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(54) Title: ANTI-CD33 ANTIBODIES AND USE THEREOF FOR IMMUNOTARGETING IN TREATING CD33-ASSOCIATED ILLNESSES

(54) Bezeichnung : ANTI-CD33 ANTIKÖRPER UND IHRE ANWENDUNG ZUM IMMUNOTARGETING BEI DER BEHANDLUNG VON CD33-ASSOZIIERTEN ERKRANKUNGEN

(57) Abstract: The invention relates to antibodies to the tumor-associated antigen CD33 and to the use thereof for immunotargeting CD33-positive cells. The antibodies according to the invention are suitable for use in the field of medicine, pharmaceuticals, and biomedical research. According to the invention, the aim is achieved by means of novel anti-CD33 antibodies comprising the complementary determining regions (CDRs) defined in the claim. The antibodies according to the invention are characterized by a high affinity for human CD33, of the order of magnitude of 10^{-10} mol/l. The CDR sequences according to the invention are suitable in particular for producing recombinant fragments (such as scFv fragments or bispecific antibodies) and for immunotargeting, due to the high affinity thereof. The invention further relates to the use of an antibody according to the invention for producing a medication for therapeutic and/or diagnostic application for illnesses associated with the expression of CD33, particularly for acute myeloid leukemia (AML). The invention thus also comprises a pharmaceutical composition comprising one or more antibodies according to the invention in association with a pharmaceutically acceptable thinning agent or carrier.

(57) Zusammenfassung: Die Erfindung betrifft Antikörper gegen das tumorassoziierte Antigen CD33 und ihre Anwendung zum Immunotargeting von CD33-positiven Zellen. Die erfindungsgemäßen Antikörper eignen sich zur Anwendung im Bereich der Medizin, der Pharmazie und in der biomedizinischen Forschung. Erfindungsgemäß wird die Aufgabe gelöst durch neue anti-CD33 Antikörper, welche die in Anspruch definierten komplementaritätsbestimmende Regionen (complementary determining regions, CDRs) enthalten. Die erfindungsgemäßen Antikörper zeichnen sich durch eine hohe Affinität für humanes CD33 aus, welche in der Größenordnung von 10^{-10} mol/l liegt. Durch die hohe Affinität eignen sich die erfindungsgemäßen CDR-Sequenzen insbesondere zur Herstellung von rekombinanten Fragmenten (wie scFv-Fragmenten oder bispezifischen Antikörpern) und zum Immunotargeting. Die Erfindung umfasst weiter die Verwendung eines erfindungsgemäßen Antikörpers zur Herstellung eines Arzneimittels zur therapeutischen und/oder diagnostischen Anwendung bei Erkrankungen, die mit der Expression von CD33 assoziiert sind, insbesondere bei akuter myeloischer Leukämie (AML). Damit umfasst die Erfindung auch eine pharmazeutische Zusammensetzung, die einen oder mehrere erfindungsgemäße Antikörper in Assoziation mit einem pharmazeutisch annehmbaren Verdünnungsmittel oder Träger enthält.

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**ANTI-CD33 ANTIBODIES AND USE THEREOF FOR IMMUNOTARGETING
IN TREATING CD33-ASSOCIATED ILLNESSES**

The invention concerns antibodies against the tumor-associated antigen CD33 and their
5 use for immunotargeting of CD33-positive cells. The antibodies according to the
invention are suitable for use in the medical field, in pharmacy and in biomedical
research.

Cancer (malignant neoplasia) is a class of illnesses in which a group of cells is
10 characterized by uncontrolled cell division, by invasion into and destruction of adjoining
tissues and sometimes also by metastasis. These malignant properties of cancer
differentiate it from benign tumors which, by their growth, displace surrounding tissue but
do not infiltrate it and do not metastasize. Most cancer types form tumors, some do not,
such as leukemia that affects the hematopoietic system. Cancer in the context of the
15 invention refers to malignant tumors as well as to hemoblastoses.

In cancer cells, the adjustment of growth, division, and destruction in the united cell
structure is not functioning. They are generated when certain genes change, these
20 changes are no longer repaired, leading to non-functionality of certain gene regions, so
that the cancer cells stimulate themselves to undergo division and ignore growth-
inhibiting signals of the cell environment.

In principle, the immune system attempts to fight the uncontrolled growth of the cells.
Problematic in this context is the fact that cancer cells are similar to normal body cells in
25 many respects because they have evolved from them. Therefore, the defense measures
of the immune system are usually not sufficient in order to control growth of the tumor.

In case of acute myeloid leukemia (AML), the myelopoiesis is affected, i.e., the part of
the hematopoietic system that is responsible for the formation of granulocytes and
30 monocytes. The myeloblasts represent in hematopoiesis an immature precursor of the
myeloid white blood cells. In AML, several genetic modifications that concern the genes

responsible for cell division result in a single myeloblast for formation of a cell that remains in the immature state and proliferates massively. This proliferation of the immature precursors in the bone marrow and also in the blood characterizes AML.

5 The modifications may be realized at several positions of the cell cycle so that AML has different phenotypic, genotypic and clinical properties. Modern classification of AML is based on the thesis that the properties and behavior of the tumor cells depend on at which stage of the cell cycle the proliferation was stopped. In many AML patients certain specific cytogenetic distinctive features can be determined which are often of
10 prognostic significance. The genetic modifications code for abnormal fusion proteins, mostly transcription factors, causing the uncontrolled proliferation.

Tumor cells differ from healthy cells by the expression of tumor antigens, i.e., of proteins that are expressed only by the tumor cells. They are generated as a result of the
15 modified genome in the cancer cells, i.e., by modified gene expression. Tumor antigens are located on the external cell membrane of the tumor cells, in the cell plasma, and in the cell nucleus. The tumor antigens as a target structure are the basis for most of the concepts of cancer immunotherapy. Currently, more than 2,000 tumor antigens are known.

20 Ideal as a target for the cancer therapy are tumor-specific antigens (TSA). i.e., antigens produced only by cancer cells but not by healthy cells. Most tumor antigens are however not tumor-specific but tumor-associated (TAA), i.e., they are also expressed by healthy cells. However, in many tumors the tumor antigens are overexpressed. By
25 mutations in the genome, structural changes in the protein sequence can occur also. Many tumor antigens occur only for certain tumor types and, in this connection, often only in certain situations.

Tumor antigens that are present in case of AML comprise inter alia TAAs that are
30 associated with a series of further tumor illnesses, for example, telomerase reverse transcriptase, Wilm's tumor 1 (WT1)-protein, and survivin. Moreover, there are also

TAA's known that are associated with other leukemia illnesses such as CD168 and M phase phosphoprotein. Moreover, there are TAA's found in particular in connection with AML, such as CD33, CD45 and mHAg's (minor histocompatibility antigen).

5 CD33 is a glycosylated transmembrane protein of 364 amino acids and belongs to the family of sialic acid binding Ig-related lectins (SIGLECs). It is expressed also in early hematopoietic precursor cells, myelomonocytic precursors, and myeloid cells while it is not present on normal pluripotent bone marrow stem cells. Approximately 85-90 % of AML patients are positive with regard to CD33. Therefore, CD33 is a particularly
10 attractive target for immunotherapy and is already used as a target in a targeted immunotherapy.

The conventional AML therapy is a chemotherapy for induction of remission; subsequently, this may be followed by several chemotherapies or transplantation of
15 hematopoietic stem cells. In the United States, a monoclonal antibody conjugated with a cytostatic agent, gemtuzumab ozogamicin, was approved in the year 2000 for patients with recurrent AML who are no candidates for the standard chemotherapy.

Antibodies are proteins of the class of globulins which in vertebrates are formed in
20 response to certain substances, i.e., antigens. In combating cancer cells, the body's own polyclonal antibodies only play a very minor role. This results from the fact that most tumor cells do not present sufficiently strongly modified antigens that are recognized as foreign by the body's own immune system so that the body's own antibodies do not bind in a sufficient number to the tumor cells. Monoclonal antibodies are with respect to their
25 structure completely identical and are specific to only one epitope of an antigen.

For cancer therapy, the employed monoclonal antibodies act almost exclusively by the antibody-dependent cell-mediated cytotoxicity (antibody dependent cellular cytotoxicity, ADCC). In this connection, antibodies recruit cytotoxic effector cells, such as NK cells,
30 macrophages, lymphocytes or granulocytes that themselves have no antigen specificity, to the tumor cell by means of their Fc region. The direct damage of the cell by bound

monoclonal antibodies, such as by triggering certain intracellular signal cascades that initiate apoptosis by cross-linking of the tumor antigens as a result of antibody binding, is a rare exception.

5 In order to reinforce the effect of monoclonal antibodies in cancer illnesses, different strategies of drug targeting have been developed in order to utilize the antibodies as carriers for more potent active ingredients, so-called immunoconjugates or antibody derivatives. For this purpose, active ingredients such as radionuclides, toxins (for example, the diphtheria toxin), cytokines or also cytostatic agents were bonded
10 (conjugated) to the corresponding antibodies.

In this connection, it is in principle possible to bind highly potent active ingredients that, in case of free systemic administration would cause intolerable side effects as a result of their high toxicity, to an antibody. The antibody derivatives are comprised of three
15 components: an antibody or antibody fragment, an active ingredient, and a linking member (linker) between antibody and active ingredient.

