【DESCRIPTION】

【Title of Invention】

USE FOR PREVENTING AND TREATING MYELOID-DERIVED SUPPRESSOR CELL-RELATED DISEASES

【Technical field】

The present invention relates to an immune-enhancing agent comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in myeloid-derived suppressor cell (MDSC), and a use of prevention, improvement or treatment for MDSC-related diseases using the immune-enhancing agent. Specifically, the present invention provides a use of prevention, improvement or treatment, or a use of diagnosis for MDSC-related diseases, by regulating production, death, or activity with a monoclonal antibody, so as to reduce an immunosuppressive activity of MDSC.

【Related Art】

The studies on immunotherapy using antibodies or immune cell vaccines have been actively conducted in the treatment of cancer recently. However, the immune evasion and suppression action of cancer cells inhibit the therapeutic effects. Cancer cells reduce the activity of various immune cells for the purpose of preventing an immune response to themselves, and induce cells with immune suppression functions such as inactive dendritic cells, regulatory T cells (Treg), and Tumor-associated macrophages (TAM). As one of the immunosuppressive cells, the role of myeloid-derived suppressor cells (MDSCs) has been recently gained great attention.

MDSC is defined as a collection of bone marrow-derived immature bone marrow cells with immunosuppressive function. It is reported that they are accumulated in peripheral blood, lymphatic organs, spleen, and cancer tissues in pathological conditions such as chronic/acute infections and cancer, .although the number of MDSC is limited in healthy individuals.

MDSC can also promote the growth of cancer cells, and induce remote metastasis of cancer cells, by inhibiting the immune response of T cells and NK cells and inducing the generation of Treg cells which are immunosuppressive cells.

The immunosuppression mechanisms of MDSC known so far can be divided into four major types. The first is to be deficient in nutrients required by lymphocytes. The second is to generate oxidative stress, which inhibits various steps such as of proliferation to function of T cell by making active oxygen or active nitrogen. The third

is to affect the trafficking and survival of lymphocytes. Specifically, the mechanisms such as inhibiting the recirculation process of T cells to lymph nodes, preventing the movement of T cells to the center of a tumor, and inducing T cell death are known. Fourth, it is known to proliferate antigen-specific natural Treg cells and promote the process of converting naïve CD4+ T cells to Tregs.

One of the greatest features of MDSC is its diversity in form, phenotype, and function. As the markers for MDSC, Lineage(-), HLA-DRLOW/(-), CD11b(+), and CD33(+) are known. Since these markers are commonly expressed in several different types of myeloid cells such as dendritic cells, macrophages, and precursor cells of granular leukocytes, MDSC has been defined as a group of myeloid-derived cells with immune suppression functions. This diversity of MDSC has led to different analyzes in studying the origins and characteristics of MDSCs, thereby causing great confusion of the study. Accordingly, a study has been conducted to clarify the subgroups of MDSC, to currently find that MDSC consists of 80% of granulocytic MDSCs and 20% of monocytic MDSCs. These two cell types differ not only in shape and phenotype, but also in the mechanism of suppressing immunity. The granulocytic MDSC induces antigen-specific immunosuppression through contact between T cells via active oxygen. The monocytic MDSC exhibits immunosuppressive function mainly by using high expression of arginase and various immunosuppressive cytokines.

Recent studies have reported that the accumulation of MDSC is involved in the immunosuppressive environment occurred in cancer patients, which is common in almost all cancer types. It is supported by many studies that the degree of increase in MDSC becomes higher as the stage of cancer progresses. Accordingly, studies to use the increase degree of MDSC as a prognostic marker for the low survival rate and treatment response rate of cancer patients are actively underway. It seems clear that MDSC plays an important role in the pathophysiology of cancer.

【Disclosure】

【Technical Problem】

An embodiment of the present invention is an immune-enhancing agent, and immune-activating agent, or a composition for reducing or eliminating an immunosuppressive activity of MDSC, comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in myeloid-derived suppressor cell (MDSC).

An embodiment of the present invention is a pharmaceutical composition or a use for prevention, improvement or treatment of MDSC-related diseases comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in MDSC.

An embodiment of the present invention is a method of enhancing or activating an immune response of a subject, comprising administering an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in MDSC, to the subject in need of.

An additional embodiment of the present invention is a method of inhibiting an activity of MDSC, comprising contacting MDSC with an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in MDSC.

