Title: BI- OR MULTISPECIFIC POLYPEPTIDES BINDING IMMUNE EFFECCTOR CELL SURFACE ANTIGENS AND HBV ANTIGENS FOR TREATING HBV INFECTIONS AND ASSOCIATED CONDITIONS

Abstract: The present invention relates to a polypeptide comprising (a) a first set of six complementarity determining regions (CDRs) configured to bind a first antigen; and (b) (ba) a second set of six CDRs configured to bind a second antigen; or (bb) a ligand capable of binding to a second antigen; wherein (i) said first antigen is selected from Hepatitis B virus (HBV) small surface antigen; HBV medium surface antigen; and HBV large surface antigen; and (ii) said second antigen is selected from surface antigens presented by immune effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). Also provided are compositions for use in a method of treating or preventing HBV infection and/or a condition caused by said HBV infection, said condition caused by said HBV infection being selected from liver cirrhosis and hepatocellular carcinoma.
The present invention relates to a polypeptide comprising (a) a first set of six complementarity determining regions (CDRs) configured to bind a first antigen; and (b) (ba) a second set of six CDRs configured to bind a second antigen; or (bb) a ligand capable of binding to a second antigen; wherein (i) said first antigen is selected from Hepatitis B virus (HBV) small surface antigen; HBV medium surface antigen; and HBV large surface antigen; and (ii) said second antigen is selected from surface antigens presented by immune effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).

In this specification, a number of documents including patent applications and manufacturer’s manuals is cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

About 350 million humans are chronically infected with hepatitis B virus (HBV). HBV infection may entail liver cirrhosis and hepatocellular carcinoma (HCC) which cause about one million casualties per year (Ganem et al., Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med; 350:1118-29 (2004)). Infections with HBV presently cannot be controlled in about 5% of adult patients and about 90% of newborns. In such a case, the HBV infection becomes chronic. The likely cause is an insufficient cellular immune response. The presently available antiviral drugs, which are used for treatment of HBV infection, inhibit viral replication. However, the covalently closed circular DNA (cccDNA) remains in the nucleus of infected hepatocytes and may cause a reactivation of the HBV infection once the patient stops to take the medication. Therefore, it would be indispensable to eliminate HBV infected cells carrying said cccDNA if the infection were to be cured completely (Protzer et al., Nat Immunol Rev 12: 2013-213 (2012)).

However, such cytotoxic elimination of HBV infected cells (be it by cytotoxic T lymphocytes or natural killer (NK) cells) does not occur or not to a sufficient degree.
Infected cells carrying a HBV cccDNA display on their surface viral surface proteins. It is presumed that this occurs although the virus is released into intracellular vesicles because a number of HBV surface proteins remain integrated into the intracellular membrane of the endoplasmatic reticulum. In the course of vesicle transport processes said intracellular membrane may fuse with the cellular membrane, the consequence being that HBV surface proteins are displayed on the surface of the infected cell.

Bohne et al. (T cells redirected against hepatitis B virus surface proteins eliminate infected hepatocytes. Gastroenterology; 134:239-247 (2008)) and Krebs et al. (T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelope Proteins Control Virus Replication in Mice. Gastroenterology (2013)) describe chimeric antigen receptors which, when retrovirally delivered and expressed on the surface of a T cell, enable primary human and murine T cells to recognize hepatocytes displaying HBV small surface antigen and lyse HBV replicating cells.

Bispecific antibodies are typically employed in the field of oncology. As an example, we refer to Hartmann et al. (Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. Blood; 89:2042-7 (1997)).

EP 2 524 699 A1 describes trifunctional antibodies. These antibodies “have a functional Fc portion” and “must consist of heavy immunoglobulin chains of different subclasses”. Hornig und Färber-Schwarz on the other hand describe in Chapter 40 of “Antibody Engineering” (ed. Patrick Channes, Humane Press, 2012) an scFv construct which is devoid of the Fc portion.

Liao et al. (Oncology Reports 3, 637-644 (1996)) describe bispecific monochlonal antibodies retargeting effector cells for lysis of human hepatoma xenografts in nude mice. The bispecific antibodies described are generated by the fusion of two hybridomas, resulting in a hybridoma cell line expressing the heavy/light chain combinations of two distinct antibodies. This may result in pairing of the two different heavy chains, but also in the pairing of identical heavy chains, giving rise to a random mixture of mono-specific parental and bi-specific antibodies. The bispecific antibodies contain heavy and light chain and dimerize to form an Ig molecule which is not a single polypeptide chain.

In view of the prior art, the technical problem could be seen in the provision of alternative or improved means and methods of treating HBV infection as well as conditions caused by HBV
infection such as liver cirrhosis or hepatocellular carcinoma. Expressed in terms of cell biology, the technical problem can be seen in the provision of means and methods for the eradication of cells bearing HBV cccDNA. This technical problem is solved by the enclosed claims.

Accordingly, the present invention relates in a first aspect to a polypeptide comprising (a) a first set of six complementarity determining regions (CDRs) configured to bind a first antigen; and (b) (ba) a second set of six CDRs configured to bind a second antigen; or (bb) a ligand capable of binding to a second antigen; wherein (i) said first antigen is selected from HBV small surface antigen; HBV medium surface antigen; and HBV large surface antigen; and (ii) said second antigen is selected from surface antigens presented by immune effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).

The term “polypeptide” defines a molecule which is a polycondensate of amino acids which form one single chain with one N-terminus and one C-terminus. The constituent amino acids include the 20 naturally occurring proteinogenic amino acids. Preferably, said polypeptide consists exclusively of said naturally occurring proteinogenic amino acids. Having said that, the term extends to molecules which, in addition to said naturally occurring proteinogenic amino acids, contain up to 20%, 10%, 5%, 2%, or 1% amino acids which are selected from non-naturally occurring α-amino acids, β-amino acids, D-amino acids, selenocysteine, selenomethionine, hydroxyproline, pyrrolysine and ornithine. It is furthermore understood that one or more such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids may be phosphorylated. The latter applies in particular to serine, threonine and tyrosine. Also other post-translational modifications as known in the art may be present including glycosylation. Glycosylations include N-linked glycosylations, typically at an asparagine and O-linked glycosylations, typically at serine or threonine residues. N- and/or C-terminus may be protected, protection groups including acetyl for the N-terminus and amine for the C-terminus. The type of linkage between the amino acids comprised in said polypeptide is confined to amide (CONH) bonds. The term “amide bond” includes peptide bonds which connect the α-carboxylate of a given amino acid to the α-amino group of the subsequent amino acid. The “amide bond” also extends to isopeptide bonds which is an amide bond that is not present on the main chain of the polypeptide. For example, instead of an α-amino group, the side chain amino group of lysine may be involved. Similarly, instead of the α-carboxyl group, the side chain carboxylate of glutamate or aspartate may be involved. The occurrence of one or more such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 isopeptide bonds is envisaged. Preference is given, though, to polypeptides wherein the constituent amino acids are linked to each other exclusively by peptide bonds.
In general, there is no upper limit on the number of amino acids in a polypeptide. As can be seen from the exemplary polypeptide sequences comprised in the sequence listing, the polypeptides of the present invention typically contain several hundred amino acids, preferably between 250 and 1000, 400 and 900, or between 700 and 800 amino acids. It is common to distinguish between peptides on the one hand and polypeptide on the other hand, wherein peptides have 30 or less amino acids and polypeptides have more than 30 amino acids.

The term “complementarity determining region”, abbreviated as “CDR” has its meaning as established in the art. These are short subsequences, typically in the range from about 3 to about 25 amino acids, which confer to an antibody the capability to specifically recognize an epitope of an antigen. In general, the variable domain of the light chain of an antibody provides three CDRs and the variable domain of the heavy chain of an antibody provides three CDRs. While CDRs are typically part of immunoglobulin domains, there is no requirement in that respect in accordance with the present invention. What is sufficient is an amino acid sequence, which comprises said CDRs provided that said amino acid sequence, when folded under physiological conditions, presents said CDRs in spatial proximity and maintains their capability to recognize the cognate antigen. The mentioned spatial proximity and capability of antigen binding is expressed by the term “configured to bind an antigen” as used in the above disclosed main embodiment. The term “immunoglobulin domain” is known in the art and refers to a sequence of typically 70 to 100 amino acids assuming a three-dimensional structure of a 2-layer sandwich of between 7 and 9 anti-parallel β-strands.

Said first set of six CDRs as well as said second set of six CDRs each define a binding site.

It is understood that beyond said first set and said second set no further CDRs are present in the polypeptide of the invention.

The term “antigen” has its art-established meaning. It refers to a molecule which is specifically recognized and bound by a set of six CDRs which typically are presented by immunoglobulin domains. The specific part of an antigen recognized and bound by said CDRs is also known as epitope.

The term “ligand” has its art-established meaning. A ligand is the counter-structure to a receptor. More specifically a ligand is capable of binding, preferably specifically binding to its cognate receptor. In accordance with the invention, said ligand is preferably an immunoligand.
An immunoligand is a ligand which is capable of binding to a receptor present on the surface of an immune effector cell. Preferred immune effector cells are, as defined above, NK cells and CTLs. Preferred are those immunoligands which, when bound to their cognate receptor on the surface of an immune effector cell, exert a stimulating and/or co-stimulating effect. The terms “activate” and “stimulate” are used equivalently in this context. Receptors bound by preferred immunoligands are specified further below.

HBV S/M/L surface proteins are the small, medium and large surface proteins in the outer envelope of HBV (Stibbe, W., and W. H. Gerlich. Structural relationships between minor and major proteins of hepatitis B surface antigen. J. Virol. 1983 46:626-628).

The three HBV surface antigens are transcribed and translated from one reading frame and differ from each other by the length of the N-terminal part. Accordingly, the large surface antigen comprises a part which is neither present in the medium nor in the small surface antigen, and the medium surface antigen comprises a part which – while being comprised in the large antigen – is not comprised in the small antigen. The small antigen consists of a sequence, which is comprised in the C-terminal part of both the medium and the large antigen.

The large HBV surface antigen may be inserted in two manners in the cytoplasmic membrane. Either the N-terminus or the C-terminus may be located on the extracellular side. Both configurations are found in HBV infected cells.

The recited second antigen is a surface antigen presented by immune effector cells, preferably specifically presented by NK cells and/or CTLs. Immune effector cells are the cells to be redirected to HBV infected cells, said HBV infected cells presenting the mentioned HBV surface antigens on their surface.

It is particularly preferred that binding in accordance with the invention, in particular between CDRs and antigens as well as between ligands and antigens is specific. The terms "specifically binds" and "specifically binding" (having the same meaning as "specifically interacting") as used in accordance with the present invention mean that these binding portions do not or essentially do not cross-react with an epitope or a structure similar to that of the target antigen. Cross-reactivity of a panel of molecules under investigation may be tested, for example, by assessing binding of said panel of molecules under conventional conditions to the epitope of interest as well as to a number of more or less (structurally and/or functionally) closely related epitopes. Only those molecules that bind to the epitope of interest in its
relevant context (e.g. a specific motif in the structure of a protein) but do not or do not 

essentially bind to any of the other epitopes are considered specific for the epitope of interest.

The first aspect comprises embodiments wherein items (a) and (ba) together are the only 

binding sites present on said polypeptide as well as embodiments wherein items (a) and (bb) 
together are only binding sites present on said polypeptide.

Chronic HBV infection is characterized by an immuno-tolerant status. More specifically, the 

patient's CTLs and NK cells perform such that a complete eradication of infected cells or a 

complete control of HBV replication or a complete elimination of HBV does not occur. The 
polypeptides according to the invention are bispecific molecules in the sense that they 
specifically recognize a HBV surface antigen on the one hand and an immune effector cell 
surface antigen on the other hand. Such bispecific molecules could be seen as conferring an 
artificial specificity to immune effector cells. In fact, CTLs and NK cells are retargeted by the 
polypeptides of the invention (also referred to as being “bispecific”) such that they are 
recruited to HBV infected cells and kill them.

Binding of the polypeptides of the invention to HBV infected cells on the one hand and 

recruiting of immune effector cells on the other hand may occur in any order or also 
simultaneously.

