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(54) Title: METHODS OF PROGNOSIS AND TREATMENT OF PATIENTS SUFFERING FROM ACUTE MYELOID LEUKEMIA

(57) Abstract: The present invention relates to methods of prognosis and treatment of patients suffering from Acute Myeloid Leukemia (AML). In particular one aspect of the present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) comprising determining the expression level of CD81 on leukemic cells and leukemic stem cells isolated from a sample obtained from the patient. One further object of the present invention relates to a method of treating acute myeloid leukemia in a patient in need thereof comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.



METHODS OF PROGNOSIS AND TREATMENT OF PATIENTS SUFFERING FROM ACUTE MYELOID LEUKEMIA

FIELD OF THE INVENTION:

The present invention relates to methods of prognosis and treatment of patients suffering from Acute Myeloid Leukemia (AML).

BACKGROUND OF THE INVENTION:

Acute Myeloid Leukemia (AML) is the most common type of acute leukemia in adults. Symptoms include fatigue, pallor, easy bruising and bleeding, fever, and infection; symptoms of leukemic infiltration are present in only about 5% of patients (often as skin manifestations). Examination of peripheral blood smear and bone marrow is diagnostic. Treatment includes induction chemotherapy to achieve remission and post-remission chemotherapy (with or without stem cell transplantation) to avoid relapse. AML has a number of subtypes that are distinguished from each other by morphology, immunophenotype, and cytochemistry. Five classes are described, based on predominant cell type, including myeloid, myeloid-monocytic, monocytic, erythroid, and megakaryocytic. Remission induction rates range from 50 to 85%. Long-term disease-free survival reportedly occurs in 20 to 40% of patients and increases to 40 to 50% in younger patients treated with stem cell transplantation. Prognostic factors help determine treatment protocol and intensity; patients with strongly negative prognostic features are usually given more intense forms of therapy, because the potential benefits are thought to justify the increased treatment toxicity. The most important prognostic factor is the leukemia cell karyotype; favorable karyotypes include t(15;17), t(8;21), and inv16 (p13;q22). Negative factors include increasing age, a preceding myelodysplastic phase, secondary leukemia, high WBC count, and absence of Auer rods. The FAB or WHO classification alone does not predict response. Initial therapy attempts to induce remission and differs most from ALL in that AML responds to fewer drugs. The basic induction regimen includes cytarabine by continuous IV infusion or high doses for 5 to 7 days; daunorubicin or idarubicin is given IV for 3 days during this time. Some regimens include 6-thioguanine, etoposide, vincristine, and prednisone, but their contribution is unclear. Treatment usually results in significant myelosuppression, with infection or bleeding; there is significant latency before marrow recovery. During this time, meticulous preventive and supportive care is vital. AML results from a pathologic deregulation of the hematopoietic

tissue homeostasis inducing the expansion of one or multiple leukemic clones and is organized as a hierarchy of malignant cells. The latter is viewed as a continuous developmental process in which self-renewing pluripotent stem cells give rise to the various lineages of blood cells via a series of characteristic cellular differentiation and expansion events. Most circulating blasts have a low proliferation potential, and cannot form colonies in clonogenic assays and do not induce AML in recipient immunodeficient mice. Leukemia stem cells (LSC) represent small subset of the total leukemic burden (i.e., 0.1 to 1%) and have self-renewal capacities. The CD34+CD38- cell fraction is in particular enriched for LSCs. Subsequently, these LSCs may differentiate into CD34+CD38+ leukemic progenitor cells which are able to give rise to either CD34+ or CD34- AML blast cells. Thus, the eradication of the LSC subset is of major clinical importance to avoid AML relapse and to achieve long-term cure of leukemia. LSCs may be further characterized with other markers such as CD123, CD90, CD81 and ALDH activity.

SUMMARY OF THE INVENTION:

The present invention relates to methods of prognosis and treatment of patients suffering from Acute Myeloid Leukemia (AML). In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Leukemia stem cells (LSC) represent small subset of the total leukemic burden (i.e., 0.1 to 1%) and have self-renewal capacities. The CD34+CD38- cell fraction is in particular enriched for LSCs. These LSCs may differentiate into CD34+CD38+ leukemic progenitor cells which are able to give rise to either CD34+ or CD34- AML blast cells. Thus, the eradication of the LSC subset is of major clinical importance to avoid AML relapse and to achieve long-term cure of leukemia.

Now the inventors show that CD81 may be better at predicting treatment outcome than CD123 or any other marker. The LSC CD81+ population is quiescent (cell cycle analysis) and drug resistant (in vitro drug resistance) and proliferates in clonogenic assays (soft agar). The inventors in particular demonstrate that the high expression of CD81 on leukemic stem cells (cd34+cd38-) determined by flow cytometry is linked to bad prognosis. CD81+ cells are more quiescent by ki67 compared to CD81-. CD81+ primary AML are significantly more potent to produce xenograft in immunodeficient mice compared to CD81- primary AML. The inventors demonstrate that blocking CD81 in AML cells activates the ERK pathway,

sensitized to chemotherapy, induces number of macrophage colonies (colony forming assay) whereas blocking CD81 in normal bone marrow cells does not change the above and neither does incubation with an non-blocking antibody. Similarly, the inventors have created AML cell lines models of stably transfected CD81 cDNA over-expression and of lentiviral shRNA mediated CD81 knock-down.