The first clinically tested antibody directed against CD33 is M195. The humanized variant of this antibody of the isotype IgG1, also known by the name lintuzumab, was
20 tested in clinical studies wherein in phase III no advantage of lintuzumab in combination with chemotherapy was however determined when compared to chemotherapy alone (E. J. Feldman et. al., Phase III Randomized Multicenter Study of a Humanized Anti-CD33 Monoclonal Antibody, Lintuzumab, in Combination With Chemotherapy, Versus Chemotherapy Alone in Patients With Refractory or First-Relapsed Acute Myeloid
25 Leukemia. J. Clin. Oncol. 23 (18) 2005, pp. 4110-4116).

Gemtuzumab ozogamicin is an immunoconjugate of the monoclonal antibody specific to CD33 of the type IgG4 that has been used in the USA for treatment of AML until 2010 but has been taken off the market in the meantime. It is comprised of a humanized
30 monoclonal antibody against CD33 which is conjugated with a cytostatic agent,

calicheamicin. The action mechanism is based on the endocytosis of the antibody derivative so that the cytostatic agent coupled thereto develops its toxic action after absorption in the cell. Gemtuzumab ozogamicin was not approved in Europe because the efficacy was not satisfactorily proven in the eyes of the European Medicines Agency EMEA.

The following table shows the amino acid sequences of the CDRs (complementary determining regions, the antigen binding sites of an antibody) of the anti-CD33 antibodies disclosed in the corresponding documents.

Document	V _H CDR1	SEQ ID NO.	V _H CDR2	SEQ ID NO.	V _H CDR3	SEQ ID NO.
US 7,557,189	SYIYIH	37	VIYYPGNDDISYNQKFXG	38	EVRLRYFDV	39
US 5,730,982	DYNMH	43	YIYPYNGGTGYNQKFKS	44	GRPAMDY	45
CN101210048	DYNMY	49	YIDPYKGGTIYNQKEKG	50	QMITAYYFDY	51
	V _L CDR1		V _L CDR2		V _L CDR3	
US 7,557,189	KSSQSVFFSSSQKNYLA	40	WASTRES	41	HQYLSSRT	42
US 5,730,982	RASESVDNYGISEMN	46	ASNQGS	47	QQSKEVPWT	48
CN101210048	KASQDINKYIA	52	TSTLQP	53	LOYDNLTT	54

A general problem for the therapeutic effectiveness of the monoclonal antibodies is the binding behavior of the antibodies on the cancer cells, i.e., the affinity of the antibodies. Even in cancer cells that present sufficient tumor antigens, the binding rate is often not satisfactorily high. With a molecular mass of approximately 150 kDa, antibodies are moreover generally limited in regard to their tissue migration. In this case, antibody fragments, such as Fab, F(ab)₂, or scFv (single chain variable fragments), have significant advantages as a result of their significantly reduced size.

Bispecific antibodies, i.e., antibody derivatives as components of two different monoclonal antibodies, provide new possibilities for therapy concepts in cancer immunotherapy.

Quadroma are bispecific antibodies of the first generation and are comprised each of a heavy chain and a light chain of two different monoclonal antibodies. The two arms of the antibody in this connection are specific to different antigens, respectively. The Fc region is formed commonly of the two heavy chains of the antibodies. By this configuration it is, for example, possible to place the paratope of an antibody specific to the tumor antigen and the paratope of another antibody specific to a lymphocyte antigen onto one arm each of the bispecific antibody. It is thus possible to form a three-cell complex that results from the respective different cells bonded to the paratopes and the effector cell bonded to the Fc region. In this connection, generally an improved activation of the body's own immune cells relative to the tumor cells is achieved.

Bispecific antibodies of the newer generation are comprised of two different scFv fragments. They are connected to each other by a linker. For example, a bispecific antibody can bind with an scFv to tumor cells and with the other scFv to effector cells.

When a paratope is specific to T cells, these cells can be activated also. This is not possible with normal monoclonal antibodies because T cells have no Fc receptor. Bispecific antibodies have moreover a higher cytotoxic potential. They bind also to antigens that are expressed relatively weakly.

Up to now, no bispecific antibodies for clinical use in humans have been approved.

Bispecific antibodies are known in which an scFv binds to the CD3 complex on T cells; these are referred to also as BiTE (bispecific T cell engager) (P. A. Baeuerle et. al., BiTE: Teaching antibodies to engage T cells for cancer therapy. *Curr Opin Mol Ther* 11, 2009, pp. 22–30).

At the moment, two different BiTE antibodies are in clinical studies. Blinatumomab, an antibody that is specific to CD3 and CD19, is tested in patients in late phases of non-Hodgkin lymphoma and in patients with acute lymphoblast leukemia of the B cell series

(B-ALL). MT110 is an antibody that is specific to CD3 and EpCAM (epithelial cell adhesion molecule) and is tested in patients with bronchial carcinoma and patients with gastrointestinal cancer illnesses (R. Bargou et al., Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science* 321, 2008, pp. 974–977; K. Brischwein et al., MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. *Mol Immunol* 43, 2006, pp. 1129–1143).

Not all monoclonal antibodies are suitable in the form of scFv fragments or for the construction of bispecific constructs. In this connection, in particular the affinity of the antibodies is decisive that is determined by the variable regions. Only antibodies that have an especially high affinity are suitable as scFv fragments because binding to the respective antigen is realized only with one pair of variable regions of the heavy and light chains, in contrast to complete IgG antibodies that have two pairs of variable regions of the heavy and light chains.

For the treatment of AML and other carcinomas, there is the need for a new therapeutic concept. Antibodies that are currently known have an affinity that is too low for tumor-associated antigens such as CD33 in order to be usable therapeutically. Therefore, there is a need for the development of specific antibodies for tumor-associated antigens, for example, CD33, that have a high affinity and are therefore suitable for use in cancer therapy.

When used in this specification and claims, the terms "comprises" and "comprising" and variations thereof mean that the specified features, steps or integers are included. The terms are not to be interpreted to exclude the presence of other features, steps or components.

The above references to and descriptions of prior proposals or products are not intended to be, and are not to be construed as, statements or admissions of common general knowledge in the art in Australia.

Object of the invention is therefore providing new anti-CD33 antibodies in particular with a high affinity to CD33 that enables use of the antibodies as recombinant fragments for immunotargeting.

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According to an aspect of the invention, there is provided an isolated anti-CD33 antibody containing regions determined by complementarity (complementary determining regions, CDRs), wherein the CDRs include the following sequences:

a) variable region of the heavy chain (V_H)

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CDR1 DYVX₁H, wherein

X₁ is selected from V, I and L,

CDR2 X₁INX₂YNX₃X₄X₅X₆YNX₇KFX₈G, wherein

X₁ is selected from Y, W, F, V, I and L,

X_2 is selected from P, G, S and T,
 X_3 is selected from D, G, E and A,
 X_4 is selected from G, D, E and A,
 X_5 is selected from T, V, S, I and L,
 X_6 is selected from K, Q, N, and R,
 X_7 is selected from E, Q, D and N,
 X_8 is selected from K, R, Q, N, L, F, I and M,

CDR3 DYRYEX₁YX₂MDY, wherein
 X_1 is selected from V, L, I, M, F, A, W, F, T, S and Y,
 X_2 is selected from G, P, V, L, I and A,

and

b) variable region of the light chain (V_L) :

CDR1 X₁AX₂SSVX₃YIH, wherein
 X_1 is selected from T and S,
 X_2 is selected from S, T, Q, H, K, R and N,
 X_3 is selected from N, Q, H, K, R, T and S,

CDR2 TSKX₁AS, wherein
 X_1 is selected from V, I, M, F, A and L,

CDR3 QQWX₁SX₂PLT, wherein

X_1 is selected from R, K, Q, N, S and T,
 X_2 is selected from Y, W, F, T, S, N, Q, K, R and H.

25 According to the invention, the object is solved by new anti-CD33 antibodies which
 contain regions determined by complementarity (complementary determining regions,
 CDRs) that are characterized in that the CDRs of the variable region of the heavy chain
 (V_H) comprise the following sequences:

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- CDR1 $DYVX_1H$, wherein
 X_1 is selected from V, I and L, preferably from V und L,
- CDR2 $X_1INX_2YNX_3X_4X_5X_6YNX_7KFX_8G$, wherein
 X_1 is selected from Y, W, F, V, I and L, preferably from Y and L,
 X_2 is selected from P, G, A, S and T, preferably from P and T,
 X_3 is selected from D, G, E and A, preferably from D and G,
 X_4 is selected from G, D, E and A, preferably from G and D,
 X_5 is selected from T, V, S, I and L, preferably from T and V,
 X_6 is selected from K and R,
 X_7 is selected from E, Q, D and N, preferably from E and Q,
 X_8 is selected from K, R, Q, N, L, F, I and M, preferably from K and M,
- CDR3 $DYRYEX_1YX_2MDY$, wherein
 X_1 is selected from V, L, I, M, F, A, W, F, T, S and Y, preferably from V
 and Y,
 X_2 is selected from G, P, V, L, I and A, preferably from G and A,
- and in that the CDRs of the variable region of the light chain (V_L) comprise the following
 sequences:
- CDR1 $X_1AX_2SSVX_3YIH$, wherein
 X_1 is selected from T and S,
 X_2 is selected from S, T, Q, H, K, R and N, preferably from S and N,
 X_3 is selected from N, Q, H, K, R, T and S, preferably from N and S,
- CDR2 $TSKX_1AS$, wherein
 X_1 is selected from V, I, M, F, A and L, preferably from V and L,
- CDR3 $QQWX_1SX_2PLT$, wherein
 X_1 is selected from R, K, Q, N, S and T, preferably from R and T,
 X_2 is selected from Y, W, F, T, S, N, Q, K, R and H, preferably from Y
 and H.