In particular, it is intended to systemically apply polypeptides of the invention by either 

injection or as an oral application form and allow them to bind to HBV-infected or HBV antigen 
expressing target cells and recruit said immune effector cells to said target cells.

Having said that, it is also envisaged to bring polypeptides of the invention into contact with 

immune effector cells (or a population of peripheral blood mononuclear cells comprising said 
effector cells) such that said effector cells get loaded with said polypeptides. Such effector 
cells (or a population of PBMCs comprising such loaded effector cells) which have been 
loaded in vitro or ex vivo may then be administered to a patient suffering from HBV infection or 
a condition associated therewith and defined below. Such administering may be effected 
intravenously, e.g. to the Arteria hepatica. An immune effector cell with a polypeptide 
according to the present invention being bound to a surface antigen of said immune effector 
cell is also an aspect of the present invention. This aspect is disclosed further below.

This killing, in particular in conjunction with antiviral immune mediators (e.g. cytokines) as
secreted by immune cells, provide for the eradication of HBV infection or for the sustained control of HBV infection or for the elimination of tumor cells expressing HBV surface antigens. Preferred or exemplary bispecific polypeptides in accordance with the present invention provide for astonishingly high killing rates of HBV-infected cells or liver tumor cells (also known as hepatoma cells) replicating HBV or expressing HBV surface antigens; see the examples enclosed herewith.

Given that bispecific polypeptides according to the present invention provide tailored specificities to immune effector cells, the naturally inherent specificity of the immune effector cells or the presentation of antigens to them becomes irrelevant. As such, a large pool of candidate effector cells is amenable to retargeting. Furthermore, the polypeptides of the invention have a bioavailability and half-life which is at least comparable to that of monoclonal antibodies.

In a preferred embodiment (a) said first set of six CDRs is comprised in a first scFv fragment; and/or (b) said second set of six CDRs is comprised in a second scFv fragment; or (bb) said ligand is an immunoligand, preferably capable of binding to NKG2D/CD314 (such as ligands MICA, MICB, ULBP1-6), NKp30/NCR3/CD337 (such as ligand B7-H6), 4-1BB/CD137 (such as ligand 4-1BB-1/L/CD137L) or OX40/CD134 (such as ligand OX40-L/CD252). A slash (/) separates alternative art-established designations. In brackets preferred representatives of a given genus of antigens are provided.

The term "scFv" is well-established in the art. The abbreviation stands for "single chain variable fragment" of an antibody and defines a polypeptide capable of specifically recognizing and binding the epitope of an antigen. As noted above, three CDRs are presented by the variable domain of an antibody light chain (V_L) and three CDRs are presented by the variable domain of a heavy chain (V_H) of an antibody. In an scFv two variable domains are connected to each other by a peptide linker. The obtained fusion construct is a single polypeptide chain. This provides for easy expression of the scFv molecule. A schematic drawing can be found in Figure 1.

The terms "V_H domain" and "V_L domain" are used according to the definitions provided in the art. Thus, they refer to the variable region of the heavy chain (V_H) and the variable region of the light chain (V_L) of immunoglobulins, respectively. Generally, V_H and V_L domains comprise three complementarity determining regions (CDRs) each, wherein CDRs are highly variable regions mainly responsible for the binding of the antigen.
A peptide linker is preferably used to link either variable regions of the scFv or to link the scFv to the dimerization and/or spacer region, preferably to the Fc. Typically the peptide linkers have a length between 3 and 30 amino acids, preferably between 5 and 25 or 10 and 20 amino acids. Preference is given to those linkers, which do not or not substantially interfere with structure and or function of the domains or polypeptides they connect (connecting yields a single continuous polypeptide chain). Linkers include Gly-rich linkers such as the (Gly₃Ser)₃ (SEQ ID NO: 47) linker which is used in the preferred polypeptides of the invention for connecting the \( V_H / V_L \) domains of CTL or NK specific scFvs, and the Yol linker (SEQ ID NO: 48; AKTTPKLEEGEFSEARV, as described in Sellrie et al., Journal of Biochemistry and Molecular Biology, Vol. 40, No. 6, November 2007, pp. 875-880) which is used in the preferred polypeptides of the invention for connecting the \( V_H / V_L \) domains of the scFvs specific for HBV surface antigens. Also the (Gly₃Ser)₄ linker (SEQ ID NO: 49) may be used for connecting the \( V_H / V_L \) domains of the scFvs specific for HBV surface antigens.

The term "antibody" as used herein has its art-established meaning. Preferably, it refers to the monoclonal antibody. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals with modifications developed by the art. Furthermore, antibodies or fragments thereof directed to the aforementioned HBV surface proteins can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. The production of chimeric antibodies is described, for example, in WO89/09622. A further source of antibodies to be utilized in accordance with the present invention are so-called xenogenic antibodies. The general principle for the production of xenogenic antibodies such as human antibodies in mice is described in, e.g., WO 91/10741, WO 94/02602, WO 96/34096 and WO 96/33735. Antibodies to be employed in accordance with the invention or their corresponding immunoglobulin chain(s) can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination. Methods for introducing such modifications in the DNA or polypeptide sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989. Modifications of the polypeptides include also post-translational modifications such as glycosylations.
In a further preferred embodiment, said first set of six CDRs binds an epitope of said first antigen which epitope is located (a) in said HBV small surface antigen; or (b) in the part of said HBV large surface antigen which is not comprised by said HBV small surface antigen; or (c) in a part of said HBV large surface antigen which part varies in structure from said HBV small surface antigen.

Item (a) refers to epitopes present in the HBV small surface antigen. Owing to the above described relation between small, medium and large HBV surface antigen, the entire sequence of the small antigen is comprised in the medium and the large antigen. In general, but not necessarily, a three-dimensional epitope presented by the small surface antigen will also be presented by the medium and/or the large surface antigen.

In accordance with item (b) it is preferred that said part of said HBV large surface antigen is also not comprised by said HBV medium surface antigen. As regards item (c), it is understood that “varying in structure” includes epitopes of said HBV large surface antigen which comprise or consist of sequences which are part of the sequence of said HBV small surface antigen, wherein said epitopes are not present on said HBV small surface antigen. In accordance with item (c) it is furthermore preferred that said epitope is in a part of said HBV large surface antigen which part varies in structure also from said HBV medium surface antigen.

Said item (a), i.e. said first antigen being said HBV small surface antigen is particularly preferred in conjunction with all aspects and embodiments of this invention.

In accordance with items (b) and (c), the polypeptide will specifically recognize the large surface antigen of HBV.

In a further preferred embodiment said surface antigen presented by immune effector cells is selected from CD3, CD28, 4-1BB, OX40, CD16, CD56, NKG2D, and Nkp30/NCR3. Accordingly, the present invention provides a polypeptide comprising (a) a first set of six complementarity determining regions (CDRs) configured to bind a first antigen; and (b) (ba) a second set of six CDRs configured to bind a second antigen; or (bb) a ligand capable of binding to a second antigen; wherein (i) said first antigen is selected from hepatitis B virus (HBV) small surface antigen; HBV medium surface antigen; and HBV large surface antigen; and (ii) said second antigen is selected from surface antigens presented by immune effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), wherein (c) said first
set of six CDRs is comprised in a first scFv fragment; and (d) (da) said second set of six CDRs is comprised in a second scFv fragment; or (db) said ligand is an immunoligand capable of binding to NKG2D such as ligands MICA, MICB, ULBP1-6; NKp30 such as ligand B7-H6, 4-1BB such as ligand 4-1BB-L; or OX40 such as ligand OX40-L; and wherein said surface antigen presented by immune effector cells is selected from CD3, CD28, 4-1BB, OX40, CD16, CD56, NKG2D, and NKp30.

CD3 stands for the CD3 epsilon chain, which is part of the CD3-T-cell receptor complex. (Borst, J. et al., The delta- and epsilon-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature. 1984. 312: 455-458).


OX40 (CD134) is a secondary costimulatory receptor. (Arch, R. H. et al., Mol. Cell. Biol. 1998. 18: 558-565). 4-1BB and OX40 are members of a tumor necrosis factor (TNF) receptor family that bind TNF receptor-associated ligands and activate nuclear factor kappaB.

CD16 (FcγRIIIa) is a low affinity Fc receptor expressed by NK cells, a subset of activated cytotoxic T cells as well by cell types from the myelomonocytic lineage, binding to the Fc domain of IgG molecules. (Lanier, L.L. et al., Functional properties of a unique subset of cytotoxic CD3+ T lymphocytes that express Fc receptors for IgG (CD16/Leu-11 antigen). J. Exp. Med. 1985. 162: 2089-2106).


CD3, CD28, 4-1BB and OX40 are present on the surface of CTLs. Binding of a polypeptide of the invention to any of these surface antigen entails stimulation or co-stimulation of CTLs.

CD16, CD56, NKG2D, NKp30/NCR3 and 4-1BB are present on the surface of NK cells. Binding of a polypeptide of the invention to any of these surface antigens entails stimulation or co-stimulation of NK cells.

With regard to human CTLs, preference is given to CD3 and CD28. With regard to human NK cells, preference is given to CD16 and CD56.

The mentioned surface antigens are designated by art-established names, (see also Kenneth Murphy, Janeway's Immunobiology, 7th edition, Garland Science; William E. Paul, Fundamental Immunology, 7th edition, Lippincott Williams & Wilkins).

In a further preferred embodiment, said polypeptide further comprises a dimerization region. Said dimerization region may provide for covalent and/or non-covalent dimerization.

Through dimerization the bispecific bivalent antibodies are rendered bispecific tetravalent (or even tetraspecific tetravalent if different bispecific antibodies are co-expressed in the producing cell). Bispecific tetravalent reagents as described here are expected to possess an augmented avidity that is similar to conventional monospecific antibodies since they are able to engage two antigen molecules of the same type with their N-terminal side and their C-terminal side, respectively.

In a particularly preferred embodiment, said dimerization region that connects two polypeptides of the invention consists of the hinge region of the IgG heavy chain or comprises the cysteine residues responsible for the dimerization of the heavy chains of an antibody. Preferably, said dimerization region consists of a subsequence of 32 amino acids in length, the so called hinge region of the heavy chain (EPKSSDKTHTCPPCPEPFEGAPSVFLPPKP, see SEQ ID NOs: 43 to 46) and comprises the two cysteine residues (underlined in above sequence) responsible for the dimerization of
the heavy chains. Preferably the single cysteine residue within the hinge region of the IgG heavy chain that mediates the intermolecular disulfide bond between the IgG heavy and light chain constant domains in a natural antibody is mutated into a serine in order to prevent aberrant disulfide bridges.

Dimerization domains suitable for non-covalent dimerization are known in the art and include leucine zippers.

In a further preferred embodiment, said polypeptide further comprises a spacer region, said spacer region preferably comprising a CH2 domain and a CH3 domain, said spacer region being located between (i) said first scFv fragment and (ii) said second scFv fragment or said recombinant ligand in the amino acid sequence of said polypeptide.

A spacer region comprising or consisting of a CH2 domain and a CH3 domain, in particular from IgG, is advantageous. Their capability to bind protein A provides for an efficient secretion from producer cells and/or the subsequent purification from the reagents.

Both said CH2 and CH3 domain on the one hand and said dimerization region on the other hand may be provided by the corresponding region of an IgG, preferably IgG1 or IgG2 molecule, even more preferred a human IgG1 or IgG2 (hlgG1, hlgG2) molecule. A preferred subsequence of a hlgG1 molecule providing CH2 domain, CH3 domain and dimerization domain can be seen in sequences 43 to 46. Preferably – and this applies to the mentioned sequences – the portion of hlgG1, in particular said CH2 domain, was mutagenized in multiple positions to diminish or abolish the binding to Fc receptors (indicated in bold-face italics in the sequences given further below). More generally, the Fc region, in particular the CH2 domain and/or the CH3 domain may be mutated in one or more positions to diminish or abolish the binding to Fc receptors. Such procedure is known in the art and described, for example, in Armour et al., Recombinant human IgG molecules lacking Fcgamma receptor I binding and monocyte triggering activities. Eur. J. Immunol. 1999. 29: 2613-2624 and Lazar et al., Engineered antibody Fc variants with enhanced effector function. Proc. Natl. Acad. Sci. U. S. A. 2006. 103: 4005-4010. This is advantageous because triggering of antibody dependent cell-mediated cytotoxicity (ADCC) is not preferred in accordance with the invention.