In addition, an embodiment of the present invention is a method of prevention, improvement or treatment of MDSC-related diseases, comprising administering an immune-enhancing agent, or immune-activating agent, to a subject with MDSC-related disease. In addition, an embodiment of the present invention is a method of prevention, improvement or treatment of MDSC-related diseases, comprising administering an immune-enhancing agent, or immune-activating agent comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in myeloid-derived suppressor cell, to a subject with MDSC-related diseases.

The antibody or antigen-binding fragment thereof specifically binding to CD66c which is expressed in myeloid-derived suppressor cell in accordance with the present invention, eliminates or reduces an immunosuppressive activity of MDSC, decreases the number of MDSC, regulates an activity, production or cell death of MDSC, or induces the cell death.

【Technical Solution】

The present invention relates a use of immune-enhancement, immune-activation, or reduction or elimination of an immunosuppressive activity of MDSC, comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in MDSC.

A further embodiment of the present invention relates to a use of prevention, improvement or treatment of MDSC-related diseases, for examples cancers, infective diseases, and the like, comprising an antibody or an antigen-binding fragment thereof specifically binding to CD66c which is expressed in MDSC.

Specifically, the present invention relates a use of prevention, treatment or

diagnosis of MDSC-related diseases, by inducing the reduction of immunosuppressive activity of MDSC. The antibody may be a polyclonal antibody or a monoclonal antibody, and may be a mouse antibody, chimeric antibody, or humanized antibody.

Another embodiment provides a nucleic acid molecule encoding the anti-CD66c antibody or antigen-binding fragment thereof.

Another embodiment provides a recombinant vector comprising the nucleic acid molecule. The recombinant vector may be used as an expression vector for expressing the nucleic acid molecule in a host cell.

Further embodiment provides a recombinant cell comprising the nucleic acid molecule or the recombinant vector. The recombinant cell may be obtained by transforming the nucleic acid molecule or the recombinant vector into a host cell.

Another embodiment provides a method of preparing the anti-CD66c antibody or antigen-binding fragment thereof. The preparing method may include a step of expressing the nucleic acid molecule in a host cell. The step of expressing may include culturing the recombinant cells, and optionally, may further include separating and/or purifying the antibody from the obtained cell culture. The method may include the following steps:

(a) preparing a recombinant cell transformed with the nucleic acid molecule or the recombinant vector;

(b) culturing the recombinant cell under conditions and/or a period for sufficient expression of the nucleic acid molecule; and

(c) separating and/or purifying the anti-CD66c antibody or antigen-binding fragment thereof from the culture obtained in step (c).

Hereinafter, the present invention will be described in more detail.

In one embodiment, the preparing method relates to a composition for reducing or eliminating an immune-suppressing ability of MDSC, an immune-enhancing agent, or an immune activating agent, including an antibody or antigen-binding fragment thereof that binds to CD66c expressed in MDSC.

MDSC is defined as a collection of bone marrow-derived immature bone marrow cells having immunosuppressive function, and is reported to be accumulated in peripheral blood, lymphatic organs, spleen, cancer tissues, etc in pathological conditions such as chronic/acute infections and cancer.

MDSC promotes the growth of cancer cells, and can also induce remote metastasis of cancer cells by inhibiting the immune response of T cells and NK cells, and inducing the generation of Treg cells, which are immunosuppressive cells. The

immunosuppression mechanisms of MDSC known so far are to deplete nutrients required by lymphocytes, to affect the trafficking and survival of lymphocytes, to generate oxidative stress, which inhibits various steps, for examples from proliferation to function of T cell by making active oxygen or active nitrogen, to inhibit recirculation of T cell to lymph node, or to block the movement of T cell to the center of cancer tissue, and to induce the cell death of T cells. In addition, MDSC has been known to proliferate antigen-specific natural Treg cells and promote the process of converting naïve CD4+ T cells to Tregs.

