of antigen to class II MHC glycoproteins; J Immunol Methods. 1993;163(2): 209-216; Boyton RJ, Lohmann T, Londei M, Kalbacher H, Halder T, Frater AJ, Douek DC, Leslie DG, Flavell RA, Altmann DM; Glutamic acid decarboxylase T lymphocyte responses associated with susceptibility or resistance to type I diabetes: analysis in disease discordant human twins, non-obese diabetic mice and HLA-DQ transgenic mice; Int Immunol. 1998 (12):1765-1776).

Additional N- and/or C-terminally located stretches of amino acids that are not necessarily forming part of the peptide that functions as an epitope for MHC molecules but may, nevertheless, be important to provide for an efficient introduction of the peptide according to the present invention into the cells. In one embodiment of the present invention, the peptide of the present invention is a fusion protein which comprises, for example, the 80 N-terminal amino acids of the HLA-DR antigen-associated invariant chain (p33, in the following "Ii") as derived from the NCBI, GenBank Accession-number X00497 (Strubin, M., Mach, B. and Long, E.O. The complete sequence of the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity EMBO J. 3 (4), 869-872 (1984)).

Preferred are pharmaceutical composition, wherein the peptides have an overall length of between 8 and 100, preferably between 8 and 30, and most preferred between 8 and 16 amino acids.

In addition the peptide or variant may be modified further to improve stability and/or binding to MHC molecules to elicit a stronger immune response. Methods for such an optimisation of a peptide sequence are well known in the art and include, for example, the introduction of reverse peptide bonds or non-peptide bonds.

Thus, according to another aspect the invention provides a pharmaceutical composition, wherein the at least one peptide or variant includes non-peptide bonds.

In a reverse peptide bond amino acid residues are not joined by peptide (-CO-NH-) linkages but the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere et al (1997) J. Immunol. 159, 3230-3237, incorporated herein by reference. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Meziere et al (1997) show that for MHC and T helper cell responses, these pseudopeptides are useful. Retro-inverse peptides, containing NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

A non-peptide bond is, for example, $-CH_2-NH$, $-CH_2S-$, $-CH_2CH_2-$, $-CH=CH-$, $-COCH_2-$, $-CH(OH)CH_2-$, and $-CH_2SO-$. United States Patent 4,897,445 provides a method for the solid phase synthesis of non-peptide bonds ($-CH_2-NH$) in polypeptide chains that involves polypeptides synthesised by standard procedures and the non-peptide bond synthesised by reacting an amino aldehyde and an amino acid in the presence of $NaCNBH_3$.

Peptides comprising the sequences of the invention described above may be synthesized with additional chemical groups present at their amino and/or carboxy termini, to enhance, for example, the stability, bioavailability, and/or affinity of the peptides. For example, hydrophobic groups such as carbobenzoxyl, dansyl, or t-butyloxycarbonyl groups may be added to the peptides' amino termini. Likewise, an acetyl group or a 9-fluorenylmethoxy-carbonyl group may be placed at the peptides' amino termini. Additionally, e.g. the hydrophobic group, t-butyloxycarbonyl, or an amido group may be added to the peptides' carboxy termini.

Further, all peptides of the invention may be synthesised to alter their steric configuration. For example, the D-isomer of one or more of the amino acid residues of the peptide may be used, rather than the usual L-isomer. Still further, at least one of the amino acid residues of the peptides of the invention may be substituted by one of the well known non-naturally occurring amino acid residues. Alterations such as these may serve to increase the stability, bioavailability and/or binding action of the peptides of the invention.

Similarly, a peptide or variant of the invention may be modified chemically by reacting specific amino acids either before or after synthesis of the peptide. Examples for such modifications are well known in the art and are summarised e.g. in R. Lundblad, Chemical Reagents for Protein Modification, 3rd ed. CRC Press, 2005, which is incorporated herein by reference. Chemical modification of amino acids includes but is not limited to, modification by acylation, amidination, pyridoxylation of lysine, reductive alkylation, trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS), amide modification of carboxyl groups and sulphydryl modification by performic acid oxidation of cysteine to cysteic acid, formation of mercurial derivatives, formation of mixed disulphides with other thiol compounds, reaction with maleimide, carboxymethylation with iodoacetic acid or iodoacetamide and carbamoylation with cyanate at alkaline pH, although without limitation thereto. In this regard, the skilled person is referred to Chapter 15 of Current Protocols In Protein Science, Eds. Coligan et al. (John Wiley & Sons NY 1995-2000) for more extensive methodology relating to chemical modification of proteins.

Briefly, modification of e.g. arginyl residues in proteins is often based on the reaction of vicinal dicarbonyl compounds such as phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione to form an adduct. Another example is the reaction of methylglyoxal with arginine residues. Cysteine can be modified without concomitant modification of other nucleophilic sites such as lysine and histidine. As a result, a large number of reagents are available for the modification of cysteine. The websites of Pierce Chemical Company, Sigma-Aldrich and others provide information on specific reagents.

Selective reduction of disulfide bonds in proteins is also common. Disulfide bonds can be formed and oxidized during the heat treatment of biopharmaceuticals.

Woodward's Reagent K may be used to modify specific glutamic acid residues. N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide can be used to form intra-molecular crosslinks between a lysine residue and a glutamic acid residue.

E.g. Diethylpyrocarbonate is a reagent for the modification of histidyl residues in proteins. Histidine can also be modified using 4-hydroxy-2-nonenal.

The reaction of lysine residues and other α-amino groups is, for example, useful in binding of peptides to surfaces or the cross-linking of proteins/peptides. Lysine is the site of attachment of poly(ethylene)glycol and the major site of modification in the glycation of proteins.

Methionine residues in proteins can be modified with e.g. iodoacetamide, bromoethylamine, chloramine T.

Tetranitromethane and N-acetylimidazole can be used for the modification of tyrosyl residues. Cross-linking via the formation of dityrosine can be accomplished with hydrogen peroxide/copper ions.

Recent studies on the modification of tryptophan have used N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide or 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole (BPNS-skatole).

Successful modification of therapeutic proteins and peptides with PEG is often associated with an extension of circulatory half-life while cross-linking of proteins with glutaraldehyde, polyethyleneglycol diacrylate and formaldehyde is used for the preparation of hydrogels. Chemical modification of allergens for immunotherapy is often achieved by carbamylation with potassium cyanate.

Generally, peptides and variants (at least those containing peptide linkages between amino acid residues) may be synthesised e.g. using the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu et al (1981) J. Org. Chem. 46, 3433 and references therein.

Purification may be effected by any one, or a combination of, techniques such as recristallisation, size exclusion chromatography, ion-exchange chromatography, hydrophobic

interaction chromatography and (usually) reverse-phase high performance liquid chromatography using e.g. acetonitril/water gradient separation.

Analysis of peptides may be carried out using thin layer chromatography, electrophoresis, in particular capillary electrophoresis, solid phase extraction (CSPE), reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis, as well as MALDI and ESI-Q-TOF mass spectrometric analysis.

A further aspect of the invention provides a nucleic acid (e.g. polynucleotide) encoding a peptide or variant of the invention. The polynucleotide may be e.g. DNA, cDNA, PNA, CNA, RNA, either single- and/or double-stranded, or native or stabilised forms of polynucleotides, such as e.g. polynucleotides with a phosphorothiate backbone, or combinations thereof and it may or may not contain introns so long as it codes for the peptide. Of course, it is only peptides containing naturally occurring amino acid residues joined by naturally occurring peptide bonds are encodable by a polynucleotide. A still further aspect of the invention provides an expression vector capable of expressing a polypeptide according to the invention. Expression vectors for different cell types are well known in the art and can be selected without undue experimentation.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Guidance can be found e.g. in Sambrook et al (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

In a particularly preferred embodiment of the invention, however, the pharmaceutical composition comprises at least two peptides consisting of amino acid sequences according to SEQ ID NO 1 to SEQ ID NO 15.

The optimum amount of each peptide to be included in the vaccine and the optimum dosing regimen can be determined by one skilled in the art without undue experimentation. For

example, the peptide or its variant may be prepared for intravenous (i.v.) injection, subcutaneous (s.c.) injection, intradermal (i.d.) injection, intraperitoneal (i.p.) injection, intramuscular (i.m.) injection. Preferred routes of peptide injection are s.c., i.d., i.p., i.m., and i.v. Preferred routes of DNA injection are i.d., i.m., s.c., i.p. and i.v. Doses of e.g. between 1 and 500 mg 50 µg and 1.5 mg, preferably 125 µg to 500 µg, of peptide or DNA may be given and will depend from the respective peptide or DNA. Doses of this range were successfully used in previous trials (Brunsvig PF, Aamdal S, Gjertsen MK, Kvalheim G, Markowski-Grimsrud CJ, Sve I, Dyrhaug M, Trachsel S, Møller M, Eriksen JA, Gaudernack G; . Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer; Cancer Immunol Immunother. 2006; 55(12):1553-1564; M. Staehler, A. Stenzl, P. Y. Dietrich, T. Eisen, A. Haferkamp, J. Beck, A. Mayer, S. Walter, H. Singh, J. Frisch, C. G. Stief; An open label study to evaluate the safety and immunogenicity of the peptide based cancer vaccine IMA901, ASCO meeting 2007; Abstract No 3017)

The inventive pharmaceutical composition may be compiled such that the selection, number and/or amount of peptides present in the composition is/are tissue, cancer, and/or patient-specific. For instance the exact selection of peptides can be guided by expression patterns of the parent proteins in a given tissue to avoid side effects. The selection may be dependent from the specific type of cancer that the patient to be treated is suffering from as well as the status of the disease, earlier treatment regimens, the immune status of the patient, and, of course, the HLA-haplotype of the patient. Furthermore, the vaccine according to the invention can contain individualised components, according to personal needs of the particular patient. Examples are different amounts of peptides according to the expression of the related TAAs in the particular patient, unwanted side-effects due to personal allergies or other treatments, and adjustments for secondary treatments following a first round or scheme of treatment.

For composition to be used as a vaccine for CRC, for example, peptides whose parent proteins are expressed in high amounts in normal tissues will be avoided or be present in low amounts in the composition of the invention. On the other hand, if it is known that the tumour of a patient expresses high amounts of a certain protein the respective pharmaceutical composition for treatment of this cancer may be present in high amounts and/or more than one peptide specific for this particularly protein or pathway of this protein may be included.
The person of skill will be able to select preferred combinations of immunogenic peptides by testing, for example, the generation of T-cells in vitro as well as their efficiency and overall

presence, the proliferation, affinity and expansion of certain T-cells for certain peptides, and the functionality of the T-cells, e.g. by analysing the IFN-γ production (see also examples below). Usually, the most efficient peptides are then combined as a vaccine for the purposes as described above.