In other words, an antibody Fc fragment may be used to implement spacer region and dimerization region. The term "Fc fragment" is known to the skilled person and defines a fragment of IgG which is obtained by cleavage with papain and comprises CH2 and CH3
domains.

Between said first scFv fragment and said spacer region and/or between said spacer region and said second scFv fragment (a) linker sequence(s) is/are present. Preferred linker sequences are disclosed herein above. As can be seen from the preferred sequences comprised in the sequence listing, in particular sequences of SEQ ID NOs: 43 to 46, such linker sequences may consist of glycines or glycines and serines.

Figure 2 illustrates the molecular architecture of those preferred polypeptides of the invention which comprise a dimerization region (hlgG hinge region) as well as a CH2 and a CH3 region separating the two scFv fragments from each other.

The terms “CH2 domain” and “CH3 domain” have its art-established meaning. They refer to the second and third constant domain of antibody heavy chains.

It is understood that a particularly preferred embodiment relates to a polypeptide comprising (a) a first set of six complementarity determining regions (CDRs) configured to bind a first antigen; and (b) (ba) a second set of six CDRs configured to bind a second antigen; or (bb) a ligand capable of binding to a second antigen; wherein (i) said first antigen is selected from hepatitis B virus (HBV) small surface antigen; HBV medium surface antigen; and HBV large surface antigen; and (ii) said second antigen is selected from surface antigens presented by immune effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), wherein (c) said first set of six CDRs is comprised in a first scFv fragment; and (d) (da) said second set of six CDRs is comprised in a second scFv fragment; or (db) said ligand is an immunoligand, preferably capable of binding to NKG2D such as ligands MICA, MICB, ULBP1-6; NKp30 such as ligand B7-H6; 4-1BB such as ligand 4-1BB-L; or OX40 such as ligand OX40-L, wherein said surface antigen presented by immune effector cells is selected from CD3, CD28, 4-1BB, OX40, CD16, CD56, NKG2D, NKp30 and 4-1BB, and wherein said polypeptide further comprises a dimerization region and a spacer region, said dimerization region and said spacer region preferably being as further defined above.

In a further preferred embodiment, (a) said first set of six CDRs has the sequences of SEQ ID NOs: 1 to 6, 7 to 12 or 13 to 18; and/or (b) said second set of six CDRs has the sequences of SEQ ID NOs: 19 to 24, 25 to 30, 31 to 36 or 37 to 42.

As common in the art, and furthermore as evident from the enclosed sequence listing, the
ordering of the CDRs in each set of six CDRs as specified above is as follows: CDR1 of heavy chain, CDR2 of heavy chain, CDR3 of heavy chain, CDR1 of light chain, CDR2 of light chain, and CDR3 of light chain.

C8, 5F9, 5A19, OKT3, 9.3, A9 and NCAM29.2 as used in the sequence listing designate the antibody from which the respective CDRs originate from and refer to a preferred anti-HBs antibody, to a second different anti-HBs antibody, an antibody against HBV large surface antigen, an antibody against human CD3, an antibody against human CD28, an antibody against human CD16, and an antibody against human CD56, respectively. “HBs” designates the HBV small surface antigen.

Particularly preferred is that said polypeptide comprises or consists of the amino acid sequence of any one of SEQ ID NOs: 43 to 46 or an amino acid sequence which exhibits at least 80% identity to any one of SEQ ID NOs: 43 to 46, provided that the CDRs of said amino acid sequence exhibiting at least 80% identity are identical to those comprised in any one of SEQ ID NOs: 43 to 46, respectively. In SEQ ID NO: 43, the last three residues “GNS” are dispensable.

Preferred levels of sequence identity include at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, and at least 99%. Means and methods for determining sequence identity are well-known in the art. A preferred algorithm for determining pairwise sequence identity is the basic local alignment search tool (BLAST) as described, for example, in McGinnis and Madden (Nucleic Acid Research 32, W20-W25 (2004)).

The location of said CDRs in a given sequence, in the present case in the sequences of SEQ ID NOs: 43 to 46 can be determined with art-established methods, known art-established methods including the systems of Chothia, Kabat and LeFranc/IMGT, respectively. In the absence of any indication to the contrary, it is understood that the CDRs according to the above defined particularly preferred embodiment are those defined above, namely a first set having the sequences of SEQ ID NOs: 1 to 6, 7 to 12 or 13 to 18, and a second set having the sequences of SEQ ID NOs: 19 to 24, 25 to 30, 31 to 36 or 37 to 42. As can be seen from the sequences as comprised in the enclosed sequence listing, these specific CDR sequences (underlined in the sequences reproduced further below) are indeed comprised in the sequences of SEQ ID NOs: 43 to 46.
The sequences of SEQ ID NOs: 1 to 6 define the CDRs and SEQ ID NOs: 37 to 40 define bispecific polypeptides capable of binding a specific epitope within the small surface antigen of HBV. This epitope is located in the a-determinant, which is exposed to the surface of infected cells and virions, respectively. The term “a-determinant” is used to designate a region within the small surface antigen of HBV where the main epitopes for induction of a protective humoral immune response are located. These CDRs as well as the polypeptides of SEQ ID NOs: 43 to 46 have the advantage they can be used for all HBV serotypes.

In a second aspect, the present invention provides a nucleic acid encoding the polypeptides defined above. Preferred embodiments of the polypeptides give rise to corresponding preferred embodiments of said nucleic acid.

The term “nucleic acid” has its art-established meaning and is not particularly limited. Preferred are DNA such as genomic DNA or cDNA as well as RNA such as mRNA. While not being preferred, the use of nucleotide derivatives is envisaged which nucleotide derivatives include 2' derivatized nucleotides such as 2' methyl nucleotides; peptide nucleotides as the occur in peptide nucleic acids and the like.

In a third aspect, the present invention provides a covalently linked complex comprising or consisting of a first and a second polypeptide, wherein there is at least one covalent linkage between said first and said second polypeptide, preferably at least one disulfide bridge between a Cys residue of said first polypeptide and a Cys residue of said second polypeptide, said first and second polypeptides being as defined in accordance with the invention.

Preferred are two covalent linkages between said first and said second polypeptide, preferably two disulfide linkages as depicted in Figure 2.

Also provided is a complex comprising or consisting of a first and a second polypeptide, wherein said first and said second polypeptide are bound to each other non-covalently.

An exemplary drawing of such covalently linked complex is shown in Figure 2. Preference is given to said complex being a dimer.

In a fourth aspect, the present invention provides a composition comprising or consisting of one or more polypeptides according to the invention and/or one or more complexes according to the invention, provided that at least two polypeptides are comprised in said composition
which two polypeptides are distinct from each other with regard to the first antigen and/or the second antigen to which they bind.

In a preferred embodiment of said fourth aspect, said two polypeptides are (a) (i) a polypeptide binding to HBV small or large surface antigen and CD3; and (ii) a polypeptide binding to HBV small or large surface antigen and CD28; or (b) (i) a polypeptide binding to HBV small or large surface antigen and CD16; and (ii) a polypeptide binding to HBV small or large surface antigen and CD56.

Both alternative (a) as well as alternative (b), in particular to the extent they relate to polypeptides binding to HBV small surface antigen, of this preferred embodiment provide for outstandingly high elimination rates of up to 95% as compared to the negative control. This is expected to provide for a complete eradication of HBV infected cells or HBV-antigen positive tumor cells, especially after repeated application in an in vivo situation.

The combined use of bispecific molecules binding to two distinct CTL markers or NK markers has been found to provide for synergistic effects. Figures 3 and 4B show a comparison of specific target cell lysis upon administration of bispecific constructs.

In a particularly preferred embodiment, said two polypeptides comprise or consist of the sequences of (a) SEQ ID NOs: 43 and 44; or (b) SEQ ID NOs: 45 and 46.

Each of sequences SEQ ID NOs: 43 to 46 allows for the formation of two disulfide bridges when a homodimer is formed. Having said that, it is deliberately envisaged to form also heterodimers. An example of a heterodimer would be a covalently linked complex of two polypeptides of the present invention, wherein a first polypeptide would bind to a HBV surface antigen and a first marker presented by an immune effector cell and a second polypeptide would bind to an HBV surface antigen and a second marker of an immune effector cell. The two markers of an immune effector cell may be, for example, CD3 and CD28, or, in the alternative CD16 and CD56.

In a further aspect, the present invention provides a pharmaceutical composition comprising or consisting of one or more polypeptides of the invention, one or more complexes of the invention and/or one or more compositions of the invention.

The pharmaceutical composition may further comprise pharmaceutically acceptable carriers,
excipients and/or diluents. Examples of suitable pharmaceutical carriers, excipients and/or diluents are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc.

Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, subcutaneous or oral administration, these three options being preferred, and furthermore by intraperitoneal, intramuscular, topical, intradermal, intranasal or intrabronchial administration. Formulations for oral administration include tablets and syrups. It is particularly preferred that said administration is carried out by injection. The compositions may also be administered directly to the target site, e.g., by biolistic delivery to an external or internal target site. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Proteinaceous pharmaceutically active matter may be present in amounts between 1 ng and 10 mg/kg body weight per dose; however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. If the regimen is a continuous infusion, it should also be in the range of 1 μg to 10 mg units per kilogram of body weight per minute.

Particularly preferred is intravenous administration.

In a further aspect, the present invention provides one or more polypeptides of any the invention, one or more complexes of the invention and/or one or more compositions of any one of the invention for use in a method of treating or preventing HBV infection and/or a condition caused by said HBV infection, said condition caused by said HBV infection being selected from liver cirrhosis, hepatocellular carcinoma, and liver cancer, said liver cancer being characterized by the expression of one or more HBV surface antigens. It is preferred that said hepatocellular carcinoma is characterized by the expression of one or more of the above defined HBV surface antigens.

In a further aspect, the present invention provides a method of treating or preventing HBV infection and/or a condition caused by said HBV infection, said condition caused by said HBV infection being selected from liver cirrhosis and hepatocellular carcinoma, said method comprising administering a therapeutically effective amount or a preventive amount,
respectively, of one or more polypeptides of the invention, one or more complexes of the invention and/or one or more compositions of the invention to a patient in need thereof.

It is preferred that said pharmaceutical composition, said polypeptide/complex/composition for use in a method of treating and said method of treating, the recited polypeptides, complexes and/or compositions are the only pharmaceutically active agents comprised or used.

Having said that, it is also deliberately envisaged to incorporate one or more further pharmaceutically active agents in a combination therapy. Such further pharmaceutically active agents may be selected from interferons or other immunomodulators (such as e.g., interferon alpha 2a or 2b, interferon lambda), directly acting antivirals such as nucleos(t)ide analogues (such as e.g., Lamivudine (Epivir-HBV, Zeffix or Heptodin), Adefovir dipivoxil (Hepsera, Preveon), Entecavir (Baraclude, Entaliv), Telbivudine (Tyzeka, Sebivo), Tenfovir (Viread)), entry inhibitors (such as e.g., Myrcludex-B), other antivirals, or cytokines such as Interleukin-2.

In a further aspect, the present invention provides an in vitro method of killing cells infected with HBV, said method comprising culturing said cells infected with HBV with (i) immune effector cells and (ii) one or more polypeptides of the invention, one or more complexes of the invention and/or one or more compositions of the invention.

In a preferred embodiment of the in vitro method, said immune effector cells (i) are comprised in peripheral blood mononuclear cells; or (ii) are or comprise NK cells and/or CTLs.