Preferred are antibodies whose CDR2 of the variable region of the heavy chain (V_H)

comprise one of the following sequences:

$GX_1INX_2YNX_3X_4X_5X_6YNX_7KFX_8G$,

$X_1INX_2YNX_3X_4X_5X_6YNX_7KFX_8GK$, or

$GX_1INX_2YNX_3X_4X_5X_6YNX_7KFX_8GK$, wherein

- 5 X_1 is selected from Y, W, F, V, I and L, preferably from Y and L,
 X_2 is selected from P, G, A, S and T, preferably from P and T,
 X_3 is selected from D, G, E and A, preferably from D and G,
 X_4 is selected from G, D, E and A, preferably from G and D,
 X_5 is selected from T, V, S, I and L, preferably from T and V,
10 X_6 is selected from K and R,
 X_7 is selected from E, Q, D and N, preferably from E and Q,
 X_8 is selected from K, R, Q, N, L, F, I and M, preferably from K and M.

Preferred are antibodies whose CDR2 of the variable region of the light chain (V_L)
15 comprises the following sequence:

$DTSKX_1AS$, wherein

X_1 is selected from V, I, M, F, A and L, preferably from V and L.

Especially preferred are antibodies whose CDRs of the variable region of the heavy
20 chain comprise the following sequences:

CDR1 SEQ ID NO. 01

CDR2 SEQ ID NO. 02

CDR3 SEQ ID NO. 03

and whose CDRs of the variable region of the light chain comprise the following
25 sequences:

CDR1 SEQ ID NO. 04

CDR2 SEQ ID NO. 05

CDR3 SEQ ID NO. 06.

Especially preferred are anti-CD33 antibodies whose CDRs of the variable region of the heavy chain comprise the following sequences:

CDR1 SEQ ID NO. 07,

5 CDR2 SEQ ID NO. 08, or

SEQ ID NO. 33, or

SEQ ID NO. 34, or

SEQ ID NO. 35,

CDR3 SEQ ID NO. 09

10 and whose CDRs of the variable region of the light chain comprise the following sequences:

CDR1 SEQ ID NO. 10,

CDR2 SEQ ID NO. 11 or SEQ ID NO. 36,

CDR3 SEQ ID NO. 12.

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The anti-CD33 antibodies according to the invention, herein also referred to as anti-CD33DRB, are characterized by their amino acid sequences of the heavy and the light chains, their CDRs, and by methods for expression of the antibody in recombinant form.

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Moreover, the anti-CD33DRB antibodies are functionally characterized and it has been demonstrated that they are distinguished by a high affinity for human CD33 (Figure 3). The antibodies according to the invention have thus advantageously a high affinity for human CD33. The affinities of the native antibodies are within an order of magnitude of 10^{-10} mol/l. The affinities of the recombinant derivatives achieve 10^{-7} to 10^{-8} mol/l. It was possible to demonstrate that the antibodies predominately are not taken up by endocytosis in the CD33-positive cells and remain on the cell surface for a long period of time. This makes them particularly suitable for a therapeutic use for immunotargeting of

25

effector cells on CD33-positive cells. Because of the high affinity the CDR sequences according to the invention are suitable in particular for preparing recombinant fragments (like scFv) and for immunotargeting.

5 The term antibody in the meaning of the invention comprises all antibodies, antibody fragments, and derivatives thereof that are capable of binding to the antigen, in this case CD33, that comprise the CDRs according to the invention completely or partially. This encompasses the complete monoclonal antibodies and also the epitope-binding fragments of these antibodies. In this connection, the epitope-binding fragments (also
10 referred to herein as antibody fragments or antibody derivatives) comprise all regions of the antibody that are capable of binding to the antigen, in this case CD33. Examples of antibody fragments that are preferred in accordance with the invention comprise, but expressly are not limited to, Fab, Fab', F(ab')₂, Fd, individual chain (single-chain) variable fragments (scFv), single-chain antibodies, disulfide-linked variable fragments
15 (sdFv), and fragments that either contain a variable region of the light chain (V_L) or a variable region of the heavy chain (V_H). Moreover, they include recombinantly prepared antibodies, such as diabodies, triabodies, and tetrabodies.

20 Preferably, the antibody carries a marker molecule, for example, biotin, dioxigenin, a radionuclide, or a fluorescent dye. Especially preferred, the antibody is conjugated with an effector group.

25 Antibody fragments contain the variable regions either alone or in combination with further regions that are selected from the hinge region and the first, second and third regions of the constant region (C_H1, C_H2, C_H3). Also, the term antibody comprises chimeric antibodies in which different regions of the antibody originate from different species, for example, antibodies with a murine variable region combined with a human constant region.

30 Antibody fragments are optionally linked with each other by a linker. The linker

comprises a short (preferably 10 to 20 amino acid residues), flexible peptide sequence that is selected such that the antibody fragment has such a three-dimensional folding of V_L and V_H that it exhibits the antigen specificity of the complete antibody. Preferred linkers, but not exclusive linkers, are glycine-serine linkers with the structure (Gly_xSer_y) with x and y selected from 1 to 10, preferably 3 to 5. Moreover, linkers are preferred that are comprised of a peptide sequence that can increase the protease resistance of the antibody derivatives.

The term variable region is defined in accordance with the invention as the regions of the heavy and light chains of the antibodies that differ in their sequence between antibodies and that determine the specificity of the antibody and binding to its antigen. The variability in this context is not distributed uniformly in the variable region. It is usually concentrated within three defined segments of the variable region that are referred to as complementary determining regions (CDRs) or also as hypervariable regions and are existing in the variable regions of the light as well as the heavy chains. The more strongly preserved portion of the variable regions are referred to as scaffolding regions (framework regions). The variable regions of the heavy and the light chains contain four framework regions that primarily assume a beta-sheet structure wherein each framework region is connected with three CDRs that form loops that connect the beta-sheet structures and in some cases are part of the beta-sheet structure. The CDRs of the respective chain are moved by the framework regions into immediate vicinity and contribute together with the CDRs of the other chain to the formation of the antigen binding region of the antibody.

The constant region (Fc) of the antibody is not participating in antigen binding but provides instead multiple effector functions that are triggered by binding to the corresponding Fc-binding receptors, for example, the induction of antibody-dependent cellular cytotoxicity (ADCC).

Preferably, the antibodies according to the invention comprise at least one variable

region of the heavy chain (V_H) and a variable region of the light chain (V_L) in the form of an scFv. The variable region of the heavy chain (V_H) and the variable region of the light chain (V_L) each contain in this context at least one of the CDR sequences according to the invention.

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According to the invention, antibodies are preferred that have the following structures that can be optionally humanized:

- a variable region of the heavy chain according to sequence SEQ ID NO. 13 and a
10 variable region of the light chain according to sequence SEQ ID NO. 14.

Moreover, preferred antibodies are antibodies that contain the following structures:

- a variable region of the heavy chain according to the sequence SEQ ID NO. 15
15 and a variable region of the light chain according to sequence SEQ ID NO. 16.

In the antibodies according to the invention, certain amino acids of the specific amino acid sequences can be exchanged in such a way that they maintain the binding properties of the CD33 antibody but differ in their sequence by exchange, deletion or
20 addition of one or several amino acids. Therefore, encompassed are also antibodies that contain structures whose amino acid sequences relative to the amino acid sequences according to the invention of the variable regions according to SEQ ID NOs. 13 to 16 have a sequence identity of preferably at least 70 %, especially preferred at least 80 %, in particular at least 90 %, and that bind the antigen CD33, wherein these
25 sequences comprise at least one of the CDRs according to the invention in accordance with SEQ ID NOs. 1 to 12.

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In preferred embodiments of the invention, the antibody comprises the following structures:

- a variable region of the heavy chain with the sequence according to SEQ ID NO. 13 and a variable region of the light chain with the sequence according to SEQ ID NO. 14, or a variable region of the heavy chain with the sequence according to SEQ ID NO. 15 and a variable region of the light chain with the sequence according to SEQ ID NO. 16; and
- a constant region of a heavy chain of a human IgG;
- a region C_L of the human kappa light chain
- and a human IgG3 hinge region.

Optionally, the antibody is existing in the form of a F(ab')₂ fragment.

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In an especially preferred embodiment of the invention, antibodies are in the form of scFv fragments which contain at least one variable region of the heavy chain (V_H) and/or the variable region of the light chain (V_L) that contain the CDR regions according to the invention. Moreover, especially preferred are antibodies in the form of scFv fragments which comprise at least one of the variable regions of the heavy and/or light chain according to the invention.

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The invention comprises murine anti-CD33 antibodies and humanized versions of these antibodies.