A suitable vaccine will preferably contain between 1 and 20 peptides, more preferably 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 different peptides, further preferred 6, 7, 8, 9, 10 11, 12, 13, or 14 different peptides, and most preferably 14 different peptides. The length of the peptide for use in a cancer vaccine may be any suitable peptide. In particular, it may be a suitable 9-mer peptide or a suitable 7-mer or 8-mer or 10-mer or 11-mer peptide or 12-mer, 13-mer, 14-mer or 15-mer. Longer peptides may also be suitable, 9-mer or 10-mer peptides as described in the attached Tables 1 and 2 are preferred for MHC class I-peptides, while 12- to 15-mers are preferred for MHC class II peptides.

The peptide(s) constitute(s) a tumour or cancer vaccine. It may be administered directly into the patient, into the affected organ or systemically, or applied ex vivo to cells derived from the patient or a human cell line which are subsequently administered to the patient, or used in vitro to select a subpopulation from immune cells derived from the patient, which are then re-administered to the patient.

The peptide may be substantially pure, or combined with an immune-stimulating adjuvant(see below) or used in combination with immune-stimulatory cytokines, or be administered with a suitable delivery system, for example liposomes. The peptide may also be conjugated to a suitable carrier such as keyhole limpet haemocyanin (KLH) or mannan (see WO 95/18145 and Longenecker et al (1993) Ann. NY Acad. Sci. 690,276-291). The peptide may also be tagged, or be a fusion protein, or be a hybrid molecule. The peptides whose sequence is given in the present invention are expected to stimulate CD4 or CD8 CTL. However, stimulation is more efficient in the presence of help provided by T-cells positive for the opposite CD. Thus, for MHC Class II epitopes which stimulate CD4 CTL the fusion partner or sections of a hybrid molecule suitably provide epitopes which stimulate CD8-positive T-cells. On the other hand, for MHC Class I epitopes which stimulate CD8 CTL the fusion partner or sections of a hybrid molecule suitably provide epitopes which stimulate CD4-positive T-cells. CD4- and CD8-stimulating epitopes are well known in the art and include those identified in the present invention.

To elicit an immune response it is usually necessary to include excipients that render the composition more immunogenic. Thus in a preferred embodiment of the invention the pharmaceutical composition further comprises at least one suitable adjuvant.

Adjuvants are substances that non-specifically enhance or potentiate the immune response (e.g., immune responses mediated by CTLs and helper-T (T_H) cells to an antigen, and would thus be considered useful in the medicament of the present invention. Suitable adjuvants include, but are not limited to 1018 ISS, aluminium salts, Amplivax, AS15, BCG, CP-870,893, CpG7909, CyaA, dSLIM, GM-CSF, IC30, IC31, Imiquimod, ImuFact IMP321, IS Patch, ISS, ISCOMATRIX, JuvImmune, LipoVac, MF59, monophosphoryl lipid A, Montanide IMS 1312, Montanide ISA 206, Montanide ISA 50V, Montanide ISA-51, OK-432, OM-174, OM-197-MP-EC, ONTAK, PepTel® vector system, PLG microparticles, resiquimod, SRL172, Virosomes and other Virus-like particles, YF-17D, VEGF trap,R848, beta-glucan, Pam3Cys, Aquila's QS21 stimulon (Aquila Biotech, Worcester, MA, USA) which is derived from saponin, mycobacterial extracts and synthetic bacterial cell wall mimics, and other proprietory adjuvants such as Ribi's Detox. Quil or Superfos. Adjuvants such as incomplete Freund's or GM-CSF are preferred. Several immunological adjuvants (e.g., MF59) specific for dendritic cells and their preparation have been described previously (Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, van Nest G, Ott G, McDonald DM; Dendritic cells internalize vaccine adjuvant after intramuscular injection; Cell Immunol. 1998; 186(1):18-27; Allison AC; The mode of action of immunological adjuvants; Dev Biol Stand. 1998; 92:3-11). Also cytokines may be used. Several cytokines have been directly linked to influencing dendritic cell migration to lymphoid tissues (e.g., TNF-α), accelerating the maturation of dendritic cells into efficient antigen-presenting cells for T-lymphocytes (e.g., GM-CSF, IL-1 and IL-4) (U.S. Pat. No. 5,849,589, specifically incorporated herein by reference in its entirety) and acting as immunoadjuvants (e.g., IL-12) (Gabrilovich DI, Cunningham HT, Carbone DP; IL-12 and mutant P53 peptide-pulsed dendritic cells for the specific immunotherapy of cancer; J Immunother Emphasis Tumor Immunol. 1996 (6):414-418).

CpG immunostimulatory oligonucleotides have also been reported to enhance the effects of adjuvants in a vaccine setting. Without bound by theory, CpG oligonucleotides act by activating the innate (non-adaptive) immune system via Toll-like receptors (TLR), mainly TLR9. CpG triggered TLR9 activation enhances antigen-specific humoral and cellular

responses to a wide variety of antigens, including peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines and polysaccharide conjugates in both prophylactic and therapeutic vaccines. More importantly it enhances dendritic cell maturation and differentiation, resulting in enhanced activation of T_{H1} cells and strong cytotoxic T-lymphocyte (CTL) generation, even in the absence of CD4 T-cell help. The TH1 bias induced by TLR9 stimulation is maintained even in the presence of vaccine adjuvants such as alum or incomplete Freund's adjuvant (IFA) that normally promote a TH2 bias. CpG oligonucleotides show even greater adjuvant activity when formulated or co-administered with other adjuvants or in formulations such as microparticles, nano particles, lipid emulsions or similar formulations, which are especially necessary for inducing a strong response when the antigen is relatively weak. They also accelerate the immune response and enabled the antigen doses to be reduced by approximately two orders of magnitude, with comparable antibody responses to the full-dose vaccine without CpG in some experiments (Arthur M. Krieg, Therapeutic potential of Toll-like receptor 9 activation, Nature Reviews, Drug Discovery, 2006, 5, 471-484). U. S. Pat. No. 6,406,705 B1 describes the combined use of CpG oligonucleotides, non-nucleic acid adjuvants and an antigen to induce an antigen-specific immune response. A commercially available CpG TLR9 antagonist is dSLIM (double Stem Loop Immunomodulator) by Mologen (Berlin, GERMANY) which is a preferred component of the pharmaceutical composition of the present invention. Other TLR binding molecules such as RNA binding TLR 7, TLR 8 and/or TLR 9 may also be used.

Other examples for useful adjuvants include, but are not limited to chemically modified CpGs (e.g. CpR, Idera), Poly(I:C) (e.g. polyI:C12U), non-CpG bacterial DNA or RNA as well as immunoactive small molecules and antibodies such as imidazoquinolines, cyclophosphamide, sunitinib, bevacizumab, celebrex, NCX-4016, sildenafil, tadalafil, vardenafil, sorafinib, XL-999, CP-547632, pazopanib, ZD2171, AZD2171, ipilimumab, tremelimumab and SC58175, which may act therapeutically and/or as an adjuvant. The amounts and concentrations of adjuvants and additives useful in the context of the present invention can readily be determined by the skilled artisan without undue experimentation.

Preferred adjuvants are dSLIM, BCG, OK432, imiquimod, PeviTer, and JuvImmune.

In a preferred embodiment the pharmaceutical composition according to the invention the adjuvant is selected from the group consisting of colony-stimulating factors, such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, sargramostim).

In a preferred embodiment of the pharmaceutical composition according to the invention, the adjuvant is imiquimod.

This composition is used for parenteral administration, such as subcutaneous, intradermal, intramuscular or oral administration. For this, the peptides and optionally other molecules are dissolved or suspended in a pharmaceutically acceptable, preferably aqueous carrier. In addition, the composition can contain excipients, such as buffers, binding agents, blasting agents, diluents, flavours, lubricants, etc.. The peptides can also be administered together with immune stimulating substances, such as cytokines. An extensive listing of excipients that can be used in such a composition, can be, for example, taken from A. Kibbe, Handbook of Pharmaceutical Excipients, 3. Ed. 2000, American Pharmaceutical Association and pharmaceutical press. The composition can be used for a prevention, prophylaxis and/or therapy of adenomateous or cancerous diseases, preferably CRC.

Cytotoxic T-cells (CTLs) recognise an antigen in the form of a peptide bound to an MHC molecule rather than the intact foreign antigen itself. The MHC molecule itself is located at the cell surface of an antigen presenting cell. Thus, an activation of CTLs is only possible if a trimeric complex of peptide antigen, MHC molecule, and APC is present. Correspondingly, it may enhance the immune response if not only the peptide is used for activation of CTLs but if additionally APCs with the respective MHC molecule are added.

Therefore, in a preferred embodiment the pharmaceutical composition according to the present invention additionally contains at least one antigen presenting cell.

The antigen-presenting cell (or stimulator cell) typically has an MHC class I or II molecule on its surface and in one embodiment is substantially incapable of itself loading the MHC class I or II molecule with the selected antigen. As is described in more detail below, the MHC class I or II molecule may readily be loaded with the selected antigen in vitro.

Preferably the mammalian cell lacks or has a reduced level or has reduced function of the TAP peptide transporter. Suitable cells which lack the TAP peptide transporter include T2, RMA-S and Drosophila cells. TAP is the Transporter Associated with antigen Processing.

The human peptide loading deficient cell line T2 is available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA under Catalogue No CRL 1992; the Drosophila cell line Schneider line 2 is available from the ATCC under Catalogue No CRL 19863; the mouse RMA-S cell line is described in Karre and Ljunggren (1985) J. Exp. Med. 162, 1745. These cell lines can be used as APCs, and due to the lack of TAP nearly all peptides presented by MHC class I will be the peptides under scrutiny used for externally loading the empty MHC class I molecules of these cell lines, hence all effects will clearly attribute to the used peptides.

Preferably, the antigen presenting cells are dendritic cells. Suitably, the dendritic cells are autologous dendritic cells which are pulsed with an antigenic peptide. The antigenic peptide may be any suitable antigenic peptide which gives rise to an appropriate T-cell response. T-cell therapy using autologous dendritic cells pulsed with peptides from a tumour associated antigen is disclosed in Murphy et al (1996) The Prostate 29, 371-380 and Tjua et al (1997) The Prostate 32, 272-278.