In a further aspect, the present invention provides an in vitro or ex vivo immune effector cell, which has a polypeptide of the invention or a complex in accordance with the invention bound to a surface antigen of said immune effector cell. Preferred immune effector cells and preferred surface antigens presented by immune effector cells are as defined above. Such immune effector cell is useful for administration to a patient suffering from HBV infection, liver cirrhosis or hepatocellular carcinoma. Accordingly provided is also a pharmaceutical composition comprising or consisting of an immune effector cell which has bound to a surface antigen thereof a polypeptide of the invention or a complex in accordance with the invention.

Also provided is an immune effector cell which has bound to a surface antigen thereof a polypeptide of the invention or a complex in accordance with the invention for use in a method of treating or preventing HBV infection, liver cirrhosis or hepatocellular carcinoma.
Sequences disclosed in this application

SEQ ID NO 1
C8 HC CDR1
Gly Phe Thr Phe Ser Gly Tyr Ala

SEQ ID NO 2
C8 HC CDR2
Ile Ser Gly Ser Gly Gly Ser Thr

SEQ ID NO 3
C8 HC CDR3
Ala Lys Pro Pro Gly Arg Gln Glu Tyr Tyr Gly Ser Ser Ile Tyr Tyr Phe Pro Leu Gly Asn

SEQ ID NO 4
C8 LC CDR1
Asn Ile Gly Ser Lys Ser

SEQ ID NO 5
C8 LC CDR2
Asp Asp Ser

SEQ ID NO 6
C8 LC CDR3
Gln Val Trp Asp Ser Ser Ser Asp Leu Val Val

SEQ ID NO 7
5F9 HC CDR1
Gly Phe Thr Phe Asn Asn Tyr Ala

SEQ ID NO 8
5F9 HC CDR2
Ile Asn Ser Asp Gly Arg Ser Thr

SEQ ID NO 9
5F9 HC CDR3
Ala Arg Thr Phe Tyr Ala Asp Tyr

SEQ ID NO 10
5F9 LC CDR1
5
Gln Asn Val Asp Thr Thr

SEQ ID NO 11
5F9 LC CDR2
10
Trp Ala Ser

SEQ ID NO 12
5F9 LC CDR3
15
Gln Gln Tyr Ser Ile Phe Pro Tyr Thr

SEQ ID NO 13
5A19 HC CDR1
20
Gly Phe Thr Phe Ser Ser Tyr Ala

SEQ ID NO 14
5A19 HC CDR2
25
Val Ser Ser Asp Gly Ser Tyr Ala

SEQ ID NO 15
5A19 HC CDR3

SEQ ID NO 16
5A19 LC CDR1
30
Gln Ser Leu Leu Asn Thr Arg Thr Arg Lys Ser Tyr

SEQ ID NO 17
5A19 LC CDR2
35
Trp Ala Ser

SEQ ID NO 18
5A19 LC CDR3
Lys Gln Ser Tyr Ser Leu Tyr Thr

SEQ ID NO 19
OKT3 HC CDR1

Gly Tyr Thr Phe Thr Arg Tyr Thr

SEQ ID NO 20
OKT3 HC CDR2
Ile Asn Pro Ser Arg Gly Tyr Thr

SEQ ID NO 21
OKT3 HC CDR3
Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr

SEQ ID NO 22
OKT3 LC CDR1
Ser Ser Val Ser Tyr

SEQ ID NO 23
OKT3 LC CDR2
Asp Thr Ser

SEQ ID NO 24
OKT3 LC CDR3
Gln Gln Trp Ser Ser Asn Pro Phe Thr

SEQ ID NO 25
9.3 HC CDR1
Gly Phe Ser Leu Ser Asp Tyr Gly

SEQ ID NO 26
9.3 HC CDR2
Ile Trp Ala Gly Gly Gly Thr

SEQ ID NO 27
9.3 HC CDR3
Ala Arg Asp Lys Gly Tyr Ser Tyr Tyr Ser Met Asp Tyr

SEQ ID NO 28
9.3 LC CDR1
Glu Ser Val Glu Tyr Tyr Val Thr Ser Leu

SEQ ID NO 29
9.3 LC CDR2
Ala Ala Ser

SEQ ID NO 30
9.3 LC CDR3
Gln Gln Ser Arg Lys Val Pro Tyr Thr

SEQ ID NO 31
A9 HC CDR1
Gly Tyr Thr Phe Thr Asn Tyr Trp

SEQ ID NO 32
A9 HC CDR2
Ile Tyr Pro Gly Gly Gly Tyr Thr

SEQ ID NO 33
A9 HC CDR3
Ala Arg Ser Ala Ser Trp Tyr Phe Asp Val

SEQ ID NO 34
A9 LC CDR1
Thr Gly Thr Val Thr Thr Ser Asn Tyr

SEQ ID NO 35
A9 LC CDR2
His Thr Asn

SEQ ID NO 36
A9 LC CDR3
Ala Leu Trp Tyr Asn Asn His Trp Val

SEQ ID NO 37
NCAM29.2 HC CDR1
Gly Phe Thr Phe Ser Ser Phe Gly

SEQ ID NO 38
NCAM29.2 HC CDR2
Ile Ser Ser Gly Ser Tyr Ala Ile

SEQ ID NO 39
NCAM29.2 HC CDR3
Val Arg Gly Arg Leu Gly Glu Gly Tyr Ala Met Asp Tyr

SEQ ID NO 40
NCAM29.2 LC CDR1
Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Asn Tyr

SEQ ID NO 41
NCAM29.2 LC CDR2
Trp Ala Ser

SEQ ID NO 42
NCAM29.2 LC CDR3
Gln Gln Tyr Ser Ser Trp Thr

SEQ ID NO 43
C8-hlgG1Fcmut-OKT3
MDFEVQIFSLLISASVIMSRMAEVQLVESGGGLPQGSLRLSCAASQFTFSGYAMSWVRQA
PKGLEWSSSGGGSTYADSVKGRFTIQRDNSKNLTLQVMNLRADTALYCAKPPGRO
EYYGSSYFYFPLCNWQGTLVTSVSASTKPKLEEGFSERVQQALTQPSVSVAPGQTARI
TCGNNISGSKSHWYQKPGQPVLVYDDSRRSPGIPERFSGSNSQNTATLTLTSRVEAGDEA
DYYCQWVDDSSDLVVFGGTTKLVLGSSGGGSSGGSSGGGSSGASEPKSSDKHTTCPCPAAPP
AAGPSVFLFPPKPKDTFLMSRTPEVTCVVVDVSHEDPEVKFNWWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWNKEYKCAVSNKGLASSIEKTISKAKGQPREPQYPVTLPPSRDELTK
NQVSITCLVKGFYPSDIAVEWESNGQPPNYKTTPVLDSDGPFFLGYKLTVDKSRWQQGNVF
SCSVMEHALHNIHTQKSLSLSPGBKPDGWSHPQFEKSRGGGQVQLQSGAEAGLPASVKMSC
SEQ ID NO 44

C8- hlgG1Fmut-9.3

MDFEVQIFSLFILLASAVIMSRMAEVLQVESGGGQLLPQGGLRLLS CAAASGFTSGYAMSWVRQA
PGKGLEWVSSTSGGSSTYADYSVKGRFTISRDNKSNTLYLMQNSLRAREDALYYCALKPQGRO
EYYGGSIYFPGLQNGWQRGTLLTVSSASTKGPKLEEGFSEARVQSAFTQPSAVSVتطبيق
TCGGNNIGSKSVWHPQQKPGQPVLPVYDSDRPSGIFPERFSNGSNNTALLTISRVEAGDEA
DYDQVWDSSSDLVFGGTTLVGLNSGGGSSGGGSLLLSEPKSSDKHTCPCPAPP
AAAGPSVLFPPPKPKDITLMISRTPETCVDVVDVSHEDEPKFNWWYGDVEVHNAKTPREEQYN
STYRVVSVLTVLVHQLDWNKGYKCAVSNKGLASSIETKISKAKQGPREPQVTLPPSPRDELTK
NQVSLTCLVKGFYPSDIAVEWESNQPPENNYKTPPPVPLDSGSFFLYSKLTVMDSRWWQQNVF
SCSVMHEALHNHTQKSLSPGDKPWSHPQEKKSSGGQVQLQSEGPGVTSQSLSTC
TVSGFSLSDQVHVRQFSGQGLEWGLGVIWAGGCTNSAMLRSKISKDNSKQVFLKMNLS
QADTDATVYCARQGSYSVYSSMDYWQGTITVTVSSRGGGSSGGGSGGGSIELTQTSPASLAV
SLQORATISCRASESVEYYYVTSLMQWYQKPGQPKLLIFAAASNVESGVPARFSGSGSTNFS

SEQ ID NO 45

C8- hlgG1Fmut-A9

MDFEVQIFSLFILLASAVIMSRMAEVLQVESGGGQLLPQGGLRLLS CAAASGFTSGYAMSWVRQA
PGKGLEWVSSTSGGSSTYADYSVKGRFTISRDNKSNTLYLMQNSLRAREDALYYCALKPQGRO
EYYGGSIYFPGLQNGWQRGTLLTVSSASTKGPKLEEGFSEARVQSAFTQPSAVSVتطبيق
TCGGNNIGSKSVWHPQQKPGQPVLPVYDSDRPSGIFPERFSNGSNNTALLTISRVEAGDEA
DYDQVWDSSSDLVFGGTTLVGLNSGGGSSGGGSLLLSEPKSSDKHTCPCPAPP
AAAGPSVLFPPPKPKDITLMISRTPETCVDVVDVSHEDEPKFNWWYGDVEVHNAKTPREEQYN
STYRVVSVLTVLVHQLDWNKGYKCAVSNKGLASSIETKISKAKQGPREPQVTLPPSPRDELTK
NQVSLTCLVKGFYPSDIAVEWESNQPPENNYKTPPPVPLDSGSFFLYSKLTVMDSRWWQQNVF
SCSVMHEALHNHTQKSLSPGDKPWSHPQEKKSSGGQVQLQSEGPGVTSQSLSTC
TVSGFSLSDQVHVRQFSGQGLEWGLGVIWAGGCTNSAMLRSKISKDNSKQVFLKMNLS
QADTDATVYCARQGSYSVYSSMDYWQGTITVTVSSRGGGSSGGGSGGGSIELTQTSPASLAV
SLQORATISCRASESVEYYYVTSLMQWYQKPGQPKLLIFAAASNVESGVPARFSGSGSTNFS

LNIHPVDDEDDVAMYFCQSQSRKVPYTFGGGTKLEIKR
SEQ ID NO 46
C8- hlgG1Fmut-NCAM29.2
MDPEVQIFSFLILASVIMSRMAEVQLVESGGGLPQGSLRLSCAASQFTSGYAMSVVRQA
PGKLEGWVSSISGSGGSTYYADSVKGRFTISRDNSKNTLYQLMNLSRAEDTALYYCAKPPGRQ
EYGGSIYFPLNGWQGTLVTSSASTKGPKEEGFSEARVQSLATQPASVSVAPGTARI
TCGGNNGISKSCHWYQQKPGQAIPVLYYDSDRPSGIPERFSGNSNGNTATLTIISRVEAGDEA
DYYCQVWDSSSDLVFFGGTKLTTLGNSGSGGGSGGGSGGGSDAPEKSSDKHTHTCPPCPAAPP
AAAPSFLPPPKPDLLMKISRTPEVTCVVDVSHEDEPVKFNWYDVGEVHNAKTPREEQNYN
STYRVSVTLTVLHODWLNGKEYKCAVSNKGLASSIEKTISSCAKGPQPREPQVYTLPPSRDELTK
NQVSLTCLVKGFYPSDIAVEVESNGQPENNYKTPPPVLDSDGSFFLYSKLTDKSRWQGGNVF
SCSVMHEALHNHYTQKSLSLPGKDPSHPQFEKSSGGVDQLVESGGGLVQPGSSRKLSCA
ASQFTSSFGMHWRQAPKEKLEWVAYISSGYSAYATYADTVKGRFTISRDNPENTLFLQMTSL
RSEDSAMYVCYGRRLGEQYANDYQWQGTSVTSSGNSGSGGGSGGGSSGAGSDIVMSQSP
SSLAVSVGKEKMTSSQSSLLYSNOKNLAWYQQKPGQPSPKLLIYWASTRGVQGDRTFGR
GSGLTFDPIISSVKAEDLAVYECQWYQSSWTFGGGTKLEIKR

SEQ ID NO 47

SEQ ID NO 48
Ala Lys Thr Thr Pro Lys Leu Glu Gly Glu Phe Ser Glu Ala Arg Val

SEQ ID NO 49

The figures illustrate the invention.