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The goal of humanizing antibodies lies in the reduction of the immunogenicity of a xenogenic antibody, in this case of the murine antibody, for use in the human system wherein the complete binding affinity and the antigen specificity are maintained. Humanized antibodies can be prepared in various ways, for example, by resurfacing and CDR grafting. In case of resurfacing, by a combination of molecular modeling, statistic analyses, and mutagenesis, all non-CDR regions on the surface of the antibody are modified so that they are similar to the surface of antibodies of the target organism. In CDR grafting, the CDR regions according to the invention are introduced into human variable regions.

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Humanized antibodies that contain the CDR regions according to the invention are expressly encompassed by the invention.

5 An aspect of the invention concerns also antibodies that are conjugated with an effector group. Conjugation in this context means the coupling of a substance to an antibody. The connection of the antibody to the effector group is preferably prepared by the expression as a fusion protein or by in vitro methods wherein the effector group preferably is bonded by a linker group to the antibody (for example, by means of thioether bonds or disulfide bonds). They may be bonded to the antibody also by an
10 intermediary carrier molecule, for example, serum albumin. Optionally, an antibody contains also several effector groups.

In this connection, the effector groups are preferably pharmaceutically active substances (active ingredients). Preferred active ingredients comprise, but are not limited to, toxins,
15 such as cytostatic agents, for example, maytansinoids and maytansinoid analogues, taxoids, CC-1065 and CC-1065 analogues, dolastatin and dolastatin analogues, methotrexate, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil, and calicheamicin. The invention comprises also antibodies which are conjugated with radionuclides as effector groups and their use for therapy and
20 diagnostics, in particular of tumors. Suitable radionuclides are preferably the radioactive isotopes of technetium, rhenium, yttrium, copper, gallium, indium, bismuth, and platinum, for example, ^{99m}Tc , ^{90}Y , ^{186}Re , ^{188}Re , ^{68}Ga and ^{111}In .

Effector groups according to the invention comprise furthermore enzymes (particularly
25 enzymes suitable for the ADEPT system), co-stimulating molecules (e.g. CpG) or also nucleic acids. The antibody that is conjugated with an effector group may exist optionally in the form of a fusion protein.

30 Further aspects of the invention are also antibodies that are conjugated with a further antibody or antibody fragment which is specific to an antigen different than CD33. The

antibody in this case is a bispecific antibody. Preferably, the antibody is a single chain bispecific diabody (scBsDb). In this case, two scFv fragments are connected to each other by means of a short linker (preferably having a length of 10 to 20 amino acid residues). It is preferred especially that the antibody is a single chain bispecific tandem antibody (scBsTaFv). In this case, the two scFv fragments are connected by a longer linker (preferably having a length of 18 to 50 amino acid residues), and this results in a particularly flexible structure.

Preferably, the bispecific antibodies contain, in addition to the CD33 antibody according to the invention, an antibody or an antibody fragment that is specific to surface antigens of effector cells, for example, T cells, in particular cytotoxic T cells, NK cells, monocytes, macrophages, dendritic cells or granulocytes. The definition of effector cells in the meaning of the invention comprises all cells of the inherent and adaptive immune system that mediate immune reactions or actively participate therein. It is particularly preferred that these antibodies are specific to the following surface structures on effector cells: CD3, CD8, CD4, CD25, CD28, CD16, NKG2D, NKp46, NKp44, activating KIR receptors (activating killer cell immunoglobulin-like receptors).

An aspect of the invention concerns also antibodies that are conjugated with a ligand that has an effect on the activity of effector cells by binding to the surface of the effector cells. The ligand is selected such that it binds specifically to surface structures of effector cells and, by the binding action, triggers signal cascades for activating the effector cells. Preferably, as a ligand a protein structure or a glycan that binds specifically to a receptor that is specifically expressed on the surface of the effector cells, wherein the ligand by its binding action to the receptor effects activation of the effector cell. It is especially preferred to select the protein structures from ULB-Ps (e.g. ULB-P2), MICA, MICB, as well as cytokines (such as IL2 and IL15) and their fusion proteins.

The connection of the antibody to the further antibody, antibody fragment, or to the protein structure is preferably produced by an expression as a fusion protein or by *in vitro* methods wherein the further antibodies, antibodies, or protein structures preferably

are bonded by linkers such as the peptide linkers to the antibody.

The invention comprises furthermore nucleic acid sequences that code for an antibody according to the invention, as well as vectors that contain the nucleic acid sequences.

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The vector (expression vector) is preferably a plasmid, an artificial chromosome, or even a virus particle or another vector that contains an expression cassette that is incorporated stably into the genome of the host (host cell or host organism).

10 Preferably, the nucleic acid sequences that code for an antibody according to the invention contain the following sequences that code for the CDR regions of the variable regions of the heavy chain::

- CDR1 SEQ ID NO. 17,
- 15 - CDR2 SEQ ID NO. 18,
- CDR3 SEQ ID NO. 19, and

the following sequences that code for the CDR regions of the variable regions of the light chains

- CDR1 SEQ ID NO. 20,
- 20 - CDR2 SEQ ID NO. 21, and
- CDR3 SEQ ID NO. 22.

It is especially preferred that the nucleic acid sequences that code for an antibody according to the invention contain the following sequences that code for the CDR
25 regions of the variable regions of the heavy chains:

- CDR1 SEQ ID NO. 23,
- CDR2 SEQ ID NO. 24,

- CDR3 SEQ ID NO. 25, and

the following sequences that code for the CDR regions of the variable regions of the light chain

- CDR1 SEQ ID NO. 26,
- CDR2 SEQ ID NO. 27, and
- CDR3 SEQ ID NO. 28.

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In a further preferred embodiment of the invention, the nucleic acid sequences that code for an antibody according to the invention contain the following sequence that is coding for the variable region of the heavy chain: SEQ ID NO.29 and/or the following sequence that codes for the variable region of the light chain: SEQ ID NO. 30.

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In yet another preferred embodiment of the invention, the nucleic acid sequences that code for an antibody according to the invention contain the following sequence that is coding for the variable region of the heavy chain: SEQ ID NO. 31 and/or the following sequence that codes for the variable region of the light chain: SEQ ID NO. 32.

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Aspects of the invention are also host cells or non-human host organisms that contain a nucleic acid sequence according to the invention.

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A host cell is a naturally occurring cell or a transformed or genetically modified cell line or a (multicellular) non-human host organism that contains the expression system according to the invention (i.e., at least one expression vector). The invention comprises in this connection transient transfectants (for example, by mRNA injection), i.e., hosts (host cells or host organisms) in which the expression system is contained as a plasmid or artificial chromosome as well as hosts in which the expression system is integrated stably into the genome of the host (or individual cells of the host). The host cell is preferably selected from prokaryotes or eukaryotes. Preferred prokaryotes are selected from *Escherichia coli* and *Bacillus subtilis*. Preferred eukaryotes are yeast cells (e.g.

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Saccharomyces cerevisiae, *Pichia pastoris*), insect cells, amphibic cells or mammalian cells, for example, CHO, HeLa, Hek293T, Hek293A. Preferred host organisms are plants, for example, maize or tobacco, invertebrates or vertebrates, in particular *Bovidae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, *Medaka*,
5 zebrafish or *Mus musculus*, or cells or embryos of the aforementioned organisms.

The invention encompasses moreover a pharmaceutical composition that contains one or several antibodies according to the invention in association with a pharmaceutically acceptable dilution agent or carrier. Preferably, the pharmaceutical composition is
10 present in a form suitable for intravenous administration.

Preferably, the composition comprises a chimeric or humanized antibody with a reduced immunogenicity which contains the CDR regions according to the invention.

15 The pharmaceutical composition according to the invention comprises various administration forms. The pharmaceutical compositions are preferably administered parenterally, particularly preferred intravenously. In one embodiment of the invention, the parenteral pharmaceutical composition exists in an administration form that is suitable for injection. Particularly preferred compositions are therefore solutions,
20 emulsions, or suspensions of the antibody that is present in a pharmaceutically acceptable dilution agent or carrier.

As a carrier, preferably water, buffered water, 0.4 % saline solution, 0.3 % glycine and similar solvents are used. The solutions are sterile. The pharmaceutical compositions
25 are sterilized by conventional well-known techniques. The compositions contain preferably pharmaceutically acceptable excipients, for example, those that are required in order to provide approximately physiological conditions and/or to increase the stability of the antibody, such as agents for adjusting the pH value and buffering agents, agents for adjusting the toxicity and the like, preferably selected from sodium acetate, sodium
30 chloride, potassium chloride, calcium chloride and sodium lactate. The concentrations

of the antibodies according to the invention in these formulations, depending on the application, are variable; they are preferably less than 0.01 % by weight, preferably at least 0.1 % by weight, further preferred between 1 and 5 % by weight and they are selected primarily on the basis of fluid volumes, viscosity etc. or in compliance with the
5 respective administration mode.

The antibodies according to the invention are preferably introduced into a composition that is suitable for parenteral administration. Preferably, the pharmaceutical composition is an injectable buffered solution that contains between 0.1 to 500 mg/ml of antibody,
10 especially preferred between 0.1 to 250 mg/ml of antibody, in particular together with 1 to 500 mmol/l (mM) of a buffer. The injectable solution can be present in liquid as well as a lyophilized administration form. The buffer can be preferably histidine (preferably 1 to 50 mM, especially preferred 5 to 10 mM) at a pH value of 5.0 to 7.0 (especially preferred at a pH of 6.0).