Thus, in a preferred embodiment of the present invention the pharmaceutical composition containing at least one antigen presenting cell is pulsed or loaded with the peptide, for instance by the method of example 4.

As an alternative the antigen presenting cell comprises an expression construct encoding the peptide. The polynucleotide may be any suitable polynucleotide and it is preferred that it is capable of transducing the dendritic cell thus resulting in the presentation of a peptide and induction of immunity.

Conveniently, a nucleic acid of the invention may be comprised in a viral polynucleotide or virus. For example, adenovirus-transduced dendritic cells have been shown to induce antigen-specific antitumour immunity in relation to MUC1 (see Gong et al (1997) Gene Ther. 4, 1023-1028). Similarly, adenovirus-based systems may be used (see, for example, Wan et al (1997) Hum. Gene Ther. 8, 1355-1363); retroviral systems may be used (Specht et al (1997)

J. Exp. Med. 186, 1213-1221 and Szabolcs et al (1997) Blood particle-mediated transfer to dendritic cells may also be used (Tuting et al (1997) Eur. J. Immunol. 27, 2702-2707); and RNA may also be used (Ashley et al (1997) J. Exp. Med. 186, 1177 1182).

Generally, a pharmaceutical composition of the invention containing (a) nucleic acid(s) of the invention can be administered in a similar manner as those containing peptide(s) of the invention, e.g. intravenously, intra-arterially, intra-peritoneally, intramuscularly, intradermally, intratumorally, orally, dermally, nasally, buccally, rectally, vaginally, by inhalation, or by topical administration.

Due to evasion mechanisms a tumour often develops resistance to the drug it is treated with. The drug resistance may occur during treatment and manifests itself in metastases and recurring tumours. To avoid such a drug resistance a tumour is commonly treated by a combination of drugs and metastases and tumours recurring after a disease free period of time often require a different combination. Therefore, in one aspect of the invention the pharmaceutical composition is administered in conjunction with a second anticancer agent. The second agent may be administered before after or simultaneously with the pharmaceutical composition of the invention. A simultaneous administration can e.g. be achieved by mixing the pharmaceutical composition of the invention with the second anticancer agent if chemical properties are compatible. Another way of a simultaneous administration is the administration of the composition and anticancer agent on the same day independently from the route of administration such that the pharmaceutical composition of the invention may be e.g. injected while the second anticancer agent is for instance given orally. The pharmaceutical composition and second anticancer agent may also be administered within the same treatment course but on different days and/or within separate treatment courses.

In another aspect the present invention provides a method for treating or preventing a cancer in a patient comprising administering to the patient a therapeutically effective amount any one of the pharmaceutical compositions of the invention.

A therapeutically effective amount will be an amount sufficient to induce an immune response, in particular an activation of a subpopulation of CTLs. A person skilled in the art may easily determine whether an amount is effective by using standard immunological methods, such as those provided in the examples of the present specifications. Another way of

monitoring the effect of a certain amount of the pharmaceutical composition is to observe the growth of the tumour treated and/or its recurrence.

In a particularly preferred embodiment of the present invention the pharmaceutical composition is used as an anti-cancer vaccine.

The composition containing peptides or peptide-encoding nucleic acids can also constitute a tumour or cancer vaccine. It may be administered directly into the patient, into the affected organ or systemically, or applied ex vivo to cells derived from the patient or a human cell line which are subsequently administered to the patient, or used in vitro to select a subpopulation from immune cells derived from the patient, which are then re-administered to the patient.

The composition of the invention may be used in a method for treating of or used as a vaccine for cancer. The cancer may be of the buccal cavity and pharynx, cancer of the digestive tract, cancer of the colon, rectum, and anus, cancer of the respiratory tract, breast cancer, cancer of the cervix uteri, vagina, and vulva, cancer of the uterine corpus and ovary, cancer of the male genital tract, cancer of the urinary tract, cancer of the bone and soft tissue, and kaposi sarcoma, melanoma of the skin, eye melanoma, and non-melanoma eye cancer, cancer of the brain and central nervous system, cancer of the thyroid and other endocrine glands , Hodgkin Lymphoma, Non-Hodgkin Lymphoma, and myeloma, preferably renal cancer, colorectal cancer, lung cancer, breast cancer, pancreatic cancer, prostate cancer, gastric cancer, brain cancer, GIST or glioblastoma.

In the most preferred embodiment of the method of treatment or vaccine according to the invention, the vaccine is a multiple peptide tumour vaccine for treatment of colorectal carcinoma. Preferably, the vaccine comprises a set of tumour-associated peptides selected from SEQ ID No. 1 to SEQ ID No. 15 which are located and have been identified on primary colorectal cancer cells. This set includes HLA class I and class II peptides. The peptide set can also contain at least one peptide, such as from HBV core antigen, used as a positive control peptide serving as immune marker to test the efficiency of the intradermal administration. In one particular embodiment, the vaccine consists of 14 individual peptides (according to SEQ ID No. 1 to SEQ ID No. 15) with between about 1500 µg to about 75 µg, preferably between about 1000 µg to about 750 µg and more preferred between about 500 µg to about 600 µg, and most preferred about about 578 µg of each peptide, all of which may be

purified by HPLC and ion exchange chromatography and appear as a white to off-white powder. The lyophilisate is preferably dissolved in sodium hydrogen carbonate, and is used for intradermal injection within 30 min after reconstitution at room temperature. According to the present invention, preferred amounts of peptides can vary between about 0.1 and 100 mg, preferably between about 0.1 to 1 mg, and most preferred between about 300 µg to 800 µg per 500 µl of solution. Herein, the term "about" shall mean +/- 10 percent of the given value, if not stated differently. The person of skill will be able to adjust the actual amount of peptide to be used based on several factors, such as, for example, the immune status of the individual patient and/or the amount of TUMAP that is presented in a particular type of cancer. The peptides of the present invention might be provided in other suitable forms (sterile solutions, etc.) instead of a lyophilisate.

The pharmaceutical preparation of the present invention comprising peptides, and/or nucleic acid(s) according to the invention is administered to a patient that suffers from an adenomateous or cancerous disease that is associated with the respective peptide or antigen. By this, a T cell-mediated immune response can be triggered.

Preferred is a pharmaceutical composition according to the invention, wherein the amount of (in particular tumour associated) peptide(s), of nucleic acid(s) according to the invention or expression vector(s) according to the invention as present in the composition is/are tissue, cancer, and/or patient-specific.

In another embodiment of the invention the vaccine is a nucleic acid vaccine. It is known that inoculation with a nucleic acid vaccine, such as a DNA vaccine, encoding a polypeptide leads to a T-cell response. It may be administered directly into the patient, into the affected organ or systemically, or applied ex vivo to cells derived from the patient or a human cell line which are subsequently administered to the patient, or used in vitro to select a subpopulation from immune cells derived from the patient, which are then re-administered to the patient. If the nucleic acid is administered to cells in vitro, it may be useful for the cells to be transfected so as to co-express immune-stimulating cytokines, such as interleukin-2 or GM-CSF. The nucleic acid(s) may be substantially pure, or combined with an immune-stimulating adjuvant, or used in combination with immune-stimulatory cytokines, or be administered with a suitable delivery system, for example liposomes. The nucleic acid vaccine may also be administered

with an adjuvant such as those described for peptide vaccines above. It is preferred if the nucleic acid vaccine is administered without adjuvant.

The polynucleotide may be substantially pure, or contained in a suitable vector or delivery system. Suitable vectors and delivery systems include viral, such as systems based on adenovirus, vaccinia virus, retroviruses, herpes virus, adeno-associated virus or hybrids containing elements of more than one virus. Non-viral delivery systems include cationic lipids and cationic polymers as are well known in the art of DNA delivery. Physical delivery, such as via a "gene-gun", may also be used. The peptide or peptide encoded by the nucleic acid may be a fusion protein, for example with an epitope from tetanus toxoid which stimulates CD4-positive T-cells.

Suitably, any nucleic acid administered to the patient is sterile and pyrogen free. Naked DNA may be given intramuscularly or intradermally or subcutaneously. Conveniently, the nucleic acid vaccine may comprise any suitable nucleic acid delivery means. The nucleic acid, preferably DNA, may also be delivered in a liposome or as part of a viral vector delivery system. It is preferred if the nucleic acid vaccine, such as DNA vaccine, is administered into the muscle, whilst peptide vaccines are preferably administered s.c. or i.d. It is also preferred if the vaccine is administered into the skin.

It is believed that uptake of the nucleic acid and expression of the encoded polypeptide by professional antigen presenting cells such as dendritic cells may be the mechanism of priming of the immune response; however, dendritic cells may not be transfected but are still important since they may pick up expressed peptide from transfected cells in the tissue ("cross-priming", e.g., Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, Laheru DA, Goggins M, Hruban RH, Jaffee EM. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. J Exp Med. 2004 Aug 2;200(3):297-306).

Polynucleotide-mediated immunisation therapy of cancer is described in Conry et al (1996) Seminars in Oncology 23, 135-147; Condon et al (1996) Nature Medicine 2, 1122-1127; Gong et al (1997) Nature Medicine 3, 558-561; Zhai et al (1996) J. Immunol. 156, 700-710; Graham et al (1996) Int J. Cancer 65, 664-670; and Burchell et al (1996) 309-313 In: Breast

Cancer, Advances in biology and therapeutics, Calvo et al (eds), John Libbey Eurotext, all of which are incorporated herein by reference in their entireties.

It may also be useful to target the vaccine to specific cell populations, for example antigen presenting cells, either by the site of injection, use of targeting vectors and delivery systems, or selective purification of such a cell population from the patient and ex vivo administration of the peptide or nucleic acid (for example dendritic cells may be sorted as described in Zhou et al (1995) Blood 86, 3295-3301; Roth et al (1996) Scand. J. Immunology 43, 646-651). For example, targeting vectors may comprise a tissue-or tumour-specific promoter which directs expression of the antigen at a suitable place.

Finally, the vaccine according to the invention can be dependent from the specific type of cancer that the patient to be treated is suffering from as well as the status of the disease, earlier treatment regimens, the immune status of the patient, and, of course, the HLA-haplotype of the patient. Furthermore, the vaccine according to the invention can contain individualised components, according to personal needs of the particular patient. Examples are different amounts of peptides according to the expression of the related TAAs in the particular patient, unwanted side-effects due to personal allergies or other treatments, and adjustments for secondary treatments following a first round or scheme of treatment.