As used herein the term “CD81” has its general meaning in the art. CD81, also named TAPA-1, is a 26 kD cell-surface glycoprotein which belongs to the tetraspanin family. CD81 is widely expressed and has been shown to regulated various cellular function as proliferation, pathogen entry and cell migration (Pileri et al., 1998, Science; Silvie et al., 2003, Nat Med; Yunta et al. 2003, Cell Signal; Levy et al.2005, Nat Rev Immunol). Tetraspanins are characterized by four transmembrane domains and conserved cysteine residues. These proteins are able to form complex with themselves or with other functional molecules as integrins and signaling proteins. However, these proteins do not have a receptor function itself and even if they are qualify of molecular organizer (Hemler, 2005, Nat Rev MCB) their function is not clearly identified.

Methods for predicting the survival time

CD81 marker is thus useful in clinical diagnostic applications including, without limitation, primary diagnosis of AML or pre-leukemic conditions from blood and/or bone marrow specimens, evaluation of leukemic involvement of the cerebrospinal and other body fluids, monitoring of interval disease progression, and monitoring of minimal residual disease status. In some embodiments, methods are thus provided for detection, classification or clinical staging of acute myeloid leukemias according to the stem cells that are present in the leukemia, wherein a high expression of CD81 is indicative of a more aggressive cancer phenotype. Staging is useful for prognosis and treatment.

In particular one aspect of the present invention relates to a method for predicting the survival time of a patient suffering from actue myeloid leukemia (AML) comprising i) determining the expression level of CD81 on leukemic cells and leukemic stem cells isolated from a sample obtained from the patient, ii) comparing the level determined at step i) with a predetermined reference value and iii) concluding that the patient will have a short survival time when the level determined at step i) is higher than the predetermined reference value or

concluding that the patient will have a long survival time when the level determined at step i) is lower than the predetermined reference value.

The predictive method of the present invention is particularly suitable for predicting the duration of the overall survival (OS), progression-free survival (PFS) and/or the disease-free survival (DFS) of the cancer patient. Those of skill in the art will recognize that OS survival time is generally based on and expressed as the percentage of people who survive a certain type of cancer for a specific amount of time. Cancer statistics often use an overall five-year survival rate. In general, OS rates do not specify whether cancer survivors are still undergoing treatment at five years or if they've become cancer-free (achieved remission). DSF gives more specific information and is the number of people with a particular cancer who achieve remission. Also, progression-free survival (PFS) rates (the number of people who still have cancer, but their disease does not progress) includes people who may have had some success with treatment, but the cancer has not disappeared completely.

Typically, the expression "short survival time" indicates that the patient will have a survival time that will be lower than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a short survival time, it is meant that the patient will have a "poor prognosis". Inversely, the expression "long survival time" indicates that the patient will have a survival time that will be higher than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a long survival time, it is meant that the patient will have a "good prognosis".

Samples for use in the predictive methods of the present invention may be obtained from a variety of sources, particularly blood, although in some instances samples such as bone marrow, lymph, cerebrospinal fluid, synovial fluid, and the like may be used. Such samples can be separated by centrifugation, elutriation, density gradient separation, apheresis, affinity selection, panning, FACS, centrifugation with Hypaque, etc. prior to analysis. Once a sample is obtained, it can be used directly, frozen, or maintained in appropriate culture medium for short periods of time. Various media can be employed to maintain cells. The samples may be obtained by any convenient procedure, such as the drawing of blood, venipuncture, biopsy, or the like. Usually a sample will comprise at least about 10^2 cells, more usually at least about 10^3 cells, and preferable 10^4 , 10^5 or more cells. An appropriate solution may be used for

dispersion or suspension of the cell sample. Such solution will generally be a balanced salt solution, e.g. normal saline, PBS, Hank's balanced salt solution, etc., conveniently supplemented with fetal calf serum or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from 5-25 mM. Convenient buffers include

5 HEPES, phosphate buffers, lactate buffers, etc.

The leukemic cells and leukemic stem cells can be prospectively isolated or identified from primary tumor samples. In particular leukemic stem cells possess the unique properties of cancer stem cells in functional assays for cancer stem cell self-renewal and differentiation.

10 Methods for isolating leukemic cells and leukemic stem cells are well known in the art and typically involve the presence or absence of specific cell surface markers. For example, the comparison can be made between leukemic stem cells and the normal counterpart cells a human hematopoietic stem cell (HSC), which include without limitation cells having the phenotype Lin-CD34+CD38-CD90+; or the phenotype

15 Lin-CD34+CD38-CD90+CD45RA- and a human hematopoietic multipotent progenitor cell (MPP), which include without limitation cells having the phenotype Lin-CD34+CD38-CD90-; or the phenotype Lin-CD34+CD38-CD90-CD45RA-.

In some embodiments, determining the presence or absence of the cell surface markers

20 involves use of a panel of binding partners specific for the cell surface markers of interest. Said binding partners include but are not limited to antibodies, aptamer, and peptides. The binding partners will allow for the screening of cellular populations expressing the marker. Various techniques can be utilized to screen for cellular populations expressing the cell surface markers of interest, and typically include magnetic separation using antibody-coated

25 magnetic beads, "panning" with antibody attached to a solid matrix (i.e., plate), and flow cytometry (See, e.g., U.S. Pat. No. 5,985,660; and Morrison et al. Cell, 96:737-49 (1999)).