**Figure 1:**
scFv fragments are obtained by fusion of two variable domains. Fusion involves the use of a flexible peptide linker which does not or not substantially interfere with the structure of each variable domain.

**Figure 2:**
Dimerization of two polypeptides of the invention by the formation of disulfide bonds. Each polypeptide comprises a bispecific bivalent antibody. Natural antibody dimerization in the endoplasmic reticulum of producer cells can result in formation of a bispecific tetravalent antibody, or a tri- or tetrascpecific, tetravalent antibody if two bispecific bivalent antibodies are
co-expressed (not shown).

**Figure 3:**
Comparison of specific elimination of HBV surface antigen producing hepatoma target cells after administration of single bispecific antibodies and synergistic effects of simultaneous administration of two CTL-specific or two NK cell-specific bispecific antibodies. The CellTiter-Blue Cell Viability Assay is used.

**Figure 4:**
A) Cytokine secretion as an indication of activation of immune effector cells in the presence of bispecific antibodies of the present invention. HBV-infected HepaRG cells were co-cultured with PBMC in the presence or absence of indicated bispecific antibodies. B) Specific elimination of HBV-infected target cells in co-culture with immune effector cells and bispecific antibodies.

**Figure 5:**
Viability of target cells co-cultured with PBMC in presence of individual HBs-reactive bispecific antibodies. Single bispecific antibodies mediate lysis of target cells. A, C, E: Effect of stimulation with αHBs x αCD3 (A), αHBs x αCD28 (C) or summarized (E). B, D, F: Effect of stimulation with αHBs x αCD3 [FcΔADCC] (B), αHBs x αCD28 [FcΔADCC] (D) or summarized (F). The arrowhead indicates addition of PBMC and bispecific antibodies. Curves with dots represent HBs-transfected HuH7-S cells, curves with rhombuses represent HuH7 parental hepatoma cells. The xCELLigence real-time cytotoxicity assay is used. Normalization time of cell index: 0h.

**Figure 6:**
Viability of target cells co-cultured with PBMC in presence of HBs-reactive bispecific antibodies. Combination of bispecific antibodies mediate massive killing of target cells. A: Effect of stimulation with αHBs x αCD3 and αHBs x αCD28. B: Effect of stimulation with αHBs x αCD3 [FcΔADCC] and αHBs x αCD28 [FcΔADCC]. C, D: Effect of individual bispecific antibodies compared to combinations. The arrowhead indicates addition of PBMC and bispecific antibodies. Curves with dots represent HuH7-S cells, curves with rhombuses HuH7 cells. Normalization time of cell index: 0h.
Figure 7:
Viability of target cells co-cultured with PBMCs in the presence of different concentrations of bispecific antibodies. 50 μl/50 μl mixtures of antibody-containing supernatants of αHBs x αCD3/ αHBs x αCD28 (A), or αHBs x αCD3 [FcΔADCC] αHBs x αCD28 [FcΔADCC] (B), induced lysis of target cells earlier than 25 μl/25 μl mixtures, indicating dose-dependent effects. The arrowhead indicates addition of PBMCs and bispecific antibodies. Curves with dots represent HuH7-S cells, curves with rhombuses HuH7 cells. Normalization time of cell index: 0h.

Figure 8:
Viability of target cells co-cultured with different amounts of PBMC in the presence of a mixture of αHBs x αCD3 and αHBs x αCD28. 2×10⁵ PBMC mediate a significantly earlier elimination of HuH7-S cells than 1×10⁵ PBMC. The arrowhead indicates addition of PBMC and bispecific antibodies. Curves with dots represent HuH7-S cells, curves with rhombuses HuH7 cells. Normalization time of cell index: 0h.

Figure 9:
Viability of target cells co-cultured with PBMCs in presence of αHBs x αCD3/ αHBs x αCD28 mixtures for various time periods. Supernatants containing bispecific antibodies were removed after the indicated periods of stimulation. 4h stimulation only led to a small decrease of target cell viability (78.5% endpoint viability). Stimulation of PBMC with bispecific antibodies for 8h or longer induced elimination of target cells. After stimulation for 8h and 12h, killing of target cells was delayed as compared to 24h or 48h stimulation, suggesting continuous activation and re-targeting of effector cells. HuH7-S endpoint viabilities at 48h were, however, comparable: 8h stim.: 14.7%; 12h stim.: 11.7%, 24h stim.: 5.1%, 48h stim.: 3.2%). The arrowhead indicates addition of PBMC and bispecific constructs. Viability kinetics for HuH7-S cells are shown. Normalization time of cell index: 0h.

Figure 10:
IL-2, IFN-γ and TNF-α secretion of PBMC after co-culture with HuH7-S/HuH7 cells in presence of αHBs x αCD3/ αHBs x αCD28 at different time points. A: IL-2 concentration increased over time and reached a plateau at approximately 24h with a concentration of about 1550 pg/ml. B: IFN-γ secretion started between 8h and 12 h and increased up to 12000 pg/ml (48h). C: TNF-α production was detectable already after 4h, increased continuously, reached its peak at 24h (1700 pg/ml) and declined to 1400 pg/ml after 48h. High background TNF-α secretion in the absence of HBs (HuH7 cells) could be detected, with the highest
concentration after 4h (~70 pg/ml) decreasing to 9 pg/ml after 48h of co-culture.

**Figure 11:**
LAMP-1 stainings after co-culture of PBMC with HuH7-S/HuH7 cells in presence of bispecific antibodies. Surface expression of the endosomal degranulation marker LAMP-1 is detected on CD4+ (A, B) and CD8+ (C, D) T cells after co-culture with HuH7-S (black line) or HuH7 (grey line) cells in the presence of either αHBs x αCD3/αHBs x αCD28 (A, C) or αHBs x αCD3 [FcΔADCC]/αHBs x αCD28 [FcΔADCC]. (B, D).

**Figure 12:**
FACS analysis of PBMC co-cultured with HuH7-S or HuH7 cells in the presence of αHBs x αCD3/αHBs x αCD28 after 8h, 12h and 24h. A, B; Percentages of IFNγ+/IL-2+/TNFα+/CD154+ CD4+ T cells (A) or IFNγ+/IL-2+/TNFα+/CD154+ CD8+ (B) T cells. C, D: Boolean combination gates of IFNγ+, IL-2+ and/or TNFα+ CD4+ (C), or IFNγ+, IL-2+ and/or TNFα+ CD8+ (D) T cells.

**Figure 13:**
FACS analysis of PBMC co-cultured with immobilized or soluble HBsAg in the presence of αHBs x αCD3 [FcΔADCC]/αHBs x αCD28 [FcΔADCC] after 24h and 48h. A, B; Percentages of IFNγ+/IL-2+/TNFα+/CD154+ CD4+ T cells (A) or IFNγ+/IL-2+/TNFα+/CD154+ CD8+ (B) T cells. C, D: Boolean combination gates of IFNγ+, IL-2+ and/or TNFα+ CD4+ (C), or IFNγ+, IL-2+ and/or TNFα+ CD8+ (D) T cells.

**Figure 14:**
HBsAg in the supernatant of HuH7-S cells (110.8 S/CO), HepG2.2.15 cells (41.7 S/CO) and HBV-infected HepaRG cells (16.5 S/CO).

**Figure 15:**
Viability of HBV-infected/uninfected HepaRG cells co-cultured with PBMC in presence of bispecific antibodies. αHBs x αCD3 (A) and αHBs x αCD3/αHBs x αCD28 (B) mediate significant target cell lysis. Endpoint viabilities of untreated cells are 65.9% (HBV+) and 62.9% (HBV-). The arrowhead indicates addition of PBMCs and bispecific constructs. Curves with dots represent HBV-infected HepaRG cells, curves with rhombuses uninfected HepaRG cells. Normalization time of cell index in xCELLigence assay: 0h.
Figure 16:
Reduction in tumor size in animal treated with bispecific antibodies. Mice bearing HBV-positive subcutaneous HepG2.2.15 tumors were treated with human PBMC and a mixture of αHBs x αCD3 and αHBs x αCD28 bispecific antibodies at four consecutive days. Mice were sacrificed and tumor size was analyzed.

The examples illustrate the invention.

Example 1

Materials and methods for Example 2

Cloning and Production of Bispecific Antibodies

Complementary DNAs coding for variable heavy and variable light chains of anti-CD3 (OKT3), anti-CD28 (9.3), anti-CD16 (A9) and anti-CD56 (NCAM29.2) were obtained by PCR amplification of reverse-transcribed mRNAs from the respective hybridoma using a set of primers covering all V Heavy and V Light subtypes. PCR products were ligated into pCR2.1-TOPO (Invitrogen, Life Technologies) and sequenced. The anti-HBsAg scFv C8 was provided in a codon-optimized form in the plasmid pMP71-C8. Using primers containing appropriate restriction sites in the 5’ and 3’ flanks variable heavy and variable light chain cDNAs coding for the above mentioned antibodies were assembled with a glycine-serine linker into scFvs. The OKT3, 9.3, A9, and NCAM29.2 scFvs (N-terminally extended by ( Gly)$_{3-4}$) were cloned at the 3’ end of a cDNA present in pBluescript KS II+ (Stratagene) that codes for the Fc domain (hinge, CH2, CH3) of human IgG1 which was extended by glycine-serine linker GlyAsnSer(Gly$_4$Ser)$_3$AlaSer at the 5’ end and a StrepTag sequence (WSHPQFEK) and, in a second series of constructs, an additional glycine-serine linker (Gly$_4$Ser)$_3$ at the 3’ end. The C8 scFv coding sequence was cloned at the 5’ end of the mentioned 5’ glycine-serine linker. The complete scFv-linker-hIgG1Fc-linker-scFv sequence was subcloned into the mammalian expression vector pcDNA3.1(-) (Invitrogen). Maxi-prep plasmid DNA was used for transfection of HEK 293 cells using the peqFECT transfection reagent (Peqlab). Stable transfectants were selected using 0.8-1.0 mg/ml G418 and expanded. Supernatants from HEK transfectants were collected and analyzed by ELISA for the concentration of secreted, bispecific antibodies and by Western blot for the integrity of the secreted antibodies using goat anti-human IgG-Fc specific, peroxidase-labeled antibodies.
Cell Culture Conditions and HBV Infection

HuH7 hepatoma cells (Nakabayashi, et al. 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. 42: 3858-3863) and HEK293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mmol/L) (all from Gibco, Life Technologies).

Peripheral blood mononuclear cells (PBMC) were isolated through density gradient centrifugation from heparinized whole blood using LSM 1077 Lymphocyte Separation Medium (PAA). 25 ml of blood was layered above 13 ml of LSM 1077. After centrifugation at 2000 rpm for 20 min (without break) at room temperature PBMC were harvested and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/ml), and L-glutamine (2 mmol/l) (all from Gibco). After an overnight resting step PBMC or sorted NK cells were used for co-culture experiments.

HepaRG cells were maintained in Williams E Medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with L-glutamine (5 mmol/l), glucose (0.06% [wt/vol]), HEPES (23 mmol/l, pH7.4), gentamycin (50 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), inosine (37 µmol/l), hydrocortisone (4.8 µg/ml), and insulin (1 µg/ml). Prior to infection HepaRG cells were differentiated for 4 weeks using differentiation medium (Williams E Medium (as described above), supplemented with DMSO (1.75%). HepaRG cells were infected using HBV stocks at a final m.o.i. of 200 and PEG (5%) in differentiation medium. Infection inoculum was removed after overnight incubation and replaced with differentiation medium and cultured for 6 days. For co-cultures with redirected T cells, we changed from differentiation medium to hydrocortison-free medium 2 days before starting the co-culture to avoid immunosuppression mediated by the hydrocortison.