In addition to being useful for treating cancer, the peptides of the present invention are also useful as diagnostics. Since the peptides were generated from glioblastoma and since it was determined that these peptides are not present in normal tissues, these peptides can be used to diagnose the presence of a cancer.

The presence of the peptides of the present invention on tissue biopsies can assist a pathologist in diagnosis of cancer. Detection of certain peptides of the present invention by means of antibodies, mass spectrometry or other methods known in the art can tell the pathologist that the tissue is malignant or inflamed or generally diseased. Presence of groups of peptides of the present invention can enable classification or subclassification of diseased tissues.

The detection of the peptides of the present invention on diseased tissue specimen can enable the decision about the benefit of therapies involving the immune system, especially if T

lymphocytes are known or expected to be involved in the mechanism of action. Loss of MHC expression is a well described mechanism by which infected of malignant cells escape immunosurveillance. Thus, presence of the peptides of the present invention shows that this mechanism is not exploited by the analyzed cells.

The peptides of the present invention might be used to analyze lymphocyte responses against those peptides of the present invention, such as T cell responses or antibody responses against the peptides of the present invention or the peptides of the present invention complexed to MHC molecules. These lymphocyte responses can be used as prognostic markers for decision on further therapy steps. These responses can also be used as surrogate markers in immunotherapy approaches aiming to induce lymphocyte responses by different means, e.g. vaccination of protein, nucleic acids, autologous materials, adoptive transfer of lymphocytes. In gene therapy settings, lymphocyte responses against the peptides of the present invention can be considered in the assessment of side effects. Monitoring of lymphocyte responses might also be a valuable tool for follow-up examinations of transplantation therapies, e.g. for the detection of graft versus host and host versus graft diseases.

The peptides of the present invention can be used to generate and develop specific antibodies against MHC/peptide complexes. These can be used for therapy, targeting toxins or radioactive substances to the diseased tissue. Another use of these antibodies can be targeting radionuclides to the diseased tissue for imaging purposes, such as PET. This use can help to detect small metastases or to determine the size and precise localization of diseased tissues. In addition, the peptides can be used to verify a pathologist's diagnosis of a cancer based on a biopsied sample.

In yet another aspect thereof, the present invention relates to a kit comprising (a) a container that contains a pharmaceutical composition as described above, in solution or in lyophilized form; (b) optionally, a second container containing a diluent or reconstituting solution for the lyophilized formulation; and (c) optionally, instructions for (i) use of the solution or (ii) reconstitution and/or use of the lyophilized formulation. The kit may further comprise one or more of (iii) a buffer, (iv) a diluent, (v) a filter, (vi) a needle, or (v) a syringe. The container is preferably a bottle, a vial, a syringe or test tube; and it may be a multi-use container. The pharmaceutical composition is preferably lyophilized.

Kits of the present invention preferably comprise a lyophilized formulation of the present invention in a suitable container and instructions for its reconstitution and/or use. Suitable containers include, for example, bottles, vials (e.g. dual chamber vials), syringes (such as dual chamber syringes) and test tubes. The container may be formed from a variety of materials such as glass or plastic. Preferably the kit and/or container contains instructions on or associated with the container that indicates directions for reconstitution and/or use. For example, the label may indicate that the lyophilized formulation is to reconstituted to peptide concentrations as described above. The label may further indicate that the formulation is useful or intended for subcutaneous administration.

The container holding the formulation may be a multi-use vial, which allows for repeat administrations (e.g., from 2-6 administrations) of the reconstituted formulation. The kit may further comprise a second container comprising a suitable diluent (e.g., sodium bicarbonate solution).

Upon mixing of the diluent and the lyophilized formulation, the final peptide concentration in the reconstituted formulation is preferably at least 0.15 mg/mL/peptide (=75μg) and preferably not more than 3 mg/mL/peptide (=1500μg). The kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

Kits of the present invention may have a single container that contains the formulation of the pharmaceutical compositions according to the present invention with or without other components (e.g., other compounds or pharmaceutical compositions of these other compounds) or may have distinct container for each component.

Preferably, kits of the invention include a formulation of the invention packaged for use in combination with the co-administration of a second compound (such as adjuvants (e.g. GM-CSF), a chemotherapeutic agent, a natural product, a hormone or antagonist, a anti-angiogenesis agent or inhibitor, a apoptosis-inducing agent or a chelator) or a pharmaceutical composition thereof. The components of the kit may be pre-complexed or each component may be in a separate distinct container prior to administration to a patient. The components of the kit may be provided in one or more liquid solutions, preferably, an aqueous solution, more preferably, a sterile aqueous solution. The components of the kit may also be provided as

solids, which may be converted into liquids by addition of suitable solvents, which are preferably provided in another distinct container.

The container of a therapeutic kit may be a vial, test tube, flask, bottle, syringe, or any other means of enclosing a solid or liquid. Usually, when there is more than one component, the kit will contain a second vial or other container, which allows for separate dosing. The kit may also contain another container for a pharmaceutically acceptable liquid. Preferably, a therapeutic kit will contain an apparatus (e.g., one or more needles, syringes, eye droppers, pipette, etc.), which enables administration of the agents of the invention that are components of the present kit.

The pharmaceutical formulation of the present invention is one that is suitable for administration of the peptides by any acceptable route such as oral (enteral), nasal, ophthal, subcutaneous, intradermal, intramuscular, intravenous or transdermal. Preferably the administration is s.c., and most preferably, i.d. Administration may be by infusion pump.

It should be understood that the features of the invention as disclosed and described herein can be used not only in the respective combination as indicated but also in a singular fashion without departing from the intended scope of the present invention. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

The invention will now be described in more detail by reference to the following Figures, the Sequence listing, and the Examples. The following examples are provided for illustrative purposes only and are not intended to limit the invention.

Brief description of the Figures

Figure 1: Tetramer analysis of microsphere driven proliferation of ODC-001 and NOX-001 specific CD8+ lymphocytes from peripheral blood. 1 x 10^6 CD8+ enriched PBMCs per well of the healthy HLA-A*0201+ donor HD100 was stimulated weekly with microspheres coupled to anti-CD28 plus high densitiy tumor antigen A*0201/ODC-001 (upper panel) or anti-CD28 plus high densitiy tumor antigen A*0201/NOX-001 (lower panel). After three stimulations in vitro, all cells were stained with antibody CD8 FITC plus tetramers A*0201/NOX-001 PE and A*0201/ODC-001 APC. Cells are gated on the lymphocyte

population or CD8+ lymphocytes (right panel) and numbers represent percentage of tetramer+ within CD8+ lymphocytes.

Figure 2 In vitro immunogenicity of TGFBI-004 as detected by IFNγ ELISPOT after five stimulation cycles. Cells were primed and restimulated repeatedly with TGFBI-004 and then incubated with relevant TGFBI-004 (Well 1, 2, 3 and 4) and irrelevant (Neg. control) peptide, respectively. The analysis after IFNγ ELISPOT was performed on an ELISPOT Reader (CTL, Cleveland, USA). PHA-Ionomycin served as positive control. Numbers indicate the count of positive spots.

Figure 3: In vitro immunogenicity of TGFBI-004 as detected by ICS after five stimulation cycles. Cells were primed with TGFBI-004-loaded autologous DCs and restimulated repeatedly with autologous PBMCs plus TGFBI-004. For the read-out cells were incubated with relevant TGFBI-004 (Well 1, 2, 3 and 4) and irrelevant (Neg. Control) peptide, respectively. Additionally to the intracellular IFNγ staining, cells were also stained with CD4-FITC and CD8-PerCP antibodies. The analysis was performed on a four-color FACSCalibur cytometer (BD Biosciences, Germany).

Figure 4: ELISPOT analysis of IFNγ production by T-cell lines upon in vitro restimulation with the NOX-001 peptide. A. T-Cell line 7+ from donor HBC-154 (sorted CD8+ NOX-001 tetramer+); B. T-Cell line 7- from donor HBC-154 (sorted CD8+ NOX-001 tetramer-). Sorted CD8+ NOX-001 tetramer+ (A.) and CD8+ NOX-001 tetramer- (B.) cells were analysed by IFNγ ELISPOT after restimulation with irrelevant (MLA-001) (upper wells) and relevant (NOX-001) (lower wells) peptide (10 µg/ml). Numbers indicate the count of positive spots.

Figure 5: Frequencies of CEA-004-specific CD8+ T cells in 4 HLA-A2 healthy donors following in vitro stimulation with CEA-004 as determined by flow cytometric analysis.

Figure 6: Affinity of HLA class I peptides of the invention to the MHC molecule coded by the HLA-A*0201 allele.

Examples

1. Synthesis and structure

Peptides were synthesized by standard and well-established solid phase synthesis using Fmoc chemistry. After purification by preparative HPLC, ion-exchange procedure was performed to incorporate physicological compatible counter ions (acetate or chloride). Finally, white to off white solids were obtained after lyophilisation. All TUMAPs are administered as acetate salts except IMA-CCN-001 which is supplied as chloride salt for technical reasons during the manufacturing procedure.

Importantly, identity and purity of the peptides can be determined easily and with high accuracy using mass spectrometry, amino acid analysis and analytical HPLC. According to analytical results, all peptides used for IMA910 vaccine show the correct structure with purities ≥ 95%.

Table : Physico-chemical characteristics of peptides in IMA910

No.	Peptide ID	Peptide length (no of amino acids)	Salt form	Physical form	Hygroscopicity
1	IMA-C20-001	9	Acetate	White to off-white powder	Stored as freeze dried powder. Lyophilized peptides generally have hygroscopic properties.
2	IMA-CCN-001	9	Chloride		
3	IMA-CEA-004	9	Acetate		
4	IMA-CEA-006	16	Acetate		
5	IMA-HBV-001	10	Acetate		
6	IMA-MET-001	9	Acetate		
7	IMA-MMP-001	16	Acetate		
8	IMA-MUC-001	9	Acetate		
9	IMA-NOX-001	9	Acetate		
10	IMA-ODC-001	9	Acetate		
11	IMA-PCN-001	10	Acetate		
12	IMA-TGFBI-001	10	Acetate		
13	IMA-TGFBI-004	15	Acetate		
14	IMA-TOP-001	10	Acetate		

Particle size distribution and particle shape measurement of the particles obtained after reconstitution have been performed by capturing direct images of each individual particle in the range of 0.25 to 100 µm followed by image analysis. As a result the majority (> 95%) of the particles have been found in the range of 0.25 to 2.7 µm. So far, no major differences in size and shape distribution could be observed within 1, 2 or 3 hours after reconstitution.