In some embodiments, the binding partners are antibodies that may be polyclonal or monoclonal, preferably monoclonal, specifically directed against one cell surface marker.

30 Polyclonal antibodies of the invention or a fragment thereof can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal

antibodies of the invention or a fragment thereof can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally; the human B-cell hybridoma technique; and the EBV-hybridoma technique.

In some embodiments, the panel of binding partners is specific for the following cell surface markers: CD33, CD34, CD36, CD38, CD45, CD81, CD90, and CD123. Accordingly quantification of CD81 is performed along with the isolation of the leukemic stem cells.

Typically, the binding partners are conjugated with a label for use in separation. Labels include magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, which can be used with a fluorescence activated cell sorter, or the like, to allow for ease of separation of the particular cell type. Fluorochromes that find use include phycobiliproteins, e.g. phycoerythrin and allophycocyanins, fluorescein and Texas red. Typically each antibody is labeled with a different fluorochrome, to permit independent sorting for each marker. Suitable fluorescent detection elements include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue™, Texas Red, IAEDANS, EDANS, BODIPY FL, LC Red 640, Cy 5, Cy 5.5, LC Red 705 and Oregon green. Suitable optical dyes are described in the 1996 Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference. Suitable fluorescent labels also include, but are not limited to, green fluorescent protein (GFP; Chalfie, et al., Science 263(5148):802-805 (Feb. 11, 1994); and EGFP; Clontech—Genbank Accession Number U55762), blue fluorescent protein (BFP; 1. Quantum Biotechnologies, Inc. 1801 de Maisonneuve Blvd. West, 8th Floor, Montreal (Quebec) Canada H3H 1J9; 2. Stauber, R. H. Biotechniques 24(3):462-471 (1998); 3. Heim, R. and Tsien, R. Y. Curr. Biol. 6:178-182 (1996)), enhanced yellow fluorescent protein (EYFP; 1. Clontech Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, Calif. 94303), luciferase (Ichiki, et al., J. Immunol. 150(12):5408-5417 (1993)), (β-galactosidase (Nolan, et al., Proc Natl Acad Sci USA 85(8):2603-2607 (April 1988)) and Renilla WO 92/15673; WO 95/07463; WO 98/14605; WO 98/26277; WO 99/49019; U.S. Pat. Nos. 5,292,658; 5,418,155; 5,683,888; 5,741,668; 5,777,079; 5,804,387; 5,874,304; 5,876,995; and 5,925,558). All of the above-cited references are expressly incorporated herein by reference. In some embodiments,

detection elements for use in the present invention include: Alexa-Fluor dyes (an exemplary list including Alexa Fluor® 350, Alexa Fluor® 405, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 500, Alexa Fluor® 514, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 610, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, and Alexa Fluor® 750), Cascade Blue, Cascade Yellow and R-phycoerythrin (PE) (Molecular Probes) (Eugene, Oreg.), FITC, Rhodamine, and Texas Red (Pierce, Rockford, Ill.), Cy5, Cy5.5, Cy7 (Amersham Life Science, Pittsburgh, Pa.). Tandem conjugate protocols for Cy5PE, Cy5.5PE, Cy7PE, Cy5.5APC, Cy7APC are known in the art. Fluorophores bound to antibody or other binding element can be activated by a laser and re-emit light of a different wavelength. The amount of light detected from the fluorophores is related to the number of binding element targets associated with the cell passing through the beam. Any specific set of detection elements, e.g. fluorescently tagged antibodies, in any embodiment can depend on the types of cells to be studied and the presence of the activatable element within those cells. Several detection elements, e.g. fluorophore-conjugated antibodies, can be used simultaneously, so measurements made as one cell passes through the laser beam consist of scattered light intensities as well as light intensities from each of the fluorophores. Thus, the characterization of a single cell can consist of a set of measured light intensities that may be represented as a coordinate position in a multi-dimensional space. Considering only the light from the fluorophores, there is one coordinate axis corresponding to each of the detection elements, e.g. fluorescently tagged antibodies. The number of coordinate axes (the dimension of the space) is the number of fluorophores used. Modern flowcytometers can measure several colors associated with different fluorophores and thousands of cells per second. Thus, the data from one subject can be described by a collection of measurements related to the number of antigens for each of (typically) many thousands of individual cells. See Krutzik et al., High-content single-cell drug screening with phosphospecific flow cytometry. *Nature Chemical Biology*, Vol. 4 No. 2, Pgs. 132-42, February 2008. Such methods may optionally include the use of barcoding to increase throughput and reduce consumable consumption. See Krutzik, P. and Nolan, G., Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nature Methods*, Vol. 3 No. 5, Pgs. 361-68, May 2006.

In some embodiments the binding partner is conjugated to a metallic chemical element such as lanthanides. Lanthanides offer several advantages over other labels in that they are stable isotopes, there are a large number of them available, up to 100 or more distinct labels,

they are relatively stable, and they are highly detectable and easily resolved between detection channels when detected using mass spectrometry. Lanthanide labels also offer a wide dynamic range of detection. Lanthanides exhibit high sensitivity, are insensitive to light and time, and are therefore very flexible and robust and can be utilized in numerous different settings. Lanthanides are a series of fifteen metallic chemical elements with atomic numbers 57-71. They are also referred to as rare earth elements. Lanthanides may be detected using CyTOF technology. CyTOF is inductively coupled plasma time-of-flight mass spectrometry (ICP-MS). CyTOF instruments are capable of analyzing up to 1000 cells per second for as many parameters as there are available stable isotope tags.