Transfection with HBV surface antigen encoding plasmids

HuH-7 cells were transfected with plasmids encoding the various surface antigens using FuGene transfection reagent (Promega). For 8 wells of a 96 well plate 3 µl of FuGENE, 1 µg of plasmid DNA were added to 100 µl OptiMEM (Gibco). The transfection solution was incubated for 15 min at room temperature in order for the FuGENE to bind the plasmid DNA. A final volume of 100 µl was applied per well, after adding further OptiMEM and incubated for at
least 24 h.

*Magnetic activated cell sorting (MACS) of NK cells*

NK cells were isolated from PBMC using a human CD56⁺CD16⁺ NK Cell Isolation Kit (Milenyi) In a first negative selection step, all non-NK cells were removed by monoclonal antibodies directed against antigens not expressed on the surface of NK cells. In a second positive selection step, the NK cells were isolated by monoclonal CD16 antibodies conjugated to iron oxide microbeads and retained inside a magnetic field. After isolation NK cells were cultured in RPMI-1640 medium as described above.

*Co-culture of HBV-positive target cells and redirected effector cells*

Target cells were cultured in a 96 well plate at full confluency. 1×10⁵ effector cells were added in a volume of 100 µl medium per well. 100 µl of the HEK supernatants containing the bispecific antibodies were applied per well. For determination of synergistic effects, 50 µl of each bispecific antibody supernatant was added per well. Untreated target cells incubated with 200 µl medium or with effector cells alone or with bispecific antibodies alone served as negative control.

*Enzyme-linked immunosorbent assay (ELISA) for effector cell activation*

Cytokine secretion resulting from activation of effector cells was detected by ELISA. Using the Human IFN-γ ELISA MAX™ (BioLegend). The absorbance at 450 nm was detected using the program Magellan6 and an InfiniteF200 (Tecan).

*Target cell viability assay*

The target cell viability after co-culture was determined using the CellTiter-Blue Cell Viability Assay (Promega). This assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) due to metabolic activity. Nonviable cells rapidly lose their metabolic capacity and thus do not generate a fluorescent signal. After removal of the supernatant 100 µl of colorless DMEM containing 20% CellTiter-Blue Reagent was added per well to the co-cultures and incubated at 37 °C for 2 hours. The fluorescence signal was recorded at 560nm using an InfiniteF200 (Tecan).
Example 2

Results

In a first line of experiments we have evaluated the activity of the bispecific antibody constructs directed against CTL surface antigens CD3 and CD28 and against NK cell surface antigens CD16 and CD56. We employed plasmid-transfected hepatoma cell lines producing HBV surface antigens. After establishing the HBV protein expression, these target cells were co-cultured together with immune effector cells, namely PBMC and isolated NK cells, and bispecific antibody constructs. PBMC contain around 70% T cells but only 7% NK-cells. Therefore, we magnetically isolated CD16⁺ CD56⁺ NK cells. As negative controls we analyzed co-cultures with HBV-negative target cells, which had been preincubated with HBV- and subviral particle-containing supernatants. This control was employed to rule out activation of effector cells due to unspecific binding of HBV particles on the surface of HBV-negative target cells. Furthermore, we co-cultured HBV-positive target cells with immune effector cells in the absence of bispecific constructs to evaluate unspecific background cytotoxicity. To exclude a cytotoxic effect of the bispecific constructs, we prepared cultures of HBV-positive target cells without immune effector cells in the presence of bispecific constructs.

These experiments showed a specific activation of CTLs upon co-culture in the presence of the CD3- or CD28-specific constructs as determined by the secretion of the proinflammatory cytokine interferon gamma (IFN-γ) of up to 7000 pg/ml. This effect was further enhanced upon co-administration of CD3- and CD28-specific constructs demonstrating a synergistic effect.

Furthermore, the bispecific constructs mediated a specific cytotoxic elimination of HBsAg-producing HuH7 hepatoma cell lines (Figure 3) of up to 90% reductions of target cell viability in comparison to controls. This cytotoxic response was observed for co-cultures of PBMC and HBV-positive target cells together with the bispecific constructs directed against CD3 and CD28 as well as for isolated NK-cells with constructs directed against CD16 and CD56. The co-administration of CTL- and NK-cell specific constructs further increased the cytotoxic effect synergistically to elimination rates above 95%. We observed unspecific background cytotoxicity of 15% to 40% for CTLs and NK cells, respectively.

In a second round of experiments we employed HBV-infected HepaRG hepatoma cells. This cell line allows for infection with HBV after a four week differentiation and mirrors the natural situation of HBV-infected tissues. Typically, infection rates of HepaRG cells never reach 100%
and this mixture of infected and non-infected cells mimics the situation in an HBV-infected individual under antiviral therapy, harboring both, infected and non-infected cells in the presence of free extracellular viral particles.

In co-cultures of immune effector cells and co-administered bispecific constructs, the HBV infected HepaRG cells mediated an efficient activation of both, CTLs and NK cells, with impressing amounts of IFN-γ secretion of up 60,000 pg/ml (Figure 4A). In this experiment we did not isolate or enrich NK cells prior to co-culture.

Furthermore the bispecific antibody constructs resulted in a cytotoxic response of the activated immune effector cells leading to the specific elimination of HBV-infected target cells (Figure 4B). We observed elimination rates of 50% to 70% for NK-cells and CTLs, respectively. Unspecific background cytotoxicity was absent in these experiments.

Example 3

Methods for Example 4

To analyze the therapeutic potential of bispecific antibody constructs to successfully retarget T cells towards HBV-positive cells, in vitro co-culturing experiments were performed and analyzed in detail. We employed bispecific antibody constructs containing single chain binding domains directed against human CD3 (αCD3) and human CD28 (αCD28) and additionally, constructs containing directed mutations in their Fc spacer domain which should abrogate antibody dependent cellular cytotoxicity (ΔADCC), by circumventing Fcγ receptor binding.

These were constructed as a safety measure to rule out unspecific activation of natural killer cells. On the other side, all bispecific antibody constructs harbored the HBV S-protein (HBsAg) specific binding domain C8. Peripheral blood mononucleated cells (PBMC) isolated from fresh venous blood of healthy donors were co-cultured with different human hepatoma cell lines as surrogate models for HBV-infection. We employed HuH7-S (HBV S-antigen transgenic) and as negative control the mother cell line HuH7 and HBV-infected or as control uninfected HepaRG cells. HepG2.2.15 (HBV genome transgenic) cell were used as controls for HBV-marker quantification. To provide bispecific antibody constructs, supernatant of producer cell lines containing bispecific antibodies was added. To visualize changes in target cell viability due to cytotoxicity mediated by bispecific antibodies over time, the xCELLigence system was employed. This technique allows for real-time monitoring of cell-viability over long time cultures. Therefore, target hepatoma cells were seeded on specially designed microtiter
plates, which contain interdigitated gold microelectrodes to noninvasively monitor the viability of adherent target cells using electrical impedance as a readout. The cytotoxic elimination results in a change of the impedance, which can be converted into the so called cell index (CI) value, which is used to monitor cell viability.

5

Co-culturing with target cells

At day zero, 3x10^4 HuH7-S/HuH7 cells were seeded per well in a 96-well plate (E-Plate 96). At day 1, the supernatant was removed and 1x10^5 primary human PBMC in 100 µl PBMC medium or only 100 µl medium for controls were added to the respective wells. Additionally, 100µl of supernatant containing bispecific antibodies, singly or in combinations were added. As negative control, 100µl DMEM medium were added to the wells, resulting in a total volume of 200 µl. Co-cultures were monitored for 48h or 72h in the xCELLigence system.

10 HepaRG cells were grown to confluence, differentiated for 21 days and infected with HBV prior to immunotherapeutic experiments.

For the infection of HepaRG cells a virus stock was prepared in differentiation medium containing PEG and 50µl were added per well. The final concentration of PEG was 5% and the MOI of the virus stock was set to 200 (7,5x10^6 virus particles/well). 16h after addition of the infection master mix, cells were washed 3 times with PBS to remove residual virus. Differentiation medium was added, and medium was changed every 3 days for a total of 12 days. Before co-culturing experiments, medium was changed to co-culturing medium (depleted of the immunosuppressant hydrocortisone). Successful HBV infection of HepaRG cells was tested by measuring HBsAg (AxSYM) and HBeAg (BEP III System) in the supernatant of infected cells.

20

PBMC preparation

PBMC for co-culturing experiments were isolated from whole blood. Heparinized fresh blood was diluted 1:1 with RPMI wash-medium. 25ml of diluted blood was over layered onto 15ml Percoll and centrifuged at 960g for 20 min without break in a swing-out centrifuge. The PBMC were isolated and transferred into 50 ml with RPMI medium. After washing, cells were resuspended in 10ml PBMC medium and cell number was determined. The concentration was adjusted to 2x10^6 cells/ml to ensure optimal conditions. PBMC were rested overnight at 37°C.
**Fluorescence activated cell sorting (FACS)**

To examine effector functions of redirected PBMCs, FACS analysis was performed. Thereby, the secretion of the pro-inflammatory cytokines IFN-γ, IL-2 and TNF-α, as well as the expression of the activation marker CD154 (CD40L) and the degranulation marker LAMP-1 (CD107a), respectively, where analyzed. The measurement of cytokine production was performed using intracellular cytokine staining. Therefore 0.2 μg/ml Brefeldin A (BFA) was applied to cells and incubated for 4 hours at 37°C.

BFA blocks the forward transport between the endoplasmic reticulum and the Golgi apparatus and, as a consequence, exocytosis of cytokines is inhibited. In the case of simultaneous staining for LAMP-1, antibody was applied 1 h before adding BFA (to enable translocation of LAMP-1 to the cellular surface). Subsequently, cells were transferred to a 96-well plate (round bottom) and washed twice in 200μl FACS buffer. For staining of viable cells and exclusion of dead cells, the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit was used. For fixation and permeabilization cells were resuspended in 100μl Cytofix/Cytoperm reagent and incubated on ice in the dark for 20 min. After washing, cells were resuspended in the prepared antibody mix or only stained with the respective single colors for systematic compensation. Staining took place on ice in the dark for 30 min. After washing, cells were resuspended in 200 μl FACS buffer and transferred into FACS tubes for acquisition. Acquisition was performed using either a FACSCanto II or LSR Fortessa. FACS Diva software was used to record data, analysis was performed using FlowJo software.

**Animal experiments**

For a first test of bispecific constructs in vivo, experiments with immunodeficient Rag2/Il2Rγcnull-mice (international nomenclature: B10.B6-Rag2tm1Fwa Il2rgtm1Wjl) were conducted. We injected 6 weeks old mice with 5x10⁶ cells of the HBV-transgenic human hepatoma cell line HepG2.2.15. Cells were injected subcutaneously into the flank of the animals. This resulted in tumor formation over a 14 day time period. HBV replication inside the tumor was monitored through determination of HBV viremia. Human PBMC were isolated from fresh human cord blood and stimulated on plates precoated with antibodies against human CD3 and CD28 at a cell concentration of 0.25x10⁶ PBMC per ml for 3 days. Subsequently cells were maintained in cell culture medium containing 300 U/ml of IL-2 for 7 days.

On day 14 after tumor induction, mice were injected with 2x10⁷ PBMCs per mouse
intraperitoneally and received 100 μl of αCD3/αCD28 bispecific antibody constructs in supernatant of HEK producer cells into the tail vein per animal at four consecutive days. Mice were sacrificed on day 18 after tumor induction and analysed for tumor size. Subsequently, serum and tissue samples were stored for further analyses.

Example 4

Bispecific antibodies mediate specific elimination of HBV surface protein expressing target cells (HuH7-S)

To examine whether bispecific antibody constructs successfully retarget T cells towards HBsAg expressing target cells and induce target cell lysis, isolated PBMC were co-cultured with HuH7-S cells in the presence of bispecific antibody constructs. HuH7-S cells were stably transfected to express HBsAg and therefore mimicked HBV-infected hepatocytes. This results in the production and secretion of subviral particles into the supernatant and the incorporation of HBsAg into the cellular membrane. Untransfected HuH7 cells served as negative control.