2. Components of the exemplary pharmaceutical composition IMA910

IMA910 is composed of a cocktail of synthetic tumor associated peptides (TUMAPs) of which the majority has been identified on primary colorectal cancer cells. The TUMAPs include 10 HLA class I-binding peptides with the capacity to activate cytotoxic T cells (CD8+ T cells) and 3 HLA class II-binding peptides with the capacity to activate T helper cells (CD4+ T cells). T helper cells play a crucial role in assisting the function of cytotoxic T cells by releasing cytokines which enhance the killer function of CD8+ T cells and may also act directly against tumor cells (Knutson, KL and Disis, ML; Augmenting T helper cell immunity in cancer, Curr.Drug Targets.Immune.Endocr.Metabol.Disord., 2005, 5, 365-371). In addition to these 13 TUMAPs IMA910 contains one viral control peptide.

Samples from surgically removed malignant and normal tissue from CRC patients and blood from healthy donors were analyzed in a stepwise approach:
First genome-wide mRNA expression analysis by microarrays was used to identify genes overexpressed in the malignant tissue compared with a range of normal organs and tissues.

In a second step, HLA ligands from the malignant material were identified by mass spectrometry.

Subsequently identified HLA ligands were compared to gene expression data. Peptides encoded by selectively expressed or overexpressed genes as detected in step 1 were considered suitable candidate TUMAPs for a multi-peptide vaccine.

A literature search was performed to identify additional evidence supporting the relevance of the identified peptides as TUMAPs.

Finally, peripheral CD8+ T cells of healthy individuals were tested for reactivity against the tumor-associated HLA ligands using several immunoassays (in vitro T-cell assays).

Table 3: IMA910 TUMAP composition.
IMA910 contains 10 HLA-A*02 (class I) and 3 HLA-DR (class II) TUMAPs. In addition, the viral marker peptide HBV-001 will be included which is not listed here.

TUMAP ID	Name	Function / Comments
*HLA-A*02 TUMAPs*		
C20-001	Chromosome 20 open reading	Poorly characterized, strong overexpression

	frame 42	
CCN-001	Cyclin D1	Cell cycle regulation, frequently upregulated in many cancer types
CEA-004	Carcinoembryonic antigen-related cell adhesion molecule 5 (CEA)	Well-established TAA in CRC, cell adhesion, metastasis
MET-001	Met proto-oncogene	Proliferation, motility, adhesion, invasion
MUC-001	Mucin 1	Well-established TAA in CRC, unmasking of epitope due to altered glycosylation in tumors
NOX-001	NADPH oxidase 1	Strong overexpression, inhibition of apoptosis
ODC-001	Ornithine decarboxylase 1	Transformation, pro-angiogenic
PCN-001	Proliferating cell nuclear antigen	Proliferation (DNA replication)
TGFBI-001	Transforming growth factor beta-induced	Tissue remodelling, angiogenesis
TOP-001	Topoisomerase (DNA) II	Proliferation (DNA replication)
HLA-DR TUMAPs		
CEA-006	Carcinoembryonic antigen-related cell adhesion molecule 5 (CEA)	Well-established TAA in CRC, cell adhesion, metastasis
MMP-001	Matrix metallopeptidase 7 (matrilysin, uterine)	Tissue remodelling, inhibition of apoptosis
TGFBI-004	Transforming growth factor beta-induced	Tissue remodelling, angiogenesis

3. Presentation of epitopes contained in IMA910 in tumour samples

Preparation

Surgically removed tissue specimens were provided by Universitätsklinik für Allgemeine, Viszeral- und Transplantationschirurgie, Tübingen after written informed consent had been obtained from each patient.

Isolation of HLA peptides from tissue samples

HLA peptide pools from shock-frozen tissue samples were obtained by immune precipitation from solid tissues according to a slightly modified protocol (Falk,K., Rotzschke,O., Stevanovic,S., Jung,G. & Rammensee,H.G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991, 351, 290-296; Seeger,F.H. et al. The HLA-A*6601 peptide motif: prediction by pocket structure and verification by peptide analysis. Immunogenetics 1999, 49, 571-576) using the HLA-A*02-specific antibody BB7.2 or the HLA-A, -B, -C-specific antibody W6/32, CNBr-activated sepharose, acid treatment and ultrafiltration.

Detection of TUMAPs by ESI-liquid chromatography mass spectrometry (ESI-LCMS)

Epitopes contained in IMA910 were systematically searched for on colorectal tumour samples by mass spectrometry. The obtained HLA peptide pools were separated according to their hydrophobicity by reversed-phase chromatography (CapLC, Waters) and the eluting peptides were analyzed in a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-TOF Ultima, Waters) equipped with an ESI source. Peptide pools were loaded onto a C18 pre-column for concentration and desalting. After loading, the pre-column was placed in line for separation by a fused-silica micro-capillary column (75 µm i.d. x 250 mm) packed with 5 µm C18 reversed-phase material (Dionex). Solvent A was 4 mM ammonium acetate/water. Solvent B was 2 mM ammonium acetate in 80% acetonitrile/water. Both solvents were adjusted to pH 3.0 with formic acid. A binary gradient of 15% to 60% B within 90 minutes was performed, applying a flow rate of 5 µl/min reduced to approximately 200 nl/min by a split-system. A gold coated glass capillary (PicoTip, New Objective) was used for introduction into the micro-ESI source. The integration time for the TOF analyzer was 1.9 s with an interscan delay of 0.1 s. For detection of defined peptides high sensitive screening was performed in this type of ESI-LCMS experiments on the basis of known molecular weights and retention times of the peptides in the chromatographic system. Therefore, an include list containing the m/z values of the previously identified peptides (singly and/or doubly charged) was applied for precursor selection. Subsequently the sequence was revealed by collisionally induced decay (CID) mass spectrometry (ESI-LCMS/MS). The TUMAP sequence was assured by comparison of the generated natural TUMAP fragmentation pattern with the fragmentation pattern of a synthetic sequence-identical reference peptide. Evaluation of the HLA peptide purification yield and reproducibility of the analytical system, including retention time stability was carried out using the intensity and retention time of an abundant endogenous HLA-A*02 peptide (YLLPAIVHI from DDX5) as internal standard. Therefore, the CRC sample inclusion criterion for detection of previously identified TUMAP in these experiments was set to a minimal intensity of 650 counts per scan of the internal doubly charged standard signal (YLLPAIVHI) in the LCMS/MS experiment to assure a successful HLA peptide isolation and the correct performance of the analytical system.

Table 3 shows the results of an analysis of colon and rectum cancer samples of different stages as well as metastases originating from either primary tumour site. All HLA-A*02 TUMAPs were found on the majority of samples. Re-detection frequencies of HLA-DR

TUMAPs are generally lower. This can be expected because for HLA class II peptides, several length variants for each core sequence may exist.

Table 3: Re-detection of TUMAPS in CRC samples

					TUMAP detected (+) or not detected (-) in mass spectrometric analysis												
					class I										class II		
No	CRC sample	Tumor mass [g]	Tumor location	Tumor stage	MET-001	C20-001	TGFBI-001	TOP-001	NOX-001	PCN-001	ODC-001	CCN-001	MUC-001	CEA-004	CEA-006	MMP-001	TGFBI-004
1	CCA062	?	colon	I	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	not feasible	not feasible	not feasible	-	-	-
2	CCA740	4,0	colon	II	-	+	+	+	+	+	+				n.a.	n.a.	n.a.
3	CCA165	10,8	colon	II	-	+	+	+	+	+	+				-	-	-
4	CCA712	1,2	colon	III	+	+	+	+	-	-	+				n.a.	n.a.	n.a.
5	CCA707	3,1	colon	III	-	+	+	+	+	+	+				n.a.	n.a.	n.a.
6	CCA718	3,4	colon	III	-	+	+	+	+	+	+				n.a.	n.a.	n.a.
7	CCA739	3,4	colon	III	-	+	+	+	+	+	+				n.a.	n.a.	n.a.
8	CCA166	5,3	colon	III	+	+	+	+	+	+	+				(+)	+	-
9	CCA734	18,1	colon	III	-	+	+	+	+	+	+				n.a.	n.a.	n.a.
10	CCA719	1,3	colon	IV	-	+	+	+	+	-	+				n.a.	n.a.	n.a.
11	CCA725	2,7	colon	IV	-	+	+	+	-	+	+				n.a.	n.a.	n.a.
12	CCA164	5,0	colon	IV	+	+	+	-	-	+	+				-	-	-
13	CCA167	5,2	colon	IV	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.				(+)	+	-
14	CCA056	1,8	colon	?	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.				-	-	-
15	CCA305	4,0	colon	?	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.				-	-	-
20	CCA708	3,2	colon metastasis	IV	-	+	+	+	+	+	+				-	+	+
16	CCA160	3,6	rectum	II	+	+	+	+	+	+	+				+	(+)	+
17	CCA754	3,6	rectum	II	-	+	+	+	-	+	+				n.a.	n.a.	n.a.
18	CCA170	4,6	rectum	III	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.				(+)	-	+
19	CCA171	10,3	rectum	IV	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.				-	+	-
21	CCA724	4,8	rectum metastasis	IV	+	+	+	-	-	-	+				-	-	+
Detected in % of analyzed samples					33%	100%	100%	87%	67%	80%	100%	-	-	-	33%	42%	33%

n.a. not analysed

4. *In vitro* immunogenicity for IMA910 MHC class I presented peptides

To get information regarding the immunogenicity of peptides included in IMA910, we performed investigations using a well established in vitro stimulation platform already described by (Walter, S, Herrgen, L, Schoor, O, Jung, G, Wernet, D, Buhring, HJ, Rammensee, HG, and Stevanovic, S; 2003, Cutting edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres, J.Immunol., 171, 4974-4978). This way we could show positive immunogenicity data for 10/10 tested HLA-A*0201 restricted peptides contained in IMA910 demonstrating that these peptides are T-cell eptiopes against which CD8+ precursor T cells exist in humans. The only other HLA class I peptide contained in IMA910 (MUC-001) could not be tested with this method due to the relative low A*0201 affinity of this TUMAP.