Typically, the binding partners are added to a suspension of cells, and incubated for a period of time sufficient to bind the available cell surface antigens. The incubation will usually be at least about 5 minutes and usually less than about 30 minutes. It is desirable to have a sufficient concentration of binding partners in the reaction mixture, such that the efficiency of the separation is not limited by lack of binding partners. The appropriate concentration is determined by titration. The medium in which the cells are separated will be any medium that maintains the viability of the cells. A preferred medium is phosphate buffered saline containing from 0.1 to 0.5% BSA. Various media are commercially available and may be used according to the nature of the cells, including Dulbecco's Modified Eagle Medium (dMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (dPBS), RPMI, Iscove's medium, PBS with 5 mM EDTA, etc., frequently supplemented with fetal calf serum, BSA, HSA, etc.

In some embodiments, the predetermined reference value is relative to a number or value derived from population studies, including without limitation, patients of the same or similar age range, patients in the same or similar ethnic group, and patients having the same severity of cancer. Such predetermined reference values can be derived from statistical analyses and/or risk prediction data of populations obtained from mathematical algorithms and computed indices of the disease.

In some embodiments, the predetermined reference value is a threshold value or a cut-off value. A "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of

ordinary skilled in the art. For example, retrospective measurement of the expression level of the marker of interest (i.e. CD81) in properly banked historical patient samples may be used in establishing the predetermined reference value.

5 In some embodiments, the predetermined reference value is the median measured in the population of the patients for the marker of interest (i.e. CD81).

In some embodiments, the threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk
10 balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression level of the marker of interest (i.e. CD81) in a group of reference, one can use algorithmic analysis for the statistic treatment of the expression levels determined
15 in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It
20 reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the
25 area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the
30 accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER

POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, the predetermined reference value is typically determined by carrying out a method comprising the steps of:

a) providing a collection of samples from patients suffering from AML;
b) providing, for each sample provided at step a), information relating to the actual clinical outcome for the corresponding patient (i.e. the duration of the disease-free survival (DFS) and/or the overall survival (OS));

c) providing a serial of arbitrary quantification values;
d) determining the level of the marker of interest (i.e. CD81) for each sample contained in the collection provided at step a);

e) classifying said samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising samples that exhibit a quantification value for said level that is higher than the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of samples are obtained for the said specific quantification value, wherein the samples of each group are separately enumerated;

f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the patients from which samples contained in the first and second groups defined at step f) derive;

g) reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested;

h) setting the said predetermined reference value as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been calculated at step g).

For example the level of the marker of interest (i.e. CD81) has been assessed for 100 samples of 100 patients. The 100 samples are ranked according to the level of the marker of interest (i.e. CD81). Sample 1 has the highest level and sample 100 has the lowest level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side

the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding cancer patient, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated. The predetermined reference value is then selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the level of the marker of interest (i.e. CD81) corresponding to the boundary between both subsets for which the p value is minimum is considered as the predetermined reference value. It should be noted that the predetermined reference value is not necessarily the median value of levels of the marker of interest (i.e. CD81). Thus in some embodiments, the predetermined reference value thus allows discrimination between a poor and a good prognosis with respect to DFS and OS for a patient. Practically, high statistical significance values (e.g. low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the invention, instead of using a definite predetermined reference value, a range of values is provided. Therefore, a minimal statistical significance value (minimal threshold of significance, e.g. maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values includes a "cut-off" value as described above. For example, according to this specific embodiment of a "cut-off" value, the outcome can be determined by comparing the level of the marker of interest (i.e. CD81) with the range of values which are identified. In certain embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum p value which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. Therefore, a patient may be assessed by comparing values obtained by measuring the level of the marker of interest (i.e. CD81), where values greater than 5 reveal an increased risk of having a poor prognosis and values less than 5 reveal a decreased risk of a poor prognosis. In some embodiments, a patient may be assessed by comparing values obtained by measuring the level of the marker of interest (i.e. CD81) and comparing the values on a scale, where values above the range of 4-6 indicate an increased risk having a poor prognosis and values below the range of 4-6 indicate

a decreased risk of having a poor prognosis, with values falling within the range of 4-6 indicating an intermediate prognosis.

In some embodiments, the predictive method of the present invention is performed during the course of treatment, where the quantification of CD81 is carried out before, during and as follow-up to a course of therapy. Desirably, therapy targeted to cancer stem cells results in a decrease in the total number, and/or percentage of CD81+ leukemic stem cells in the patient's sample.

Methods of treatment

CD81 can be used as a target for eradicating leukemic stem cells. In particular CD81 is useful as target of therapeutic monoclonal antibodies for treatment of patients with de novo, relapsed, or refractory acute myeloid leukemia. CD81 monoclonal antibodies are indeed useful for depleting the leukemic stem cells. Such monoclonal antibodies are also useful in the treatment of pre-leukemic conditions, such as myelodysplastic syndromes (MDS) and myeloproliferative disorders (MPDs) including: chronic myelogenous leukemia, polycythemia vera, essential thrombocytosis, agnogenic myelofibrosis and myeloid metaplasia, and others.

Accordingly one further object of the present invention relates to a method of treating acute myeloid leukemia in a patient in need thereof comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.