Other suitable buffers encompass, but are explicitly not limited to, sodium succinate, sodium citrate, sodium phosphate, or potassium phosphate. Preferably, sodium chloride
15 between 0 to 300 mM, especially preferred 150 mM, is used for a liquid administration form. For a lyophilized administration form, the pharmaceutical composition contains preferably a cryoprotectant, preferably 0 – 10 % sucrose (especially preferred 0.5 – 1.0 %). Other cryoprotectants comprise trehalose and lactose. For a lyophilized administration form, the pharmaceutical composition contains preferably swelling agents, preferably 1 to 10% mannitol. Other swelling agents encompass glycine and arginine. In liquid as well as lyophilized administration forms, stabilizers are preferably
20 used, especially preferred between 1 to 50 mM of L-methionine (preferably between 5 and 10 mM).

In a preferred embodiment, the pharmaceutical composition comprises the antibody in a dosage quantity of 0.1 mg/kg to 10 mg/kg per administration. Especially preferred
30 dosage quantities comprise 1 mg/kg of body weight.

Pharmaceutical compositions must be sterile and stable under the manufacturing and storage conditions. The composition can be formulated as a solution, microemulsion, dispersion, in liposomes or in other ordered structures that are suitable for high concentrations of antibodies. Sterile injectable solutions can be produced in that the antibody is taken up in the required quantity of the suitable solvents, with one or with a combination of the aforementioned ingredients as needed, followed by sterilization by filtration. For sterile lyophilized powders for producing sterile injectable solutions, the preferred manufacturing methods are vacuum drying and spray drying; this produces a powder of the antibody plus possible additionally desired ingredients from a prior sterile-filtered solution thereof.

The invention comprises the use of the antibody according to the invention as a medication.

The invention comprises further the use of an antibody according to the invention for preparing a medication for therapeutic and/or diagnostic use in case of illnesses that are associated with the expression of CD33, in particular in acute myeloid leukemia (AML).

The invention also encompasses a method for treatment of a human having a disease that is associated with the expression of CD33 by administration of an antibody according to the invention.

The diseases that are associated with the expression of CD33 comprise cancer illnesses such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and pro-myeloid leukemia (PML), and further diseases such as the myelodysplastic syndrome (MDS). The application as a medication comprises also further not explicitly mentioned illnesses that are associated with the expression of CD33.

For therapeutic applications, a sterile pharmaceutical composition, containing a pharmacologically effective quantity of one or several antibodies according to the invention, is administered to a patient in order to treat the aforementioned illnesses.

According to the invention, in particular a use of the antibodies is preferred that leads to targeting of the CD33 expressing cells. For this purpose, by application of the antibody, binding by means of CD33 to the target cells is achieved and killing of the target cells is achieved through the Fc region or through the effector groups on the antibodies. This is realized either by recruiting effector cells or by targeted transport of pharmacological active ingredients (for example, toxins) to the CD33-positive cell and the release thereof.

In a special embodiment of the invention, an antibody in the form of a bispecific antibody is used for treatment of CD33-associated diseases. In this connection, the bispecific antibody in an especially preferred embodiment of the invention comprises an scFv fragment according to the invention and an scFv fragment that is specific to a surface structure of NK cells, preferably against ULB-P2. Preferably, the bispecific antibody is used for treatment of AML.

In a further especially preferred embodiment of the invention, the bispecific antibody contains an scFv fragment according to the invention and an scFv fragment that is specific to the surface structure of T cells, preferably against CD3 or CD8. Preferably, the bispecific antibody is used for treatment of AML.

In addition to the use in the medical field for therapeutic purposes, the antibodies according to the invention are suitable for diagnostics, biological research, and other applications in which the detection of CD33 is of interest. Such applications are in particular Western blot, immunostaining of cells (for example, for flow cytometry and microscopy), and ELISA.

The invention also encompasses a method for preparing an antibody according to the invention, in which a host cell or non-human host organism is exposed to conditions that are favorable for the expression and optionally secretion of the antibody, and optionally purifies at least partially the antibody. Preferably, for this purpose, host cells are cultivated in a selective medium that selects with regard to the growth of the cells that

contain an expression vector. The expression of the gene sequences of the expression vector results in the production of the antibodies or fusion proteins. The expressed antibodies or fusion proteins are then isolated and purified by any conventional method, including extraction, precipitation, chromatography, electrophoresis, but also by affinity chromatography.

The antibodies according to the invention are endocytosed only slowly by CD33-positive cells (Fig. 7). This has the advantage that the antibodies according to the invention or the antibody fragments in a therapeutic application stay for an extended period of time on the surface of the CD33-positive cells. In this way, they are accessible for a long period of time for effector cells that, in turn, mediate the immune reaction against the CD33-positive cells (target cells). This increases the effectiveness of a therapy. Antibodies that are quickly endocytosed are not suitable for such an application. Accordingly, because of these special properties, the antibodies according to the invention are distinguished from the antibodies known from the prior art. In addition to their high affinity to CD33, they offer the possibility of an effective therapeutic application whose action mechanism is based on recruiting effector cells.

The invention will be explained in more detail based on the following Figures and embodiments without limiting the invention to them.

Figures

Figure 1: Figure 1 shows a sequence comparison of the CDR regions of two antibodies according to the invention, anti-CD33DRB1 and anti-CD33DRB2, with the CDR regions of the variable regions of the light and heavy chains of the anti-CD33 antibodies known in the prior art. Illustrated is also the percentage of sequence identity (% SI) of the amino acid sequences to the sequences according to the invention.

Figure 2: Figure 2 shows binding of two anti-CD33DRB antibodies of the supernatant of the hybridomas on CD33-Hek293T cells. In this connection, (A) shows

binding of an antibody with the CDR regions of the anti-CD33DRB1. (B) shows binding of an antibody with the CDR regions of the anti-CD33DRB2. Illustrated is the median fluorescence intensity (MFI) of the flow cytometry analysis wherein, to the left, binding on wild type cells Hek293T cells (wt) and, to the right, binding to the CD33⁺-Hek293T cells (Hek293T-CD33) is illustrated.

Figure 3: Figure 3 shows binding of anti-CD33 antibodies to HL60 cells. (A) shows in this context binding of a commercial anti-CD33 antibody, (B) shows binding of the antibody with the CDR regions of anti-CD33DRB1, and (C) shows binding of the antibody with CDR regions of anti-CD33DRB2. Illustrated is the mean fluorescence intensity (MFI) of the flow cytometry analysis.

Figure 4: Figure 4 shows the comparison of binding of (A) monoclonal anti-CD33DRB1 and (B) of an scFv fragment with the CDR regions of the anti-CD33DRB2 on CD33⁺-Hek293T cells. Illustrated is the mean fluorescence intensity (MFI) of flow cytometry analysis.

Figure 5: Figure 5 shows the T cell-mediated specific lysis of CD33⁺-Hek293T cells which is controlled by two different scBsTaFv that are specific to CD33 (with the CDR regions of the anti-CD33DRB2) and CD3. Illustrated is the proportion of the CD33⁺-Hek293T cells that are affected by the lysis that is mediated by the T cells.

Figure 6: Figure 6 shows the NK cell-mediated specific lysis of CD33⁺-P815 cells which is controlled by a bispecific recombinant protein of ULB-P2 and scFv CD33 (with the CDR regions of the anti-CD 3DRB2. Illustrated is the proportion of the CD33⁺-Hek293T cells that are affected by the NK cell-mediated specific lysis. The experiments were carried out in different ratios between effector cells (NK cells) and target cells (CD33⁺-P815 cells) (E:T ratio, ratio effector cells to target cells).

Figure 7: Figure 7 shows the residence time of the anti-CD33 antibodies according to the invention on the surface of CD33-positive blasts of a leukemia patient with AML. At the top: binding of anti-CD33DRB2 (from Example 1), at 1, 6, 24 and 48 hrs after incubation with the CD33-positive blasts (black), respectively. The detection was realized by staining with PE-marked anti-murine IgG antibody (white: negative control without anti-DC33 antibody). Bottom: binding of CD3xCD33 scBsTaFv (from Example 4), at 1, 6, 24, and 48 hrs after incubation with CD33-positive blasts (black), respectively. The detection was realized by staining with PE-marked anti-myc antibody (white: negative control without CD 3xCD33 scBsTaFv).

Example 1: preparation and characterization of monoclonal murine anti-CD33DRB antibodies

For preparing new anti-CD33 antibodies, the murine fibroblast cell line A9 (ATCC CCL-1.4, American Type Culture Collection, Rockville, MD, U.S.A.) was transduced stably with human CD33. With the thus obtained cells, three mice were immunized. After immunization, sera of the immunized mice were applied to with Hek293T cells (ATCC CRL-11268) that were also transduced with human CD33 (CD33⁺-Hek293T cells) in various degrees of dilution and immunofluorescence staining was carried out. The serum of the mouse #3 showed at the highest dilution of 1:1,000 significantly the highest reactivity so that the splenocytes of this mouse were used for the hybridoma fusion. Accordingly, the murine myeloma cell line X63Ag8.653 (ATCC CRL 1580) was fused in the logarithmic growth phase with splenocytes of the mouse #3 and subsequently cultured in a selection medium (HAT medium). After two weeks of culturing, the antibody production of the hybridomas was tested. Two hybridomas were identified that are characterized by an especially high affinity to CD33 (anti-CD33DRB1 and anti-CD33DRB2).