Recent evidence severely challenges the usefulness of CEA-005 for a cancer vaccine. In a recent comprehensive study (Iero, M, Squarcina, P, Romero, P, Guillaume, P, Scarselli, E, Cerino, R, Carrabba, M, Toutirais, O, Parmiani, G, Rivoltini, L; Low TCR avidity and lack of tumor cell recognition in CD8(+) T cells primed with the CEA-analogue CAP1-6D peptide, Cancer Immunol Immunother. 2007 Dec; 56(12): 1979-91) the authors for the first time systematically characterized effector functions of CEA-005-primed T cells against the native sequence CEA-004. For a large number of blood samples from CRC patients and healthy donors it was observed that T-cell priming with CEA-005 reproducibly promoted the generation of low-affinity T cells lacking the ability to recognize CEA-expressing colorectal carcinoma cells presenting the native sequence. Such non-effective low-affinity cross-recognition of native sequences might be a general problem in vaccination protocols using altered peptide ligands, as corroborated by recently reported similar results for another CEA peptide and its altered agonists (Alves, PM, Viatte, S, Fagerberg, T, Michielin, O, Bricard, G, Bouzourene, H, Vuilleumier, H, Kruger, T, Givel, JC, Levy, F, Speiser, DE, Cerottini, JC, Romero, P; Immunogenicity of the carcinoembryonic antigen derived peptide 694 in HLA-A2 healthy donors and colorectal carcinoma patients, Cancer Immunol. Immunother., 2007, 56, 1795-1805). Furthermore, such results have also been reported for a native sequence of the well-established melanoma antigen Melan-A/MART-1 and its agonist (D. Speiser, personal communication).

Altogether, despite the generally enhanced immunogenicity of altered agonist peptides, recent evidence suggests that native peptides might be more attractive vaccine candidates due to inefficient cross-recognition of the native sequence by T cells stimulated with altered agonists. This suggests that CEA-004 (CAP1) should be preferred to its agonists described in WO9919478A1, like CEA-005 (CAP1-6D) or CAP1-6D,7I.

In fact, ample data demonstrate considerable in vivo immunogenicity of the native CEA-004 sequence itself. Spontaneously induced T cell responses against this peptide among cancer patients but not healthy donors have been observed in several studies (Nagorsen, D, Keilholz, U, Rivoltini, L, Schmittel, A, Letsch, A, Asemissen, AM, Berger, G, Buhr, HJ, Thiel, E, Scheibenbogen, C; Natural T-cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer, Cancer Res. 2000, 60, 4850-4854; Weihrauch, MR, Ansen, S, Jurkiewicz, E, Geisen, C, Xia, Z, Anderson, KS, Gracien, E, Schmidt, M, Wittig, B, Diehl, V, Wolf, J, Bohlen, H,

Nadler, LM; Phase I/II combined chemoimmunotherapy with carcinoembryonic antigen-derived HLA-A2-restricted CAP-1 peptide and irinotecan, 5-fluorouracil, and leucovorin in patients with primary metastatic colorectal cancer, Clin Cancer Res. 2005, 11, 5993-6001; Babatz, J, Rollig, C, Lobel, B, Folprecht, G, Haack, M, Gunther, H, Kohne, CH, Ehninger, G, Schmitz, M, Bornhauser, M; Induction of cellular immune responses against carcinoembryonic antigen in patients with metastatic tumors after vaccination with altered peptide ligand-loaded dendritic cells, Cancer Immunol. Immunother. 2006, 55, 268-276). Furthermore, vaccination approaches in CRC patients using CEA-004 or CEA protein have demonstrated efficient stimulation of T-cell responses against CEA-004 (Tsang, KY, Zaremba, S, Nieroda, CA, Zhu, MZ, Hamilton, JM, Schlom, J; Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine, J Natl. Cancer Inst. 1995, 87, 982-990; Morse, MA, Deng, Y, Coleman, D, Hull, S, Kitrell-Fisher, E, Nair, S, Schlom, J, Ryback, ME, Lyerly, HK; A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen, Clin Cancer Res. 1999, 5, 1331-1338; Zhu, MZ, Marshall, J, Cole, D, Schlom, J, Tsang, KY; Specific cytolytic T-cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine, Clin Cancer Res. 2000, 6, 24-33; Weihrauch, MR, Ansen, S, Jurkiewicz, E, Geisen, C, Xia, Z, Anderson, KS, Gracien, E, Schmidt, M, Wittig, B, Diehl, V, Wolf, J, Bohlen, H, Nadler, LM; Phase I/II combined chemoimmunotherapy with carcinoembryonic antigen-derived HLA-A2-restricted CAP-1 peptide and irinotecan, 5-fluorouracil, and leucovorin in patients with primary metastatic colorectal cancer, Clin Cancer Res. 2005, 11, 5993-6001).

In vitro priming of CD8+ T cells

To perform *in vitro* stimulations by artificial antigen presenting cells (aAPC) loaded with peptide-MHC complex (pMHC) and anti-CD28 antibody, first we isolated PBMCs (peripheral blood mononuclear cells) from fresh HLA-A*02+ buffy coats by using standard density gradient separation medium (PAA, Cölbe, Germany). Buffy coats were either obtained from the Blood Bank Tübingen or from the Katharinenhospital Stuttgart. Isolated PBMCs were incubated overnight in T-cell medium (TCM) for human *in vitro* priming consisting of RPMI-Glutamax (Invitrogen, Karlsruhe, Germany) supplemented with 10% heat inactivated human AB serum (PAA, Cölbe, Germany), 100 U/ml Penicillin / 100 µg/ml Streptomycin (Cambrex, Verviers, Belgium), 1 mM sodium pyruvate (CC Pro, Neustadt,

Germany) and 20 µg/ml Gentamycin (Cambrex). CD8+ lymphocytes were isolated using the CD8+ MACS positive selection kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Obtained CD8+ T-cells were incubated until use in TCM supplemented with 2.5 ng/ml IL-7 (PromoCell, Heidelberg, Germany) and 10 U/ml IL-2 (Chiron, Munich, Gemany). Generation of pMHC/anti-CD28 coated beads, T-cell stimulations and readout was performed as described before (Walter, S, Herrgen, L, Schoor, O, Jung, G, Wernet, D, Buhring, HJ, Rammensee, HG, and Stevanovic, S; Cutting edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres, J. Immunol., 2003, 171, 4974-4978) with minor modifications. Briefly, biotinylated recombinant HLA-A*0201 molecules lacking the transmembrane domain and biotinylated at the carboxy terminus of the heavy chain were produced following a method described by (Altman, JD, Moss, PA, Goulder, PJ, Barouch, DH, Heyzer-Williams, MG, Bell, JI, McMichael, AJ, and Davis, MM; Phenotypic analysis of antigen-specific T lymphocytes, Science, 1996, 274, 94-96). The purified costimulatory mouse IgG2a anti human CD28 Ab 9.3 (Jung, G, Ledbetter, JA, and Muller-Eberhard, HJ; Induction of cytotoxicity in resting human T lymphocytes bound to tumor cells by antibody heteroconjugates, Proc Natl Acad Sci USA, 1987, 84, 4611-4615) was chemically biotinylated using Sulfo-N-hydroxysuccinimidobiotin as recommended by the manufacturer (Perbio, Bonn, Germany). Beads used were 5.60 µm large streptavidin coated polystyrene particles (Bangs Labooratories, Illinois/USA). pMHC used as positive and negative controls were A*0201/MLA-001 (peptide ELAGIGILTV from modified Melan-A/MART-1) and A*0201/DDX5-001 (YLLPAIVHI from DDX5) or A*0201/HBV-001 (FLPSDFFPSV), respectively.

800.000 beads / 200 µl were coated in 96-well plates in the presence of 600 ng biotin anti-CD28 plus 200 ng relevant biotin-pMHC (high density beads) or 2 ng relevant plus 200 ng irrelevant (pMHC library) MHC (low density beads). Stimulations were initiated in 96-well plates by conincubating $1x10^6$ CD8+ T cells with $2x10^5$ washed coated beads in 200 µl TCM supplemented with 5 ng/ml IL-12 (PromoCell) for 3-4 days at 37°C. Half of the medium was then exchanged by fresh TCM supplemented with 80 U/ml IL-2 and incubating was continued for 3-4 days at 37°C. This stimulation cycle was performed for a total of three times. Finally, tetrameric analyses were performed with fluorescent MHC tetramers (produced as described by (Altman, JD, Moss, PA, Goulder, PJ, Barouch, DH, Heyzer-Williams, MG, Bell, JI, McMichael, AJ, and Davis, MM; Phenotypic analysis of antigen-specific T lymphocytes,

Science, 1996, 274, 94-96)) plus antibody CD8-FITC clone SK1 (BD, Heidelberg, Germany) on a four-color FACSCalibur (BD). Peptide specific cells were calculated as percentage of total CD8+ T cells. Evaluation of tetrameric analysis was done using the software FCS Express (De Novo Software). In vitro priming of specific tetramer+ CD8+ lymphocytes was detected by appropriate gating and by comparing to negative control stimulations. Immunogenicity for a given antigen was detected if at least one evaluable in vitro stimulated well of one healthy donor was found to contain a specific CD8+ T-cell line after in vitro stimulation (i.e. this well contained at least 1% of specific tetramer+ among CD8+ T-cells and the percentage of specific tetramer+ cells was at least 10x the median of the negative control stimulations).

In vitro immunogenicity for 10 IMA910 peptides

For 10/10 tested HLA class I peptides, in vitro immunogenicity could be demonstrated by generation of peptide specific T-cell lines. A representative staining showing generation of T-cell lines specifc for NOX-001 and ODC-001 is shown in Figure 1. The results are summarized in table 4. The only other HLA class I peptide contained in IMA910 (MUC-001) could not be tested with this method due to relative low A*0201 affinity of this TUMAP, therefore rendering it methodologically impossible to perform in vitro stimulations using pMHC monomers.