One further object of the present invention relates to a method of eradicating the leukemic cells and leukemic stem cells in a patient suffering from acute myeloid leukemia comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.

One further object of the present invention relates to a method of preventing relapse in a patient suffering from acute myeloid leukemia comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.

One further object of the present invention relates to a method of sensitizing leukemic cells and leukemic stem cells to chemotherapy comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.

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In some embodiments, antibodies for depleting the leukemic cells and leukemic stem cells are added to patient blood *in vivo*.

As used herein, the term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity. The term includes antibody fragments that comprise an antigen binding domain such as Fab', Fab, F(ab')₂, single domain antibodies (DABs), TandAbs dimer, Fv, scFv (single chain Fv), dsFv, ds-scFv, Fd, linear antibodies, minibodies, diabodies, bispecific antibody fragments, bibody, tribody (scFv-Fab fusions, bispecific or trispecific, respectively); sc-diabody; kappa(lamda) bodies (scFv-CL fusions); BiTE (Bispecific T-cell Engager, scFv-scFv tandems to attract T cells); DVD-Ig (dual variable domain antibody, bispecific format); SIP (small immunoprotein, a kind of minibody); SMIP ("small modular immunopharmaceutical" scFv-Fc dimer; DART (ds-stabilized diabody "Dual Affinity ReTargeting"); small antibody mimetics comprising one or more CDRs and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art (see Kabat et al., 1991, specifically incorporated herein by reference). Diabodies, in particular, are further described in EP 404, 097 and WO 93/1 161; whereas linear antibodies are further described in Zapata et al. (1995). Antibodies can be fragmented using conventional techniques. For example, F(ab')₂ fragments can be generated by treating the antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')₂, scFv, Fv, dsFv, Fd, dAbs, TandAbs, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques or can be chemically synthesized. Techniques for producing antibody fragments are well known and described in the art. For example, each of Beckman et al., 2006; Holliger & Hudson, 2005; Le Gall et al., 2004; Reff & Heard, 2001 ; Reiter et al., 1996; and Young et al., 1995 further describe and enable the production of effective antibody fragments.

Antibodies suitable for practicing the methods of the invention are preferably monoclonal and multivalent, and may be human, humanized or chimeric antibodies, comprising single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and/or binding fragments of any of the above. In certain embodiments of the invention, the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH₁, CH₂, CH₃ and CL domains. Also included in the invention are antigen-binding fragments comprising any combination of variable region(s) with a hinge region, CH₁, CH₂, CH₃ and CL domains. Preferably, the antibodies are human, murine (e.g., mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulins. The antibodies suitable for practicing the methods of the present invention may be bispecific, trispecific or of greater multispecificity. Further, the antibodies of the present invention may have low risk of toxicity against granulocyte (neutrophil), NK cells, and CD4⁺ cells as bystander cells.

In some embodiments, the antibody is a "chimeric" antibody in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see, e.g., U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)). Chimeric antibodies include PRIMATTZED® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, e.g., immunizing macaque monkeys with the antigen of interest.

In some embodiments, the antibody is a humanized antibody. "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient
5 are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These
10 modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an
15 immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, e.g., Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994);
20 and U.S. Pat. Nos. 6,982,321 and 7,087,409.

In some embodiments, the antibody is a human antibody. A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies
25 as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole
30 et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.*, 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized

xenomice (see, e.g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al., Proc. Natl. Acad. Sci. USA, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

5 In some embodiments, the antibody is a single domain antibody. The term “single domain antibody” (sdAb) or “VHH” refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called “nanobody®”. According to the invention, sdAb can particularly be llama sdAb.

10 In some embodiments, the antibodies are used to induce antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) against CD81-expressing cells. In some embodiments, the anti-CD81 antibody may be suitable for disturbing the expression of CD81 at the cell surface (e.g. by provoking internalization of CD81) so
15 signaling pathway mediated by CD81 is inhibited. In some embodiments, an anti-CD81 monoclonal antibody of the invention is used to induce antibody dependent cellular cytotoxicity (ADCC). In ADCC, monoclonal antibodies bind to a target cell (e.g., cancer cell) and specific effector cells expressing receptors for the monoclonal antibody (e.g., NK cells, CD8+ T cells, monocytes, granulocytes) bind the monoclonal antibody/target cell complex
20 resulting in target cell death. Accordingly, in some embodiments, an anti-CD81 monoclonal antibody comprising an Fc region with effector function is used to induce antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) against a CD81-expressing cell. Methods for inducing ADCC generally include contacting the CD81-expressing cell with an effective amount an anti-CD81 monoclonal antibody comprising an Fc
25 region having ADCC activity, wherein the contacting step is in the presence of a cytolytic immune effector cell expressing an Fc receptor having cytolytic activity. Immune effector cells expressing cytolytic Fc receptors (e.g., CD16) include, for example, NK cells as well certain CD8+ T cells. Methods for inducing CDC generally include contacting the CD81-expressing cell with an effective amount an anti-CD81 monoclonal antibody comprising an Fc
30 region having CDC activity, wherein the contacting step is in the presence of complement.