Sequencing of the variable regions of the light and heavy chains show the following sequences:

- anti-CD33DRB1: V_H a sequence according to SEQ ID NO. 31, V_L a sequence according to SEQ ID NO. 32, and
- anti-CD33DRB2: V_H a sequence according to SEQ ID NO. 29, V_L a sequence according to SEQ ID NO. 30.

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The CDR regions of the variable regions of the light and heavy chains have the following gene sequences:

- anti-CD33DRB1: V_H: CDR1 a sequence according to SEQ ID NO. 23, CDR2 a sequence according to SEQ ID NO. 24, CDR3 a sequence according to SEQ ID NO. 25; V_L: CDR1 a sequence according to SEQ ID NO. 27, CDR2 a sequence according to SEQ ID NO. 28, CDR3 a sequence according to SEQ ID NO. 29 and
- anti-CD33DRB2: V_H: CDR1 a sequence according to SEQ ID NO. 17, CDR2 a sequence according to SEQ ID NO. 18, CDR3 a sequence according to SEQ ID NO. 19; V_L: CDR1 a sequence according to SEQ ID NO. 20, CDR2 a sequence according to SEQ ID NO. 21, CDR3 a sequence according to SEQ ID NO. 22.

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They code for the following amino acid sequences that contain the specific CDR regions:

- anti-CD33DRB1: V_H a sequence according to SEQ ID NO. 15, V_L a sequence according to SEQ ID NO. 16, and
- anti-CD33DRB2: V_H a sequence according to SEQ ID NO. 13, V_L a sequence according to SEQ ID NO. 14,
- and the CDR regions of
- anti-CD33DRB1: V_H: CDR1 a sequence according to SEQ ID NO. 7, CDR2 a sequence according to SEQ ID NO. 8, CDR3 a sequence according to SEQ ID

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NO. 9; V_L: CDR1 a sequence according to SEQ ID NO. 10, CDR2 a sequence according to SEQ ID NO. 11, CDR3 a sequence according to SEQ ID NO. 12, and

- 5 - anti-CD33DRB2: V_H: CDR1 a sequence according to SEQ ID NO. 1, CDR2 a sequence according to SEQ ID NO. 2, CDR3 a sequence according to SEQ ID NO. 3; V_L: CDR1 a sequence according to SEQ ID NO. 4, CDR2 a sequence according to SEQ ID NO. 5, CDR3 a sequence according to SEQ ID NO. 6.

10 The amino acid sequences of the CDR regions of the two antibodies according to the invention differ significantly from anti-CD33 antibodies (Figure 1) known up to now.

Binding of the antibodies from the hybridoma supernatants on CD33⁺-Hek293T cells and on the CD33⁺ human cell line HL60 was verified by FACS analyses (Figures 2 and 3).

15 For providing monoclonal antibodies from the hybridoma cells, the latter were recloned.

Example 2: preparation of an scFv construct of anti-CD33DRB2

20 The coding areas of the heavy and light chains of the variable regions of the antibodies anti-CD33DRB2 from Example 1 were amplified with specific primers and cloned into a pSecTag2B vector for expression in eukaryote cells. In order to test the binding strength of the scFv (single chain variable fragments), the latter were expressed in Hek293T cells. For this purpose, the vector (pSecTag2B containing the coding areas) was transformed into *E. coli Top10F* bacteria and the plasmids prepared. The obtained
25 expression vectors that code for the scFv antibodies were introduced by transfection with polyethylene imine (PEI) into Hek293T cells. After culturing the transfected cells for five days, the supernatant was removed and the antibodies were purified.

The obtained scFv antibodies, in the following referred to as anti-CD33DRB2 scFv, were

tested by immunofluorescence staining with respect to their binding to CD33⁺-Hek293T cells and compared with the monoclonal anti-CD33DRB2 (Figure 4). The monoclonal antibody as well as the scFv antibody exhibited a comparable binding action to CD33⁺-Hek293T cells wherein the number of bonded cells was identical, but the binding strength that is expressed by the mean fluorescence intensity was slightly reduced for the scFv antibody. This can be explained in that the scFv antibody binds only with one pair of variable region of the light and heavy chain to the antigen.

Example 3: preparation of different bispecific CD3xCD33 antibodies and immunotargeting of T cells

For use as a targeting construct for targeting CD33⁺ cells, a bispecific antibody (single chain bispecific diabody, scBsDb) was produced that bind with one arm on CD33 and with the other arm on CD3. The domain that binds to CD33 contains the variable region of the anti-CD33DRB2 of Examples 1 and 2. It serves for binding to the target cells, for example, tumor cells. The other domain binds to CD3, a component of the T cell receptor complex, and serves for activation of T cells. In this way, recruiting of T cells on the target cells is enabled and permits in this way combating of the target cells by the T cells.

For producing the antibodies, an outer cassette for the first antibody, containing the variable region of the heavy chain (V_H) which is positioned proximal to the variable region of the light chain (V_L), wherein V_H and V_L are connected by a glycine-serine linker of (Gly₄Ser)₃. The inner cassette contains the sequence of the variable regions of light and heavy chain of the second antibody in reversed form and a glycine-serine linker of (Gly₄Ser)₅ in order to ensure correct folding of scBsDb. In this way, two different bispecific antibodies were produced which differ in the arrangement of variable regions of the anti-CD33 and anti-CD3 antibodies.

CD33xCD3 scBsDb (outer cassette CD33 – inner cassette CD3):

V_HCD33-(Gly₄Ser)-V_LCD33.(Gly₄Ser)₅- V_HCD3-(Gly₄Ser)-V_LCD33

CD33xCD3 scBsDb (outer cassette CD3 – inner cassette CD33):

V_HCD3-(Gly₄Ser)-V_LCD33.(Gly₄Ser)₅- V_HCD33-(Gly₄Ser)-V_LCD3.

5 The coding sequences of the CD33xCD3 scBsDb and CD3xCD33 scBsDb were cloned into a retroviral pcz-CFG 5.1 vector. The two constructs were introduced into a packaging cell line and the obtained viral particles were introduced into Hek293T cells where their coding sequence was introduced stably into the genome and in this way stable cell lines were obtained that express the two antibodies, CD33xCD3 scBsDb and CD3xCD33 scBsDb. The latter were purified by Ni-NTA column.

10 The thus obtained bispecific antibodies contained in addition to the complete form a further 37 kDa size product which most likely was produced by proteolysis and has a significant negative effect on the cytotoxicity. The cytotoxicity was determined by ⁵¹Cr release assay with ⁵¹Cr-CD33⁺-Hek293T cells. CD33xCD3 scBsDb and CD3xCD33 scBsDb in *in vitro* co-cultures of CD33⁺-Hek293T cells and PBMC achieved a specific T cell mediated lysis of the CD33⁺-Hek293T cells of 27 % or 23 %.

20 Example 4: preparation of CD3xCD33 tandem antibodies (scBsTaFv) and immunotargeting of T cells

25 In order to further increase the T cell mediated cytotoxicity, tandem antibodies with larger linker structures were constructed in order to avoid proteolysis. In the bispecific tandem antibodies (scBsTaFv) the variable regions of the anti-CD33 antibody and the anti-CD3 antibody are arranged on a polypeptide chain wherein the two scFv fragments are linked with a linker of a length of 18 amino acids for proper folding. A further construct contained in addition to the L18 also the green-fluorescent protein (GFP). Both constructs, CD33-L18-CD3 and CD33-L18-GFP-L18-CD3, were expressed and purified in analogy to Example 3. The cytotoxicity was also determined in analogy to Example 3 with a ⁵¹Cr release assay (Figure 5). The specific lysis of the CD33⁺-Hek293T cells for

these antibody constructs is up to 86 % for a 20-hour *in vitro* culture.

Example 5: preparation of ULB-P2xCD33 tandem antibodies (scBsFv) and immunotargeting of NK cells

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In order to achieve immunotargeting of NK cells, tandem antibodies were constructed that contain CD33 and ULB-P2. ULB-P2 is the ligand of the activated NK cell receptor NKG2D. By combination of an activating ligand and an anti-CD33 scFv antibody in a tandem antibody, immunotargeting of NK cells on CD33⁺ target cells is mediated. The
10 construct of the tandem antibody contains ULB-P2, a short (Gly₄Ser) linker, and the V_H and V_L regions of the anti-CD33DRB2 that are linked by a long linker structure (Gly₄Ser)₃ as well as a Myc tag and six His tags for purification. The antibodies were introduced in analogy to Examples 3 and 4 into an expression vector, expressed and purified.

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The NK cell-mediated cytotoxicity was determined by ⁵¹Cr release assay in CD33⁺-P815 (ATCC TIB-6) (Figure 6). In the presence of the tandem antibody, a significantly increased NK cell-mediated cytotoxicity was determined.

20 Example 6: anti-CD33 antibodies according to the invention are endocytosed only weekly by cancer cells

It is desirable that in a therapeutic treatment of CD33-associated illnesses, for example, acute myeloid leukemia (AML), the residence time of the antibodies used for therapy on
25 the cell surface is high. This has the effect that effector cells can be effectively recruited on the cancer cells. In case of a rapid endocytosis of the antibodies by the cancer cells, the binding sites for the effector cells would no longer be accessible after a short period of time and targeting of the effector cells would therefore be less effective.