Table 4: Immunogenicity of 10 HLA class I peptides included in IMA910

Antigen	Positive donors / donors tested	Positive wells / wells tested
IMA-HBV-001	7/16 (44%)	10/107 (9%)
IMA-TGFBI-001	3/4 (75%)	4/22 (18%)
IMA-NOX-001	3/5 (60%)	9/60 (15%)
IMA-PCN-001	3/4 (75%)	4/42 (10%)
IMA-TOP-001	2/5 (40%)	7/72 (10%)
IMA-C20-001	1/5 (20%)	1/60 (2%)
IMA-ODC-001	1/5 (20%)	1/60 (2%)
IMA-HBV-001	2/5 (40%)	10/54 (19%)
IMA-CEA-004	4/4 (100%)	50/60 (83%)
IMA-CCN-001	5/5 (100%)	42/54 (78%)
IMA-MET-001	4/6 (67%)	30/72 (42%)

Results of *in vitro* immunogenicity experiments conducted by immatics for 10 of 11 HLA class I peptides included in IMA910 are summarised here. Results shown have been obtained

by stimulation of CD8+ cells with high density beads. As different human serum lots may highly affect the immunogenicity results, only assays in which one and the same serum lot was used , were evaluated together.

IMA-CEA-004 in vitro primed T-cells

4/6 donors were evaluable. In all four donors we were able to show successfully the induction of CEA-004-directed T cell response in vitro upon stimulation with CEA-004 (see Table and Figure). Thus, CEA-004 peptide proved to be a potent inducer of human CD8+ T-cell responses in vitro. Importantly, CEA-004 was reproducibly capable of eliciting higher frequencies of CEA-004 specific T cell responses as compared to CEA-005 (83% of wells as compared to 64% of wells, see Table 4). Frequencies of CEA-004 specific cells within individual positive wells were also higher after CEA-004 priming as compared to CEA-005 priming (see Figure 5).

*Peptide-specific in vitro CD8+ T-cell response of 4 healthy HLA-A*02 donors determined by flow cytometric analysis*

CD8+ T cells were primed using artificial antigen presenting cells loaded with CEA-004, CEA-005 or irrelevant peptide (IMA-RSL-001), respectively. After three cycles of stimulation, the detection of peptide-reactive cells was performed by double staining with CEA-004- plus CEA-005 tetramers (table 5 A.) and with CEA-004- plus irrelevant A*0201-tetramer (table 5 B.). The numbers indicated in the table represent percentages of wells containing either CEA-004+ or CEA-005+ CTLs. The lot of human serum used for all experiments was C02104-0167.

Table 5 A.

Antigenic stimulus	Wells with CEA-004+ tetramer+ cells
CEA-004	50/60 (83%)
CEA-005	19/72 (26%)

Table 5 B.

Antigenic stimulus	Wells with CEA-004+ tetramer+

	cells
CEA-004	50/60 (83%)
CEA-005	46/72 (64%)

CD8+ T cells were isolated from PBMCs, primed in vitro using artificial antigen presenting cells loaded with CEA-004, RSL-001 or DDX5-001 peptide, respectively. After three cycles of stimulation, the detection of peptide-reactive cells was performed by staining with CEA-004- plus irrelevant A*0201- tetramers. The values indicated above represent the percentages of CEA-004 specific cells of each stimulated well. RSL-001 and DDX5-001 stimulations served as negative controls. Figure 5 shows the frequencies of CEA-004-specific CD8+ T cells in 4 HLA-A2 healthy donors following in vitro stimulation with CEA-005 as determined by flow cytometric analysis. Threshold values for positive wells are indicated for each donor separately (—) and were defined as 10 fold the median of the negative controls and at least 1%. Wells with percentage values above threshold (≥ 1%) were considered positive and are represented by the pink rhombs, while negative wells are shown by black rhombs.

5. *In vitro* immunogenicity for IMA910 MHC class II presented peptides

T helper cells play an important role in supporting CTLs to activate and sustain immune responses against tumor cells. Therefore, MHC class II peptides were included in IMA910. TGFBI-004, one of the three class II peptides contained in IMA910, was tested for its immunogenic potential in vitro and proved to be an inducer of both specific CD4+ and CD8+ T cells. The generation of CD4+ and functional CD8+ T lymphocytes was shown in experiments using stimulations performed in an autologous system.

Principle of test

Priming and expansion of specific human CD4+ and CD8+ cells were assayed in vitro by priming of monocyte-depleted PBMCs with autologous DCs and restimulation with autologous PBMCs. Briefly, to generate antigen-specific CD4+ T cells, monocyte-depleted PBMCs of one healthy donor (HLA genotype class I: A1/A25/B8/B18 and class II: DQB1*02/DQB1*06/DRB1*03/DRB1*15/DRB3/DRB5) were stimulated using peptide-pulsed autologous DCs and restimulated with autologous PBMCs plus peptide. As a read-out system, IFNγ production upon short term restimulation was assessed by ELISPOT and flow cytometry. T cells were analysed after eight stimulations by ELISPOT and intracellular IFNγ

staining plus CD4-FITC and CD8-PerCP to determine the percentage of IFNγ-producing cells in specific T-cell subpopulations. In this experiment, cells stimulated with TGFBI-004 peptide from different wells were pooled, incubated with irrelevant peptide for the read-out and performed as negative controls.

Generation of dendritic cells (DCs)

Human DCs were obtained from monocytes cultered in DC medium consisting of RPMI 1640-Glutamax/25mM Hepes (Invitrogen, Germany) supplemented with 10% autologous plasma // 100 U/ml penicillin and 100 µg/ml streptomycin. First, buffy coat and plasma was obtained by centrifugation of the blood from a healthy donor (Bloodbank Tübingen). PBMCs were then isolated from the buffy coat by standard density gradient separation (Lymphocyte Separation Medium, PAA, Austria) and resuspended in DC medium to determine total cell number. 100-120 Mio of PBMCs were washed, resuspended in 15 ml X-Vivo 20 medium (BioWhittaker, Belgium) and transferred to a cell culture flask. After 2 hours at 37°C, media containing peripheral blood leukocytes (PBL) was removed, adherent monocytes were washed twice with 10 ml PBS and cultured for 6 days in 10 ml DC medium with 100 ng/ml GM-CSF and 30 ng/ml IL-4 (ImmunoTools, Germany) or 20 ng/ml (R&D systems, Germany). On day 3 and 5 100 ng/ml GM-CSF and 30 ng/ml IL-4 (Immunotools) or 20 ng/ml IL-4 (R&D Systems, Germany) was added. On day 7 immature DCs were activated with 10 ng/ml TNF-α (R&D Systems, Germany) and 20 µg/ml poly(IC) (Sigma Aldrich, Germany) or 100 ng/ml LPS for 24 hours. Remaining PBMCs and obtained PBLs were aliquoted and frozen.

In vitro priming of specific T cells

To generate CD4+ T cells, 3 Mio PBMCs/PBLs were stimulated with 2 × 105 autologuous DCs. DCs were harvested on day 8 (see chapter 3.1, Generation of DCs). PBS with 5 mM EDTA was used for this purpose to gain as many cells as possible (including adherent cells). After washed with DC medium, cell number was determined. For loading with peptide, DCs were resuspended in 1 ml DC medium and incubated with 25 µg/ml peptide for 2 hours at 37° C. Peptides used for pulsing of DCs were TGFBI-004, Posmix (mix of EBV and CMV related peptides), Padre and CMV. Autologous PBMCs/PBLs were thawed, washed with DC medium (at least twice) and plated in a 24 well plate at a density of 3 Mio cells/ml in 1 ml. DCs loaded with peptide were then added (as 1 ml suspension containing the peptide) to the plated PBMCs/PBLs and incubated for 7 days at 37° C. After priminig, obtained CTLs were first

restimulated with cryopreserved autologous peptide-loaded PBMCs which have been irradiated (30 Gy; Gammacell 1000 Elite, Nordion International, Canada). 5 x 10^5 CTLs and 2,5 x 10^6 PBMCs were added per well for this purpose. Pulsing of PBMCs with peptide was performed as aforementioned (for DCs). On day 1 after the first restimulation, IL-2 (R&D Systems, Germany) and IL-7 was added to a final concentration of 2 ng/ml and 5 ng/ml, respectively. Afterwards, every 2nd day and every 7th day IL-2 and IL-7 were added to the media. Second restimulation was done 7 days later, but this time peptide was added alone (without PBMCs) to the cultured CTLs. Restimulations were performed in a 7 day cycle, with peptide-loaded PBMCs and petide alone added alternatively. Analyses were performed after the eight stimulation by intracellular IFNγ staining and IFNγ ELISPOT.

Results

It was possible to prime CD4+ T cell lines specifically reacting to the peptide of interest (Figure 2 and Figure 3). T-cell responses could be detected via ELISPOT in 2 out of 4 T-cell lines, whereas in 3 out of 4 T-cell lines TGFBI-004 specific IFNγ producing CD4+ and/or CD8+ cells were shown via ICS.

Thus, TGFBI-004 was able to elicit CD4+ and CD8+ T cell responses in one donor tested with the above described experimental system. According to this promising result, it is likely that this peptide is immunogenic and has the capacity to induce T-cell responses.

6. Functional validation exemplified by NOX-001 and TGFBI-001

Immunogenicity of peptides included in IMA910 vaccine was demonstrated in vitro by using immatics` TUMAP validation platform. The induction of specific T cells is an indication for the ability of peptides to successfully activate the immune system. Since efficient anti-tumor immune response is only possible when activated T cells are of high avidity and functional, we further investigated the TUMAPs to prime high avidity, functional T lymphocytes by their ability to produce IFNγ or to kill tumor cell lines. Two peptides, NOX-001 and TGFBI-001, were chosen for deeper validation due to their capacity to induce high avidity CTLs in vitro. We were able to prove that high avidity precursor T cells exist against both peptides in humans and that functional CD8+ T cell lines could be generated by NOX-001.

Principle of test

To get additional insight on the immunogenicity of IMA910 peptides and the properties of specific T cells, two peptides, NOX-001 and TGFBI-001, were selected for further evaluation.

The experiments performed for this purpose were conducted at immatics (cell sorting was performed at the University of Tübingen, lab of Dr. Bühring).

Dependent on their ability to be activated by high- or low-density antigen, T cell lines can be divided into high- or low-avidity. As it has been shown before (Walter, S, Herrgen, L, Schoor, O, Jung, G, Wernet, D, Buhring, HJ, Rammensee, HG, and Stevanovic, S; Cutting edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres, J. Immunol., 2003, 171, 4974-4978), human high-avidity CTLs can be raised successfully by using less peptide for activation compared to low-avidity CD8+ T cells. It has also been demonstrated that cells expanded this way are more efficient in recognizing antigen-expressing tumor cell lines, hereby constituting a possible major tool in the development of therapy starategies.