Individual bispecific antibodies provoke killing of target cells

To analyze if the individual bispecific antibodies are able to stimulate T cell activation and mediate target cell lysis, PBMCs were co-cultured with HuH7-S/HuH7 cells in the presence of αHBs x αCD3, αHBs x αCD28, αHBs x αCD3 [FcΔADCC] or αHBs x αCD28 [FcΔADCC] bispecific tetravalent antibodies. The stimulation of effector cells by the single bispecific antibodies resulted in specific killing of HBsAg expressing target cells (Fig. 5). Bispecific antibodies directed against CD3 mediated elimination of target cells earlier and stronger than constructs directed against CD28, as the endpoint viability of HuH7-S cells treated with αCD3 only accounted for 6.4% (αCD3ΔADCC: 15.5%) compared to 44.42% for αCD28 (αCD28ΔADCC: 48.9%). Furthermore αCD3ΔADCC and αCD28ΔADCC required more time to induce lysis of target cells compared to αCD3 and αCD28, respectively. αCD3ΔADCC-mediated killing started approximately 35h after starting the co-culture, whereas αCD3 led to a decrease of target cell viability already after 12h. A time shift of about 20h could also be observed between αCD28ΔADCC and αCD28-mediated target cell lysis. The stimulation with αCD3 also led to detectable lysis of HBsAg-negative HuH7 cells with an endpoint viability of 78.1%, indicating unspecific activation. The same was true for stimulation with αCD3ΔADCC in some experiments even if not seen here. The viability of HuH7 cells during co-culture in presence of the other bispecific constructs remained at 100%.
This data demonstrates that stimulation with each of the individual bispecific antibodies provokes elimination of target cells without further co-stimulation.

**Bispecific antibodies mediate target cell lysis in a synergistic manner**

To further analyze whether combination of bispecific constructs leads to an enhanced activity and therefore cytotoxicity of effector cells, PBMCs were co-cultured with HuH7-S/HuH7 cells either in presence of the combinations of αCD3/αCD28 or αCD3ΔADCC/αCD28ΔADCC. As shown in Figure 6, the combination of bispecific constructs led to massive killing of HBsAg expressing target cells with a remaining viability of 1.2% (αCD3/αCD28) and 4.4% (αCD3ΔADCC/αCD28ΔADCC), whereas nearly no HuH7 cells were eliminated (endpoint viability of HuH7 cells: αCD3/αCD28: 92.4%; αCD3ΔADCC/αCD28ΔADCC: 100.4%).

Again αCD3/αCD28-mediated lysis of target cells was faster than the killing induced by constructs with mutated Fc region, even if killing of target cells started at approximately the same time after about 11h (Fig. 6A, B). Combination of bispecific antibodies led to a faster elimination of target cells compared to lysis induced by individual bispecific constructs (Fig. 6C, D). This was expected, as T cells receive not only one signal as in the presence of individual constructs, but obtain both, activation and co-stimulatory signal if an antibodies directed against CD3 and CD28 are present.

Thus, combination of bispecific constructs mediate specific lysis of HBV surface protein expressing target cells in a synergistic manner.

**Bispecific antibodies provoke elimination of target cells in a concentration dependent manner**

To examine if the amount of bispecific antibodies had an effect on target cell lysis, two different amounts of bispecific constructs were used for co-culture. Therefore, the usual amount of antibodies (100μl supernatant in total = high) and the half of it (50μl supernatant in total = low) were used. The lower amount of bispecific antibodies could also induce lysis of target cells (endpoint viability of HuH7-S cells: αCD3/αCD28: 12.6%; αCD3ΔADCC/αCD28ΔADCC: 15.9%), whereas the higher amount caused elimination of target cells faster (Fig. 7) with only 1.5% (αCD3/αCD28) and 2.1% (αCD3ΔADCC/αCD28ΔADCC) of remaining viable cells. HuH7 cells were not affected in any case. Combination of either αCD3/αCD28 or αCD3ΔADCC/αCD28ΔADCC provoked killing of
target cells in a concentration dependent manner.

*Increased concentrations of effector cells enhance lysis of target cells*

5 It was of further interest if the number of effector cells had an impact on the elimination of target cells. Thus, the usual amount of PBMCs used for co-culture (1x10^5) was compared to the double amount (2x10^5). As it is demonstrated in Figure 8, the higher number of PBMCs induced lysis of HuH7-S cells in the presence of αCD3/αCD28 significantly faster with an endpoint viability of 4.5% compared to 11.7%, but also more HuH7 cells were killed, if the double amount of PBMCs was present (endpoint viability of HuH7 cells: 2x10^5 PBMCs: 83.8%; 1x10^5 PBMCs: 102.7%).

This data indicates that the elimination of target cells is dependent on the amount of effector cells.

*Bispecific antibodies mediate killing of target cells after only 8h of co-culture*

To investigate the question, how long bispecific antibodies have to be present during co-culture to activate T cells and therefore induce cytotoxicity, the supernatant of co-cultures containing the bispecific antibodies was removed after different time periods and new DMEM standard medium was added. If supernatant containing αCD3/αCD28 was removed after 4h, PBMCs only induced a small decrease in target cell viability (78.5%), but were not able to provoke lysis of all target cells (Fig. 9). If supernatant containing bispecific antibodies was present for 8h or longer, PBMCs were able to cause elimination of target cells. As it is illustrated in figure 10, PBMCs needed more time to induce target cell lysis if stimulation with αCD3/αCD28 lasted for 8h or 12h compared to 24h or 48h, but the effect after 48h was nearly similar (HuH7-S endpoint viability: 8h: 14.7%; 12h: 11.7%, 24h: 5.1%, 48h: 3.2%).

*Bispecific antibodies mediate effector functions of T cells during co-culture with either HBsAg or HuH7-S cells*

To investigate the activation and functionality of T cells during co-culture experiments, the secretion of cytokines was examined either by ELISA or FACS analysis.
Bispecific constructs mediate the secretion of IFN-γ, TNF-α and IL-2

In a time line experiment it was analyzed, when PBMCs start to secret cytokines upon contact with bispecific antibodies and how dynamics develop over time. Therefore, supernatant of co-cultures was removed 4h, 8h, 12h, 24h and 48h after addition of PBMCs and αCD3/αCD28. Cytokine production was measured by ELISAs for IL-2, IFN-γ and TNF-α. The secretion of IL-2 increased over time, but after 4h almost no IL-2 was detectable, after 8h the concentration was already 316 pg/ml and during the following 4 hours, the concentration almost quadrupled (1119pg/ml). There was no further rise between 24h and 48h and IL-2 concentration seemed to reach a plateau at about 1550pg/ml (Fig. 10A). IFN-γ secretion (Fig. 10B) needed more time, after 8h still very low levels were detected. Between 8h and 12h, T cells started to secret IFN-γ, because its concentration accounted already for 1800 pg/ml after 12h. Subsequently (24h) an increase in IFN-γ production was observed, the concentration increased to around 10000 pg/ml. The highest amount was detected after 48h (12000 pg/ml). For both, IL-2 and IFNγ, the concentration on HBsAg negative cells increased over time, with the highest amount after 48h (IL-2: 45pg/ml; IFNγ: 200pg/ml) which also corresponds to observations concerning cell viability.

The secretion of TNF-α (Fig. 10C) increased up to 24h, where it reached its peak concentration (1700 pg/ml). Then it declined and accounted for only 1400 pg/ml after 48h. In contrast TNF-α secretion started earlier than the others, with around 100 pg/ml after 4h followed by a steady rise up to 24h. Interestingly, TNF-α production on HuH7 cells behaved in exactly the opposite way. With a relatively high background concentration compared to other cytokines, it showed the highest concentration after 4h (~70 pg/ml) which declined over time and accounted for only 9 pg/ml after 48h. PBMCs are induced to secret IL-2, IFN-γ and TNF-α upon contact with αCD3/αCD28 during co-culture with HBsAg-expressing cells, whereas the secretion dynamics differ among the individual cytokines.

Bispecific constructs activate CD8+ T cells as well as CD4+ T cells

To analyze if PBMCs also show degranulation of cytotoxic vesicles, the translocation of LMAP-1 (CD107a), a degranulation marker, was investigated. After co-culture with HuH7-S/HuH7 cells in presence of αCD3/αCD28 or αCD3ΔADCC/αCD28ΔADCC, CD8+ T cells showed a clear shift in LAMP-1 staining, whereas the signal was stronger in samples stimulated with αCD3ΔADCC/αCD28ΔADCC compared to αCD3/αCD28 (Fig. 11 C, D). Interestingly, the same observation could be made for CD4+ T cells (Fig. 11 A, B). For
αCD3/αCD28 the translocation of LAMP-1 was more prominent in CD8⁺ T cells, for αCD3ΔADCC/αCD28ΔADCC exactly the opposite.

This data demonstrates, that not only CD8⁺ T cells, but also CD4⁺ are induced to secret cytotoxic granules upon contact with the bispecific antibodies and HBsAg.

To examine polyfunctionality of T cells after co-culture experiments, PBMC were stained for IFN-γ, IL-2 and TNF-α, as well as for the activation marker CD154 (CD40L) which is predominantly expressed on CD4⁺ T cells, at 8h, 12h and 24h after addition of PBMC and αCD3/αCD28 (Fig. 12). CD4⁺ T cells showed a steady increase of IFN-γ⁺ T cells (9.3% after 24h), IL-2⁺ T cells (11.3% after 24h), TNF-α⁺ T cells (14.7% after 24h) and CD154⁺ T cells (28.0% after 24h), whereas the major rise occurred between 12h and 24h (Fig. 12A).

The same was true for CD8⁺ T cells, whereas the percentage of IFN-γ⁺ and IL-2⁺ cells with 18.4% and 11.3% outnumbered CD4⁺ T cells. The amount of TNF-α⁺ and CD154⁺ CD8⁺ T cells was decreased with 10.1% and 6.25% compared to CD4⁺ T cells (Fig. 12B). PBMC on HuH7 cells showed no activation in any sample. Boolean combination gates were used for further analysis of T cells secreting cytokines (Fig. 12C, D). After 24h 3.1% of CD4⁺ T cells and 2.1% of CD8⁺ T cells were IFNγ⁺, IL-2⁺ and TNFα⁺, indicating polyfunctionality of T cells.

Therefore, αCD3/αCD28 mediates activation of PBMCs during co-culture with HuH7-S/HuH7 cells resulting in polyfunctional CD4⁺ and CD8⁺ T cells.

To exclude the possibility that false positive signals were detected due to unspecific binding of antibodies to dead target cells during FACS analysis, PBMC were cultured in the presence of immobilized HBsAg. Additionally the effect of soluble HBsAg was examined, as HBV infected patients exhibit high amounts of HBsAg in their blood. PBMCs were again stained for IFN-γ, IL-2 and TNF-α, as well as for CD154, but only 24h and 48h after addition of PBMC and αCD3ΔADCC/αCD28ΔADCC (Fig. 13). Again CD4⁺ T cells showed an increase of IFN-γ⁺ T cells (3.4% after 24h, 6.8% after 48h), and CD154⁺ T cells (17.2% after 24h, 19.9% after 48h). There were less IL-2⁺ T cells after 48h (4.9%) compared to 24h (5.5%), TNF-α⁺ T cells also decreased (14.9% after 24h, 8.1% after 48h) (Fig. 13A). CD8⁺ T cells only showed a decrease in TNF-α⁺ T cells (12.6% after 24h, 7.4% after 48h), whereby this reduction was also observed in ELISA (Fig. 10). The percentage of IFN-γ⁺, IL-2⁺ and CD154⁺ CD8⁺ T cells increased between 24h and 48h (IFNγ⁺: 4.7% after 24h, 8.5% after 48h, IL-2⁺: 5.1% after 24h, 7.2% after 48h, CD154⁺: 8.3% after 24h, 10.4% after 48h) (Fig. 13B). Again the percentage of IFN-γ⁺ and IL-2⁺ CD8⁺ T cells outnumbered CD4⁺ T cells and the amount of TNF-α⁺ and CD154⁺ CD8⁺ T
cells was decreased compared to CD4\(^+\) T cells. After 48h also some T cells seemed to be activated by the soluble HBsAg, as TNF\(\alpha\) T cells reached 1.1% (CD4\(^+\) T cells) and 1.0% (CD8\(^+\) T cells), CD154\(^+\) T cells 2.7% (CD4\(^+\) T cells) and 3.1% (CD8\(^+\) T cells), IFN\(\gamma\) CD8\(^+\) T cells 1.2% and IL-2\(^+\) CD8\(^+\) T cells 1.4%. Again boolean gates were used for further analysis of T cells secreting cytokines (Fig. 13C, D). 0.35% (after 24h) and 0.63% (after 48h) of CD4\(^+\) T cells, 0.3% (after 24h) and 1.0% (after 48h) of CD8\(^+\) T cells were IFN-\(\gamma\), IL-2\(^+\) and TNF-\(\alpha\), indicating polyfunctionality of T cells. \(\alpha\)CD3\(\Delta\)DCC/\(\alpha\)CD28\(\Delta\)ADCC mediates activation of PBMCs during co-culture with immobilized HBsAg cells resulting in polyfunctional CD4\(^+\) and CD8\(^+\) T cells. The activation due to soluble HBsAg remains poor.