30 In order to demonstrate that the antibodies according to the invention are endocytosed

only at a minimal rate by the CD33-positive cancer cells, CD33-positive blasts of leukemia patients were incubated at 4 degrees C for 1 hr with a monoclonal anti-CD33DRB2 antibody (as disclosed in Example 1) or with a CD3xCD33 scBsTaFv tandem antibody (as disclosed in Example 4). By FACS analysis, the presence of the anti-CD33 antibodies on the surface of the CD33-positive blasts was detected in that staining of the anti-CD33 antibodies was performed with a PE-marked anti-murine IgG antibody (for the monoclonal anti-CD33DRB2, Figure 7 at the top, black histogram) or with a PE-marked anti-myc antibody (for the tandem antibody, Figure 7 at the bottom, black histogram). In parallel, as a negative control without incubation with anti-CD33 antibodies, CD33-positive blasts of the same patient, were stained with the same antibodies, respectively (Figure 7, white histogram, respectively)..

Even 48 hours after contacting with the CD33-positive blasts, the antibodies could be detected on the surface. The antibodies, the monoclonal antibody as well as the bispecific antibody derivative, were predominantly not endocytosed during this time by the CD33-positive blasts. It is thus ensured for 48 hrs that the antibodies are present for 48 hrs on the surface of the cells to be killed and therefore, over an extended period of time, are capable of serving for recruiting and binding effector cells (in case of the monoclonal antibodies, for example, by means of Fc receptors; in case of the bispecific antibody derivatives by specific binding of the further antibodies).

The claims defining the invention are as follows:

1. An isolated anti-CD33 antibody containing regions determined by complementarity (complementary determining regions, CDRs), wherein the CDRs include the following sequences:

a) variable region of the heavy chain (V_H)

CDR1 DYVX₁H, wherein

X₁ is selected from V, I and L,

CDR2 X₁INX₂YNX₃X₄X₅X₆YNX₇KFX₈G, wherein

X₁ is selected from Y, W, F, V, I and L,

X₂ is selected from P, G, S and T,

X₃ is selected from D, G, E and A,

X₄ is selected from G, D, E and A,

X₅ is selected from T, V, S, I and L,

X₆ is selected from K, Q, N, and R,

X₇ is selected from E, Q, D and N,

X₈ is selected from K, R, Q, N, L, F, I and M,

CDR3 DYRYEX₁YX₂MDY, wherein

X₁ is selected from V, L, I, M, F, A, W, F, T, S and Y,

X₂ is selected from G, P, V, L, I and A,

and

b) variable region of the light chain (V_L):

CDR1 X₁AX₂SSVX₃YIH, wherein

X₁ is selected from T and S,

X₂ is selected from S, T, Q, H, K, R and N,

X₃ is selected from N, Q, H, K, R, T and S,

CDR2 TSKX₁AS, wherein

X₁ is selected from V, I, M, F, A and L,

CDR3 QQWX₁SX₂PLT, wherein

25

30

X₁ is selected from R, K, Q, N, S and T,

X₂ is selected from Y, W, F, T, S, N, Q, K, R and H.

2. An isolated anti-CD33 antibody according to claim 1, containing the following structure:

- a variable region of the heavy chain with the sequence according to SEQ ID NO. 13
- and a variable region of the light chain with a sequence according to SEQ ID NO. 14

and/or

- a variable region of the heavy chain with a sequence according to SEQ ID NO. 15
- and a variable region of the light chain with a sequence according to SEQ ID NO. 16.

3. An isolated anti-CD33 antibody according to claim 1 containing a humanized structure.

4. An isolated anti-CD33 antibody according to any one of claims 1 to 3, containing additionally at least one of the following structures

- a constant region of a heavy chain of a human IgG,
- a region C_L of the human kappa light chain

and/or

- a human IgG3 hinge region,

optionally in the form of a F(ab')₂ fragment.

25 5. An isolated anti-CD33 antibody according to any one of claims 1 to 4 in the form of an scFv fragment or in the form of a F(ab')₂ fragment.

6. An isolated anti-CD33 antibody according to any one of claims 1 to 5, wherein said antibody is conjugated with an effector group or a further antibody or antibody fragment that is specific to another antigen than CD33.

30

7. An isolated anti-CD33 antibody according to claim 6, wherein the effector group is

selected from toxins, enzymes, co-stimulating molecules, radionuclides and nucleic acids, optionally in the form of a fusion protein.

8. An isolated anti-CD33 antibody according to any one of the claims 1 to 7, wherein said antibody is conjugated with a ligand that binds specifically to effector cells and in this way affects their activity.

9. An isolated nucleic acid sequence coding for an isolated anti-CD33 antibody according to any one of claims 1 to 8.

10. A vector containing an isolated nucleic acid sequence according to claim 9.

11. A host cell or non-human host organism containing an isolated nucleic acid sequence according to claim 9 or a vector according to claim 10.

12. A pharmaceutical composition, containing an isolated anti-CD33 antibody according to any one of claims 1 to 8 in association with a pharmaceutically acceptable dilution agent or carrier, optionally in a form suitable for intravenous administration.

13. Use of an isolated anti-CD33 antibody according to any one of claims 1 to 8 as medication.

14. A method for preparing an isolated anti-CD33 antibody according to any one of claims 1 to 8, in which:

- 25
- a. a host cell or a non-human host organism according to claim 11 is exposed to conditions under which an expression and optionally a secretion of the antibody takes place, and optionally
 - b. the antibody is at least partially purified.

	V _H CDR1	%SI	V _H CDR2	%SI	V _H CDR3	%SI
anti-CD33 DRB1	DYVLH		GLINTYNGDVRYNQKFMGK		DYRYEYYAMDY	
US 7,557,189	SY Y I H	40	VIY P GN D DISYNQKFXG	66	E V RL R Y F DV	11
US 5,730,982	DYNMH	60	YIY P YNGG T GYNQKFKS	72	GR E ----AMDY	36
CN101210048	DYNMY	40	GYIDPYKGG T IYNQKFKG	56	Q-MITAY F FDY	27
anti-CD33 DRB2	DYVVH		YINPYNDGTYNEKFKG		DYRYEVYCMDY	
US 7,557,189	SY Y I H	40	VIY P GN D DISYNQKFXG	58	E V RL R Y F DV	11
US 5,730,982	DYNMH	60	YIY P YNGG T GYNQKFKS	75	GR E ----AMDY	27
CN101210048	DYNMY	40	GYIDPYKGG T IYNQKFKG	70	Q-MITAY F FDY	27

	V _L CDR1	%SI	V _L CDR2	%SI	V _L CDR3	%SI
anti-CD33 DRB1	SA-----NSSVS-YIH		DTSKLAS		QQWTSHELT	
US 7,557,189	KSSQSVFFS-----SSQKN-YLA	20	WASTRES	66	HQYLSSRT	25
US 5,730,982	RASEVDNYGISFMN	20	ASNQGS	33	QQSKEVPWT	44
CN101210048	KA-----SQDINKYIA	27	TSTLQP	33	LQYDNL-LT	33
anti-CD33 DRB2	TASSSVN-----YIH		TSKVAS		QQWRSYELT	
US 7,557,189	KSSQSVFFSSSQKN-----YLA	71	WASTRES	66	HQYLSSRT	25
US 5,730,982	RASEVDNYGISFMN	75	ASNQGS	33	QQSKEVPWT	44
CN101210048	KASQDINK-----YIA	36	TSTLQP	33	LQYDNL-LT	33