In some embodiments, the antibodies for depletion are bispecific antibodies. “Bispecific antibody” and “bispecific antibodies,” also known as bifunctional antibodies, refers to antibodies that recognize two different antigens by virtue of possessing at least one

first antigen combining site specific for a first antigen or hapten, and at least one second antigen combining site specific for a second antigen or hapten. Such antibodies can be produced by recombinant DNA methods or include, but are not limited to, antibodies produced chemically by methods known in the art. Bispecific antibodies include all antibodies or conjugates of antibodies, or polymeric forms of antibodies which are capable of recognizing two different antigens. Bispecific antibodies include antibodies that have been reduced and reformed so as to retain their bivalent characteristics and to antibodies that have been chemically coupled so that they can have several antigen recognition sites for each antigen. Bispecific antibodies for use in the methods of the present invention bind to CD81 and a second cell surface receptor or receptor complex that mediates ADCC, phagocytosis, and/or CDC, such as CD16/FcγRIII, CD64/FcγRI, killer inhibitory or activating receptors, or the complement control protein CD59. In a typical embodiment, the binding of the portion of the multispecific antibody to the second cell surface molecule or receptor complex enhances the effector functions of the anti-CD81 antibody. In some embodiment, the anti-CD81 antibody is a bispecific antibody. The term “bispecific antibody” has its general meaning in the art and refers to any molecule consisting of one binding site for a target antigen on tumor cells (i.e. a CD81 receptor) and a second binding side for an activating trigger molecule on an effector cell, such as CD3 on T-cells, CD16 (FcγRIII) on natural killer (NK) cells, monocytes and macrophages, CD89 (FcαRI) and CD64 (FcγRI) on neutrophils and monocytes/macrophages, and DEC-205 on dendritic cells. According to the invention, the bispecific antibody comprises a binding site for CD81. tApart from the specific recruitment of the preferred effector cell population, bispecific antibodies avoid competition with endogenous immunoglobulin G (IgG) when the selected binding site for the trigger molecule on the effector cell does not overlap with Fc-binding epitopes. In addition, the use of single-chain Fv fragments instead of full-length immunoglobulin prevents the molecules from binding to Fc-receptors on non-cytotoxic cells, such as FcγRII on platelets and B-cells, to Fc-receptors that do not activate cytotoxic cells, including FcγRIIIb on polymorphonuclear leukocytes (PMN), and to inhibitory Fc-receptors, such as FcγRIIb on monocytes/macrophages. Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (see, e.g., Milstein et al., 1983, Nature 305:537-39). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the

correct bispecific structure. Similar procedures are disclosed in International Publication No. WO 93/08829, and in Traunecker et al., 1991, EMBO J. 10:3655-59. Other examples of bispecific antibodies include Bi-specific T-cell engagers (BiTEs) that are a class of artificial bispecific monoclonal antibodies. BiTEs are fusion proteins consisting of two single-chain variable fragments (scFvs) of different antibodies, or amino acid sequences from four different genes, on a single peptide chain of about 55 kilodaltons. One of the scFvs binds to tumor antigen (i.e. CD81) and the other generally to the effector cell (e.g. a T cell via the CD3 receptor. Other bispecific antibodies those described in WO2006064136. In particular the bispecific antibody is a Fab format described in WO2006064136 comprising one VH or VHH specific for CD81 and one VH or VHH specific for an effector cell.

In some embodiments, the antibody is an anti-CD81 monoclonal antibody-drug conjugate. An "anti-CD81 monoclonal antibody-drug conjugate" as used herein refers to an anti-CD81 monoclonal antibody according to the invention conjugated to a therapeutic agent. Such anti-CD81 monoclonal antibody-drug conjugates produce depleting of leukemic stem cells. In some embodiments, an anti-CD81 monoclonal antibody is conjugated to a cytotoxic agent, such that the resulting antibody-drug conjugate exerts a cytotoxic or cytostatic effect on a CD81-expressing tumor cell when taken up or internalized by the cell. Any cytotoxic agent well known by the skilled person may be used. In some embodiments, the cytotoxic or cytostatic agent is auristatin E (also known in the art as dolastatin-10) or a derivative thereof. Typically, the auristatin E derivative is, e.g., an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatin derivatives include AFP (dimethylvaline-valine-dolaisoleuine-dolaproine-phenylalanine-p-phenylenediamine), MMAF (dovaline-valine-dolaisoleuine-dolaproine-phenylalanine), and MAE (monomethyl auristatin E). The synthesis and structure of auristatin E and its derivatives are described in U.S. Patent Application Publication No. 20030083263; International Patent Publication Nos. WO 2002/088172 and WO 2004/010957; and U.S. Patent Nos. 6,884,869; 6,323,315; 6,239,104; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; 4,879,278; 4,816,444; and 4,486,414.

In some embodiments, the antibodies are added to the patient blood *ex vivo*. Beads coated with the antibody of interest can be added to the blood, target cells bound to these

beads can then be removed from the blood using procedures common in the art. In some embodiments the beads are magnetic and are removed using a magnet. Alternatively, when the antibody is biotinylated, it is also possible to indirectly immobilize the antibody onto a solid phase which has adsorbed avidin, streptavidin, or the like. The solid phase, usually agarose or sepharose beads are separated from the blood by brief centrifugation. Multiple methods for tagging antibodies and removing such antibodies and any cells bound to the antibodies are routine in the art. Once the desired degree of depletion has been achieved, the blood is returned to the patient. Depletion of target cells ex vivo decreases the side effects such as infusion reactions associated with the intravenous administration.