Bispecific antibodies mediate IFN\(\gamma\) secretion and killing of HBV infected HepaRG cells

Finally, it was of interest, if bispecific antibodies are able to retarget T cells towards HBV infected HepaRG cells. Success of infection was tested by the measurement of HBsAg in the supernatant of infected cells. Compared to results of HuH7-S or HepG2.2.15 cells, the concentration of HBsAg produced by HBV infected HepaRG cells was very low. Additionally the values in different wells varied a lot, indicated by the relatively high standard deviation (Fig. 14).

Nevertheless, the infection was successful and co-culture of PBMCs with HepaRG cells in presence of bispecific antibodies was performed. As can be seen in Figure 15, viability of untreated cells decreased over time, with a remaining viability of 65.9% (HBV+) and 62.9% (HBV-) after 56h. In comparison, \(\alpha\)CD3 and the combination of \(\alpha\)CD3/\(\alpha\)CD28 mediated specific lysis of HBV infected HepaRG cells. \(\alpha\)CD28 alone could not induce specific elimination of target cells. If \(\alpha\)CD3 was present during co-culture, the viability of HBV infected cells decreased to 25.3%, while non-infected HepaRG cells remained at 53.5% (Fig.15A). The stimulation of effector cells by \(\alpha\)CD3/\(\alpha\)CD28 also led to significant killing of HBV infected HepaRG cells (Fig.15B), whereby 37.5% of target cells remained viable (not infected HepaRG cells: 62.4%).

Therefore, \(\alpha\)CD3 or \(\alpha\)CD3/\(\alpha\)CD28 induce specific lysis of HBV infected HepaRG cells.

Bispecific antibodies mediate reduction of HBV-positive tumors in vivo

Immunodeficient mice injected with human HBV-transgenic hepatoma cell line HepG2.2.15 to develop subcutaneous HBV-positive tumors were injected with human PBMC and bispecific
constructs directed against CD3 and CD28 (Fig. 16). The treatment resulted in a marked reduction in tumor size in comparison to not-treated or mock treated (animals receiving PBMC and PBS) animals. The tumor size was reduced by about fifty percent in treated animals.
1. A polypeptide comprising
   (a) a first set of six complementarity determining regions (CDRs) configured to bind
       a first antigen; and
   (b) (ba) a second set of six CDRs configured to bind a second antigen; or
       (bb) a ligand capable of binding to a second antigen;
   wherein
   (i) said first antigen is selected from Hepatitis B virus (HBV) small surface antigen;
       HBV medium surface antigen; and HBV large surface antigen; and
   (ii) said second antigen is selected from surface antigens presented by immune
        effector cells such as natural killer (NK) cells and cytotoxic T lymphocytes
        (CTLs).

2. The polypeptide of claim 1, wherein
   (a) said first set of six CDRs is comprised in a first scFv fragment; and/or
   (b) (ba) said second set of six CDRs is comprised in a second scFv fragment; or
       (bb) said ligand is an immunoligand, preferably capable of binding to NKG2D,
        Nkp30/NCR3, 4-1BB or OX40.

3. The polypeptide of claim 1 or 2, wherein said first set of six CDRs binds an epitope of
   said first antigen which epitope is located
   (a) in said HBV small surface antigen;
   (b) in the part of said HBV large surface antigen which is not comprised by said
       HBV small surface antigen; or
   (c) in a part of said HBV large surface antigen which part varies in structure from
       said HBV small surface antigen.

4. The polypeptide of any one of claims 1 to 3, wherein said surface antigen presented
   by immune effector cells is selected from CD3, CD28, 4-1BB, OX40, CD16, CD56,
   NKG2D, and Nkp30/NCR3.

5. The polypeptide of any one of the preceding claims, wherein said polypeptide further
   comprises a dimerization region.

6. The polypeptide of any one of claims 2 to 5, wherein said polypeptide further
comprises a spacer region, said spacer region preferably comprising a CH2 domain and a CH3 domain, said spacer region being located between
(i) said first scFv fragment and
(ii) said second scFv fragment or said recombinant ligand in the amino acid sequence of said polypeptide.

7. The polypeptide of any one of the preceding claims, wherein
(a) said first set of six CDRs has the sequences of SEQ ID NOs: 1 to 6, 7 to 12 or 13 to 18; and/or
(b) said second set of six CDRs has the sequences of SEQ ID NOs: 19 to 24, 25 to 30, 31 to 36 or 37 to 42,

wherein within each set of six CDRs the order of CDRs is as follows: CDR1 of heavy chain, CDR2 of heavy chain, CDR3 of heavy chain, CDR1 of light chain, CDR2 of light chain, and CDR3 of light chain.

8. The polypeptide of any one of the preceding claims, wherein said polypeptide comprises or consists of the amino acid sequence of any one of SEQ ID NOs: 43 to 46 or an amino acid sequence which exhibits at least 80% identity to any one of SEQ ID NOs: 43 to 46, provided that the CDRs of said amino acid sequence exhibiting at least 80% identity are identical to SEQ ID NOs: 1 to 6, 7 to 12, 13 to 18, 19 to 24, 25 to 30, 31 to 36 or 37 to 42, respectively.

9. A nucleic acid encoding the polypeptide of any one of the preceding claims.

10. A covalently linked complex comprising or consisting of a first and a second polypeptide, wherein there is at least one covalent linkage between said first and said second polypeptide, preferably at least one disulfide bridge between a Cys residue of said first polypeptide and a Cys residue of said second polypeptide, said first and second polypeptides being as defined in any one of claims 1 to 8.

11. A composition comprising or consisting of one or more polypeptides according to any one of claims 1 to 8 and/or one or more complexes according to claim 10, provided that at least two polypeptides are comprised in said composition which two polypeptides are distinct from each other with regard to the first antigen and/or the second antigen to which they bind.
12. The composition of claim 11, wherein said two polypeptides are
   (a) (i) a polypeptide binding to HBV small or large surface antigen and CD3; and
   (ii) a polypeptide binding to HBV small or large surface antigen and CD28;
   or
   (b) (i) a polypeptide binding to HBV small or large surface antigen and CD16; and
   (ii) a polypeptide binding to HBV small or large surface antigen and CD56.

13. A pharmaceutical composition comprising or consisting of one or more polypeptides
   of any one of claims 1 to 8, one or more complexes of claim 10 and/or one or more
   compositions of claim 11 or 12.

14. One or more polypeptides of any one of claims 1 to 8, one or more complexes of
   claim 10 and/or one or more compositions of claim 11 or 12 for use in a method of
   treating or preventing HBV infection and/or a condition caused by said HBV infection,
   said condition caused by said HBV infection being selected from liver cirrhosis,
   hepatocellular carcinoma, and liver cancer, said liver cancer being characterized by
   the expression of one or more HBV surface antigens.

15. An in vitro or ex vivo immune effector cell which has a polypeptide of any one of
   claims 1 to 8 or a complex according to claim 10 bound to a surface antigen of said
   immune effector cell.
Figure 1

IgG → 2 x Fv → scFv

Insertion of linker

Linker:
A) (Gly₃Ser)₃ for CTL- and NK-specific scFvs
B) YOL-linker for HBV surface protein specific scFvs (C8, 5F9 and 5A19)

Figure 2

scFv

anti-huCD3

CH₃

CH₂

anti-HBs-Protein (scFv-C8)

scFv

anti-huCD3

CH₃

CH₂

scFv

anti-huCD3

CH₃

CH₂
Figure 3

**NK-cell mediated cytotoxicity**

- Bispecific antibody construct: antiCD56 + antiCD16, antiCD56, antiCD16
- PBMC
  - HBV negative
  - HBV positive

**T-cell mediated cytotoxicity**

- Bispecific antibody construct: antiCD28 + antiCD3, antiCD28, antiCD3
- PBMC
  - HBV negative
  - HBV positive
Figure 4

A

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<thead>
<tr>
<th>T-cell specific</th>
<th>NK-cell specific</th>
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<tr>
<td>IFN-γ [pg/ml]</td>
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</tr>
<tr>
<td>60000</td>
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PBMC: untreated

bispecific construct: CD3/CD28  CD16/CD56

B

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<tbody>
<tr>
<td>T-cell specific</td>
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<tr>
<td>NK-cell specific</td>
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<tr>
<td>Viability in %</td>
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PBMC: untreated

bispecific construct: CD3/CD28  CD16/CD56
Figure 11

A  CD4⁺ T cells

αCD3/αCD28

% of Max

HuH7-S

B  CD4⁺ T cells

αCD3ΔADCC/αCD28ΔADCC

% of Max

HuH7

C  CD8⁺ T cells

αCD3/αCD28

% of Max

D  CD8⁺ T cells

αCD3ΔADCC/αCD28ΔADCC

% of Max

Lamp-1 (CD107a)
Figure 13

A. αCD3ΔADCC/αCD28ΔADCC

- IFNγ
- IL-2
- TNFα
- CD154

B. αCD3ΔADCC/αCD28ΔADCC

- IFNγ
- IL-2
- TNFα
- CD154

C. Cytokine CD4+ T cells

- IFNγ+
- IL-2+
- TNFα+
- IFNγ+ IL-2+
- IFNγ+ TNFα+
- TNFα+ IL-2+
- IFNγ+ TNFα+ IL-2+

D. Cytokine CD4+ T cells

- IFNγ+
- IL-2+
- TNFα+
- IFNγ+ IL-2+
- IFNγ+ TNFα+
- TNFα+ IL-2+
- IFNγ+ TNFα+ IL-2+
Figure 16

Tumor size [mm]

not treated  PBMC  PBMC
           PBS  αCD3/αCD28
Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
   a. (means)
      - on paper  
      - X in electronic form  
   b. (time)
      - X in the international application as filed  
      - [ ] together with the international application in electronic form  
      - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/08 C07K16/28 C07K16/46 A61K39/395 A61P31/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched: (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Relevant to claim No.</th>
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<td>X</td>
<td>EP 2 524 699 A1 (TRION RES GMBH [DE]) 21 November 2012 (2012-11-21) page 7, paragraph 35; claim 1</td>
<td>1-15</td>
</tr>
</tbody>
</table>

X Further documents are listed in the continuation of Box C. X See patent family annex.

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier application or patent but published on or after the international filing date
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  * "A" document member of the same patent family

Date of the actual completion of the international search 1 December 2014

Date of mailing of the international search report 05/01/2015

Name and mailing address of the ISA/ European Patent Office, P.B. 5618, Patentlaan 2 NL-2280 HV Rijswijk, Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer Saame, Tina
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<th>Relevant to claim No.</th>
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### INTERNATIONAL SEARCH REPORT

**Information on patent family members**

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<tr>
<td>EP 2524699</td>
<td>A1</td>
<td>21-11-2012</td>
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Form PCT/ISA/210 (patent family annex) (April 2005)