To be able to determine the ability of peptides to generate high-avidity CTL lines, isolated human CD8+ cells were primed and expanded by repeated in vitro stimulations with beads coated with low-density pMHC (peptide-MHC-complex) and anti-CD28 antibody in the presence of IL-12 and IL-2. After three stimulations, a fraction of in vitro primed T cells were pMHC-tetramer stained and detected by cytometric analysis. Tetramer-positive cells of each donor were pooled afterwards according to the antigen specificity, stained with pMHC-tetramer and human anti-CD8-FITC antibody and finally subjected to FACS sorting on a FACSAria. Sorted cells were cultured and expanded in the presence of irradiated feeder cells, cytokines and mitogen. As a read-out for the generation of primed high avidity antigen specific cells, pMHC-tetramer staining was performed. To determine their functionality, IFNγ production was assayed by ELISPOT and killing of tumor cell lines was examined using a cytotoxicity assay based on live/dead staining after restimulation of the cells with the corresponding peptide and tumor cell lines.

Generation of specific CD8+ T-cell lines

In vitro stimulations using artificial antigen presenting cells (aAPC) loaded with peptide-MHC complex (pMHC) and anti-CD28 antibody were conducted as detailed above. The only difference to the method described there was the fact that stimulations were performed with beads loaded with 2 ng relevant plus 200 ng irrelevant library (pMHC)MHC (low density beads) instead of 200 ng relevant MHC (high density beads). Thus, predominantly high avidity T cells were generated for deeper validation of peptides. After three stimulations, a

fraction of in vitro primed T cells was pMHC-tetramer stained and detected by cytometric analysis. Immunogenicity for a given antigen was detected if at least one evaluable in vitro stimulated well of one healthy donor was found to contain a specific CD8+ T-cell line after in vitro stimulation (i.e. this well contained at least 1% of specific tetramer+ among CD8+ T-cells and the percentage of specific tetramer+ cells was at least 10x the median of the negative control stimulations). Tetramer-positive cells of each donor were pooled afterwards according to the antigen specificity, stained with the corresponding pMHC-tetramer and human anti-CD8-FITC antibody clone SK1 and finally subjected to FACS sorting on a FACSAria (BD Biosciences, Germany). Sorted cells were cultured in T cell medium (RPMI-Glutamax supplemented with 10% heat inactivated human AB serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate and 20 µg/ml Gentamycin) in the presence of 5 x 105 cells/ml irradiated fresh allogeneic PBMCs, 5 x 104 cells/ml irradiated LG2-EBV cells, 150 U/ml IL-2 (Chiron, Munich, Germany) and 0,5 µg/ml PHA-L (Roche Diagnostics, Mannheim, Germany). Expansion of these cells occurred in T cell medium containing 150 U/ml IL-2. As a read-out for the generation of primed high avidity antigen specific cells, pMHC-tetramer staining was performed as above and analyzed on a four-color FACSCalibur (BD Biosciences, Germany).

Functionality tests

To determine their functionality, IFNγ production was assessed by ELISPOT (IFNγ ELISPOT Set, BD, Germany) after restimulation of the cells with the corresponding peptide. Additionally, cell-mediated cytotoxicity of specific CTLs was investigated by killing of tumor cell lines using the LIVE/DEAD cell-mediated cytotoxicity Kit (L7010, Invitrogen, Germany). Both assays were performed according to manufacturer's instructions, except noted otherwise.

Results

Both peptides, NOX-001 and TGFBI-001, were immunogenic in vitro as shown by successful priming with low pMHC density aAPCs. For NOX-001 as well as for TGFBI-001 specific T-cell lines could be established by FACS, thus demonstrating that high-avidity CD8+ T cell precursors exist in healthy donors.

Additionally, for NOX-001, one T-cell line could be established that also proved to be functional by ELISPOT since it was specifically expressing IFNγ after restimulation with this peptide (Fig. 4).

7. Binding of HLA class I-restricted peptides of the invention to HLA-A*0201

The objective of this analysis was to evaluate the affinity of the HLA class I peptides to the MHC molecule coded by the HLA-A*0201 allele as this is an important parameter for the mode of action of IMA910. Affinities to HLA-A*0201 were high for 9 of 10 HLA class I-restricted peptides in IMA910, dissociations constants (KD) being in the range from 0.001 to 0.2 nM. Also the viral marker peptide IMA-HBV-001 showed strong binding. Affinity for IMA-MUC-001 was about two decades weaker. These results confirmed the strong binding affinity of 9 out of 10 HLA class I peptides of the IMA910 vaccine candidate to MHC molecules.

Principle of test

Stable HLA/peptide complexes consist of three molecules: HLA heavy chain, beta-2 microglobulin (b2m) and the peptidic ligand. The activity of denatured recombinant HLA-A*0201 heavy chain molecules alone can be preserved making them functional equivalents of "empty HLA-A*0201 molecules". When diluted into aqueous buffer containing b2m and an appropriate peptide, these molecules fold rapidly and efficiently in an entirely peptide-dependent manner. The availability of these molecules is used in an ELISA-based assay to measure the affinity of interaction between peptide and HLA class I molecule (Sylvester-Hvid et al., 2002).

Purified recombinant HLA-A*0201 molecules were incubated together with b2m and graded doses of the peptide of interest. The amount of de novo-folded HLA/peptide complexes was determined by a quantitative ELISA. Dissociation constants (KD values) were calculated using a standard curve recorded from dilutions of a calibrant HLA/peptide complex.

Results

Results are shown in Figure 6. A lower KD value reflects higher affinity to HLA-A*0201. Most of the IMA910 peptides and the viral control peptide IMA-HBV-001 had similar and strong affinities to HLA-A*0201 within the range from 0.001 (IMA-TGFBI-001) to 0.2 nM (IMA-ODC-001). Affinity of IMA-MUC-001 was about two to three decades lower as

compared to the majority of the included ligands. However, vaccination with IMA-MUC-001 led to immune responses in renal cell carcinoma patients in an earlier clinical trial conducted by immatics, thus the lower binding affinity of IMA-MUC-001 gives no cause for concern.

Claims

1. A pharmaceutical composition comprising at least two peptides containing an amino acid sequence selected from the group consisting of SEQ ID NO 1 to SEQ ID NO 7, and/or containing a variant amino acid sequence that is at least 80 % identical to that of SEQ ID NO 1 to SEQ ID NO 7, and/or a polynucleotide containing a nucleic acid encoding SEQ ID NO 1 to SEQ ID NO 7 or the variant amino acid sequence, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition according to claim 1, further comprising at least one additional peptide containing an amino acid sequence selected from the group consisting of SEQ ID NO 8 to SEQ ID NO 15, or containing a variant amino acid sequence that is at least 80 % identical to that of SEQ ID NO 8 to SEQ ID NO 15, or polynucleotide containing a nucleic acid encoding SEQ ID NO 8 to SEQ ID NO 15 or the variant amino acid sequence.

3. The pharmaceutical composition according to claim 1 or 2, wherein the peptides have an overall length of between 8 and 100, preferably between 8 and 30, and most preferred between 8 and 16 amino acids.

4. The pharmaceutical composition according to any of claims 1 to 3, wherein the at least one peptide includes non-peptide bonds.

5. The pharmaceutical composition according to any of claims 1 to 4, comprising at least two peptides consisting of amino acid sequences according to SEQ ID NO 1 to SEQ ID NO 15.

6. The pharmaceutical composition according to any of claims 1 to 5, wherein the selection, number and/or amount of peptides present in the composition is/are tissue, cancer, and/or patient-specific.

7. The pharmaceutical composition according to any of claims 1 to 6, further comprising at least one suitable adjuvant, selected from the group comprising 1018 ISS, aluminium salts, Amplivax, AS15, BCG, CP-870,893, CpG7909, CyaA, dSLIM, GM-CSF, IC30, IC31, Imiquimod, ImuFact IMP321, IS Patch, ISCOMATRIX, JuvImmune, LipoVac, MF59, monophosphoryl lipid A, Montanide IMS 1312, Montanide ISA 206, Montanide ISA 50V, Montanide ISA-51, OK-432, OM-174, OM-197-MP-EC, ONTAK, PepTel vector system, PLG microparticles, resiquimod, SRL172, Virosomes and other Virus-like particles, YF-17DBCG, Aquila's QS21 stimulon, Ribi's Detox. Quil, Superfos, Freund's, GM-CSF, cholera toxin, immunological adjuvants, MF59, and cytokines.

8. The pharmaceutical composition according to claim 7, wherein the adjuvant is selected from the group consisting of colony-stimulating factors, such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF).

9. The pharmaceutical composition according to any one of claims 1 to 8, additionally containing at least one antigen presenting cell.

10. The pharmaceutical composition according to claim 9, wherein the antigen presenting cell is a dendritic cell.

11. The pharmaceutical composition according to claim 9 or 10, wherein the at least one antigen presenting cell is
    a. pulsed or loaded with the peptide or
    b. comprises an expression construct encoding the peptide.

12. The pharmaceutical composition of any one of the preceding claims, wherein the composition is administered wherein the vaccine is administered intravenously, intra-arterially, intra-peritoneally, intramuscularly, intradermally, intratumorally, orally, dermally, nasally, buccally, rectally, vaginally, by inhalation, or by topical administration.

13. A method for treating or preventing a cancer in a patient comprising administering to the patient a therapeutically effective amount of the pharmaceutical composition of any one of the preceding claims.

14. The method according to claim 13, wherein the pharmaceutical composition is an anti-cancer vaccine.

15. The method of claim 14, wherein the cancer is cancer of the buccal cavity and pharynx, cancer of the digestive tract, cancer of the colon, rectum, and anus , cancer of the respiratory tract, breast cancer, cancer of the cervix uteri, vagina, and vulva, cancer of the uterine corpus and ovary, cancer of the male genital tract, cancer of the urinary tract, cancer of the bone and soft tissue, and Kaposi sarcoma, melanoma of the skin, eye melanoma, and non-melanoma eye cancer, cancer of the brain and central nervous system, cancer of the thyroid and other endocrine glands , Hodgkin Lymphoma, Non-Hodgkin Lymphoma, and myeloma, preferably renal cancer, colorectal cancer, lung cancer, breast cancer, pancreatic cancer, prostate cancer, gastric cancer, GIST or glioblastoma.

16. The method according to claim 15, wherein the cancer is colorectal cancer.