By a "therapeutically effective amount" is meant a sufficient amount of the antibody at a reasonable benefit/risk ratio applicable to the medical treatment. It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In some embodiments, the antibody is used in combination with a chemotherapeutic agent. The term "chemotherapeutic agent" refers to chemical compounds that are effective in

inhibiting tumor growth. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratiostatine; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin (11 and calicheamicin 211, see, e.g., Agnew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabacin, caninomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomicin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitestosterone, mepitosterone, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins;

mitoguazone; mitoxantrone; mopidamol; nitracrine; pento statin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogennanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylarnine; trichothecenes (especially T-2 toxin, verracurin A, roridinA and anguidine); urethan; vindesine; dacarbazine; 5 mannomustine; mitobromtol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); 10 ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-1 1 ; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are antihormonal agents that act to regulate or inhibit hormone action on tumors 15 such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

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The antibody is typically combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. The term "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other 25 untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In the pharmaceutical compositions of the present invention, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, 30 as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and

rectal administration forms. Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or
5 dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or
10 dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as
15 hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The active ingredient can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the
20 protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also
25 be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of
30 microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and

gelatin. Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE 1: CD81 AS NEW POTENTIAL MARKER IN ACUTE MYELOID LEUKEMIA

Background: AML is a heterogeneous disease at both the phenotypic and molecular level with a variety of distinct genetic alterations giving rise to the disease. This heterogeneity extends to the leukemic stem cell (LSC), with this dynamic compartment evolving to

overcome various selection pressures imposed upon it during disease progression. Since LSC are thought to be resistant to current chemotherapeutic regimens and mediate disease relapse, their study may have profound clinical implications. Various markers have been described to characterize the LSC. CD81 antigen belongs to the tetraspanin family (33 members in mammals) which are cell surface transmembrane proteins and may be involved in the re-entry of hematopoietic stem cells into quiescence.

Aims: In this study, we investigated the role of CD81 in the heterogeneity of AML, its potential to induce tumor dormancy and if this is characterized by resistance to chemotherapy.

Methods: In order, to study CD81 expression on primary leukemic blasts, we designed a MFC panel (CD36, CD81, CD33, CD90, CD123, CD34, CD38, CD45). Cell surface marker expression was measured in fresh or thawed bone marrow samples from adult patients with AML at diagnosis (n=122) or at relapse (n=10) using the above mentioned panel. This is a retrospective study to test the prognostic value of CD81, in comparison with other markers of LSC/HSC, for survival (overall survival, event free survival, relapse free survival). AML was diagnosed between 2010 and 2013 at the CHRU of Lille. To further study the function of CD81, we will use AML xenografts. Blasts obtained from newly diagnosed AML, were FACS sorted based upon high CD45 intensity and were then directly injected into NSG mice. After engraftment, the mice were sacrificed, and the bone marrow and spleen were analyzed by flow cytometry. Serial engraftment is performed by injecting one part of the blast cells into a subsequent NSG mouse.

Results: We performed the analysis on 122 diagnosis bone marrow samples and we found that 35% of the samples were positive for CD81 expression. Classification by CD81 expression predicts OS and EFS in AML (EFS: $p=0.012$; OS: $p=0.0066$). 23 primary AMLs have been injected and 8 of them produced serial engraftments. Yet, we did not find any difference in phenotype between the AML xenografts and the AML diagnosis. However, the phenotype of the blasts differ between bone marrow and spleen ($p=0.027$).

Conclusion: Our results show that CD81 may have an important role in AML as their expression on blast cells predicts worse clinical outcome (OS and EFS) in this pathology.

EXAMPLE 2:

Material and methods:

CD81 blocking antibody:

CD81 blocking antibody (Santa cruz, sc-7637), described as blocking by Machida et al., 2005, J. Virol. ; Roccasecca et al., 2003, J. Virol., Bitzegeio J et al., 2010, PLoS Pathog and its relative isotype. For all CD81 blocking antibody experiments, cells are incubated with 20 µg/mL antibodies for 4h.

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In vitro drug resistance:

For in vitro drug resistance (MTT) (Cheok et al., 2003; Holleman et al., 2004), cells are cultured in a 96-well micro culture plates in the presence of a range of drug concentrations (n=8). After 3 days of culture, the dye MTT (3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) is added. The drug concentration that is lethal to 50% of the cells (IC50) is then estimated from the dose response curve (Holleman et al., 2004). 5 million cells were also kept to do western blotting.

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Xenograft:

After pretreatment with blocking antibody or isotype, cells are washed three times to eliminate serum and then reinjected by intravenous in immunodeficient mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ). At time of death, cells of bone marrow, spleen and liver were analyzed for the presence of human cells.

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Colony forming assays with blocking CD81 antibody

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As previously described, mononuclear cells are isolated from bone marrow aspirates by sucrose density gradient centrifugation (Lymphoprep, density 1.077 mg/ml; Nycomed Pharma.), within hours after sampling. Blast cell enrichment using immunomagnetic beads is performed to further enrich the sample for greater than 85% blast cells. We use the indirect labelling method, unwanted cells (normal contaminating cell fractions of >5%) are depleted from the sample and the negative fraction contains untouched blast cells.

25

Methylcellulose colony-forming assays were performed in MethoCult™ H4434 Classic (StemCell Technologies). In brief, after pretreatment with blocking antibody or isotype, normal or AML bone marrow cells are plated at a density of 625×10^3 . The cultures were incubated at 37°C in 5% CO₂ for 14 days. All of the cultures were done in duplicate.