Fig. 1

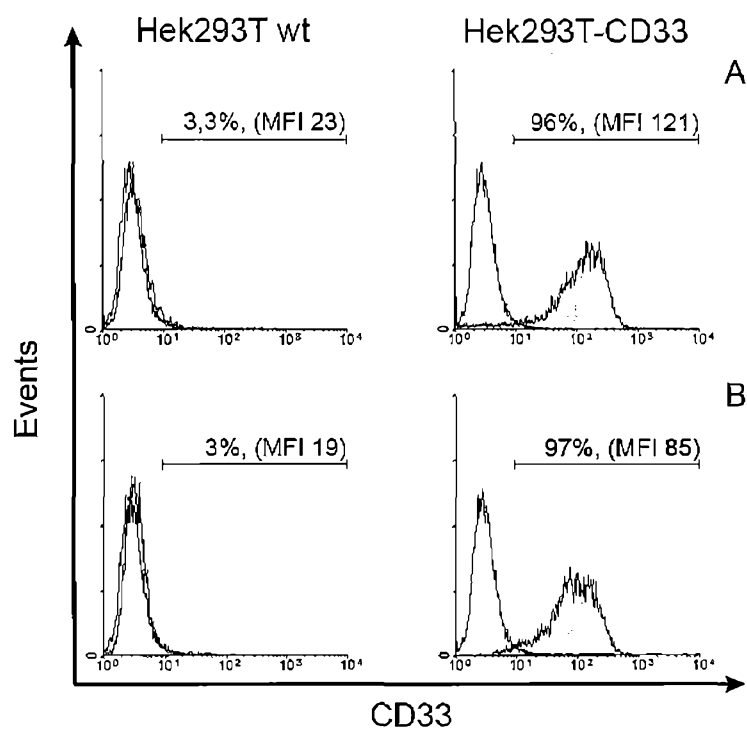


Fig. 2

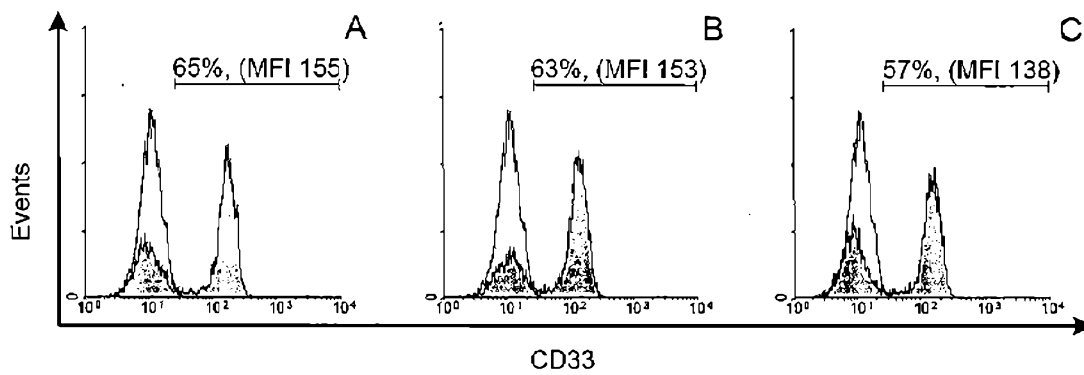


Fig. 3

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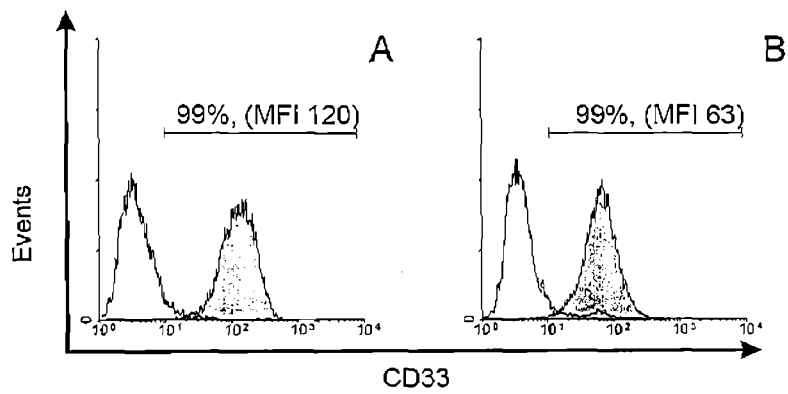


Fig. 4

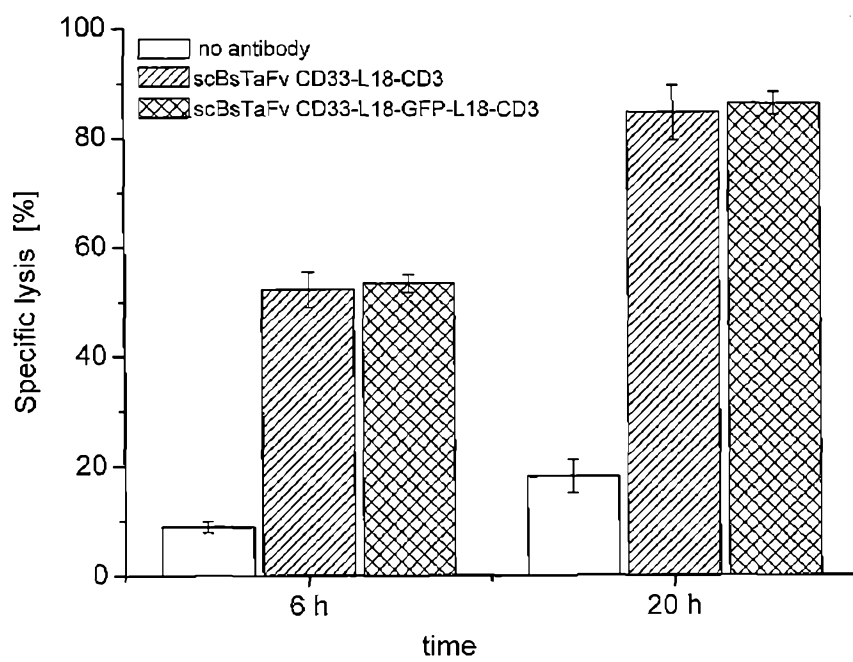


Fig. 5

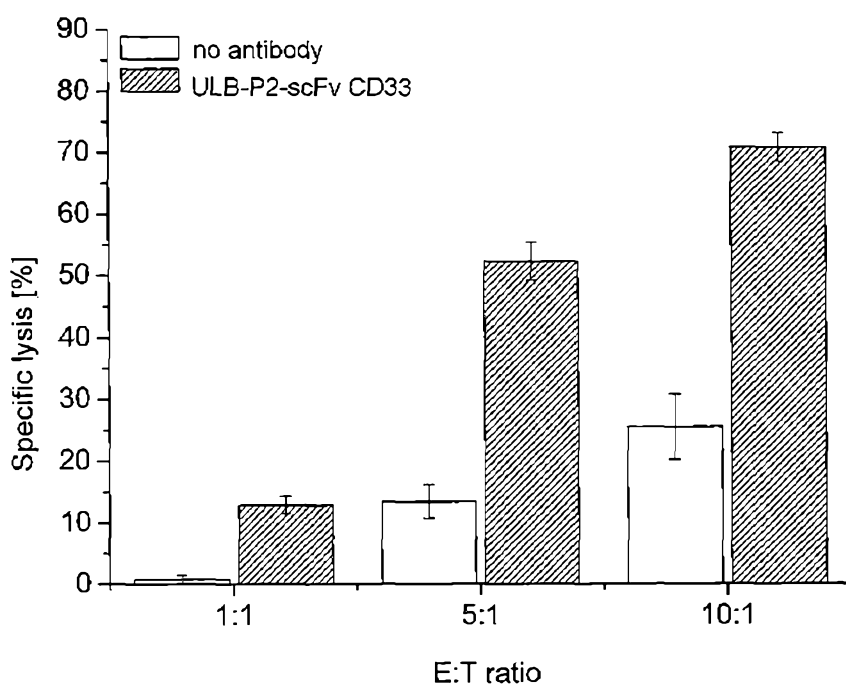


Fig. 6

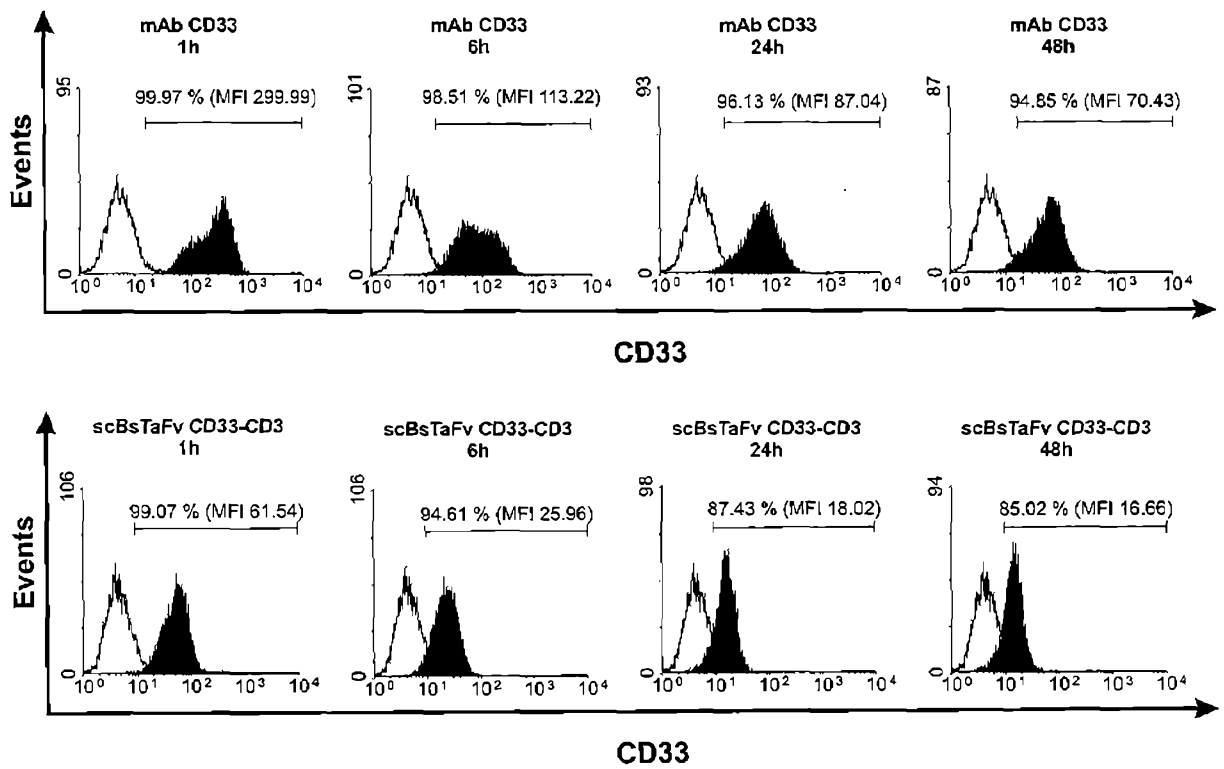


Fig. 7