30

Cell cycle:

Bone marrow or blood mononuclear cells were labelled with a combination of antibodies CD45, CD34, CD38, CD81 then fixed and permeabilized. Next, Ki-67 and DNA-labelling were realized. Cells were analyzed on a LSR FORTRESSA X20 (BD Biosciences).

5 Overexpressing CD81 cell line:

U937 cell line which do not express CD81 were transfected by Amara Nucleofactor technologies (Lonza) with a pCDM8 human CD81 plasmid (Addgene). Stably transfected cell were doubled sorted on ARIAIII (BD Biosciences) and CD81 expression were regularly checked.

10

Results:

Cells lines.

15 *In vitro* drug's resistance experiments showed that two AML cell lines: Oci-AML3 and Molm13 acquired sensitivity to multiple drugs (Amsa, Mito, DNR, Eto and Ida) when cells were pretreated for 4 hours in the presence of blocking CD81 antibody. The western blot made from the cell pellets after pretreatment, showed an increase of ERK phosphorylation with CD81 blocking antibody.

20 Oci-AML3 cells pretreated with CD81 blocking antibody or control isotype were injected into immunodeficient mice NSG. At the time of death, analysis of human chimerism showed that cells pretreated with CD81 blocking antibody remains in the bone marrow compared to control animals, while a small percentage was found in liver and spleen. Blockade of CD81 seems therefore to influence spreading of the disease.

25 Conversely, overexpressing CD81-U937 and control cells injected into NSG mice indicated an elevation of circulating blasts in blood of mice injected with overexpressing CD81 cells. More blastic invasion was also found in liver, spleen and bone marrow.

Patients.

30 In mice, CD81 protein plays a role into return in quiescence of hematopoietic stem cells. CD81 could thus have a role on leukemic stem cells quiescence. So we looked at the effect of CD81 on CD34⁺CD38⁻ AML patients in cell cycle. Our first results show that CD34⁺CD38⁻CD81⁺ are more into a quiescent state than CD34⁺CD38⁻CD81⁻ cells.

Clonality assays in methylcellulose done on AML patient's bone marrow samples in the presence of CD81 blocking antibody showed an increase in total number of colonies

compared to the control isotype. This increase was explained by increase in the number of CFU-M (macrophages), the number of the other colonies remaining unchanged compared to the control.

Importantly, same experiment on a normal marrow sample showed no difference to control in the presence of CD81 blocking antibody, highlighting the importance of CD81 in AML blast cells. These results will be to be confirmed on more bone marrow samples of AML and healthy donors.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS:

1. A method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) comprising i) determining the expression level of CD81 on leukemic cells and leukemic stem cells isolated from a sample obtained from the patient, ii)
5 comparing the level determined at step i) with a predetermined reference value and iii) concluding that the patient will have a short survival time when the level determined at step i) is higher than the predetermined reference value or concluding that the patient will have a long survival time when the level determined at step i) is lower than the predetermined reference value.
- 10 2. The method of claim 1 wherein the expression level of CD81 is determined with a binding partner specific for CD81.
3. The method of claim 2 wherein the expression level of CD81 is determined a panel of binding partners specific for the following cell surface markers CD33, CD34, CD36, CD38, CD45, CD81, CD90, and CD123.
- 15 4. The method of claim 2 wherein the binding partner is an antibody.
5. The method of claim 4 wherein the antibody is conjugated with a label.
6. The method of claim 5 wherein the label is a fluorochrome.
7. The method of claim 1 wherein the expression level of CD81 is determined by flow cytometry.
- 20 8. A method of treating acute myeloid leukemia in a patient in need thereof comprising administering to the patient a therapeutically effective amount of an anti-CD81 antibody.
9. The method of claim 8 wherein the antibody is a chimeric antibody, humanized antibody or human antibody.
- 25 10. The method of claim 8 wherein the antibody is a single domain antibody.
11. The method of claim 8 wherein the antibody is bispecific antibody.

12. The method of claim 8 wherein the antibody is an anti-CD81 monoclonal antibody-drug conjugate.
13. The method of claim 8 wherein the antibody is used in combination with a chemotherapeutic agent.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/061978

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574
ADD.

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)
G01N

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE, INSPEC, COMPENDEX, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 2013 116866 A (DAINIPPON SUMITOMO PHARMA CO) 13 June 2013 (2013-06-13) paragraphs [0023], [0048] - [0050]; claims 1,4	8-13
A	----- WO 2013/023015 A2 (EINSTEIN COLL MED [US]; STEIDL ULRICH G [US]; BARREYRO DE PUJATO LAURA) 14 February 2013 (2013-02-14) claims 1-26	1-13
A	----- WO 2015/069935 A1 (SLOAN KETTERING INST CANCER [US]) 14 May 2015 (2015-05-14) paragraphs [0025], [0217]; claims 16-48; figure 7	1-13
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/061978

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	LUO R F ET AL: "CD81 protein is expressed at high levels in normal germinal center B cells and in subtypes of human lymphomas", HUMAN PATHOLOGY, SAUNDERS, PHILADELPHIA, PA, US, vol. 41, no. 2, 1 February 2010 (2010-02-01), pages 271-280, XP026850369, ISSN: 0046-8177 [retrieved on 2009-12-08] abstract; table 2 -----	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/061978

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