MDSC is defined as a collection of bone marrow-derived immature bone marrow cells with immunosuppressive function. Although the number is limited in healthy individuals, it is accumulated in peripheral blood, lymphatic organs, spleen, and cancer tissues in pathological conditions such as chronic/acute infections and cancer. MDSC accumulation and immunosuppressive function in carcinoma have been reported in colon cancer, fibrosarcoma, thymoma, lung cancer, mesothelioma, lymphoma, prostate cancer, head and neck cancer, melanoma and the like (Gabrilovich DI, et al., Coordinated regulation of myeloid cells by tumors, Nat Rev Immunol. 12(4):253-68 (2012)). Besides the cancers, MDSC accumulation has been known to induce immunosuppression in infections such as Trypanosoma cruzi, Listeria monocytogenes, Leishmania major, helminths, Candida albicans, Porphyromonas gingivalis, and the like, or diseases of toxoplasmosis and polymicrobic sepsis (Garbrilovich DI, et al. al., Myeloid-derived suppressor cells as regulators of the immune systems. Nat Rev Immunol. 9(3):162-74 (2009)).

In the present disclosure, MDSC which is a phenotype of a non-lymphatic HLA-DRLow/(-), CD11b+, and CD33+, and expresses CD66c, can be a target of the anti-CD66c antibody or antigen-binding fragment thereof according to the present invention. Particularly, the present invention can target for the accumulation of CD66c positive MDSCs among MDSCs which is a phenotype of a non-lymphatic HLA-DRLow/(-), CD11b+ and CD33+, and thus present a plan for improvement or treatment of immunity deficiency, immunity decrease, immunity damage caused by MDSC. For example, MDSC can be designated by designating monocytic region and granulocytic regions in reference to the cell size in dot plot, except lymphocyte, selecting groups of no or lower expression level of HLA-DR, and selecting groups of CD11b and CD33 positive.

The present invention can provide a pharmaceutical composition or a use thereof for prevention, improvement or treatment of MDSC-related diseases, by using

an antibody or an antigen-binding fragment thereof specifically binding to CD66c expressed in MDSC in accordance with the present invention.

The lysis effect of MDSC by the anti-CD66c antibody according to the present invention can induce a decreased number of MDSC cells or apoptosis in CEACAM6-positive cells in both whole blood and PMBC. Preferably, the anti-CD66c antibody can induce a decreased number or cell death in a ADCC manner. In whole blood, neutrophils positive for CEACAM6 target antigen and MDSC are mixed, and thus it is difficult to say that only MDSCs are selectively lysed. However, it is possible.to perform selective lysis of MDSC by using anti-CD66c antibody, in peripheral blood mononuclear cells (PBMC) obtained after removing the neutrophil.

The MDSC-related diseases is a disease that exhibits immunosuppressive activity by MDSC, and is a disease in which the level of CD66c-positive MDSCs are increased compared to those of normal cells, which is a criterion used for determining the disease. For example, the number or the activity of CD66c-positive MDSCs in a subject with a specific disease is about 200% or more, about 300% or more, about 500% or more, about 700% or more, about 1,000% or more, or about 1,500% or more, for example, about 200 to 5,000%, or 200% to 3,000%, 200 to 1,500%, and the like, based on 100% of the number or activity of CD66c-positive MDSCs per unit volume of the corresponding normal subject sample. For example, the increase in the number of MDSCs can be determined by taking samples, such as bloods from a subject suspected of having MDSC-related diseases and a normal subject, analyzing the number of MSDCs in sample with a flow cytometer, and comparing the number of MDSCs of the subject suspected of having MDSC-related diseases, with that of normal subject. Specifically, in the subject having MDSC-related diseases, the number of MSDCs per unit volume of a sample (e.g. blood) may be increased compared to that of a normal subject, and for example, it may be about 200% or more, about 300% or more, about 500% or more, about 700% or more, about 1,000% or more, about 1,500% or more, for example, about 200 to 5,000%, or 200% to 3,000%, 200 to 1,500%, and the like, based on 100% of the number or activity of MDSCs per unit volume of the sample of corresponding normal subject.

Specifically, the MDSC-related diseases are for example, diseases in which MDSCs expressing CD66c among MDSCs showing the phenotype of non-lymphatic HLA-DRLow/(-), CD11b+, and CD33+ are increased or accumulated or diseases in which the number of MDSCs are increased compared to that of normal cells. The examples of MDSC-related diseases include chronic/acute infections, cancers and the

like, specifically chronic/acute infections, cancers and the like which shows the immunosuppressive activity of MDSC. For example, the diseases may be chronic/acute infections, cancers and the like in which CD66c-positive MDSC among the MDSCs showing the phenotype of non-lymphatic HLA-DRLow/(-), CD11b+, and CD33+ are accumulated.

The MDSC-related infective diseases may be infections such as Trypanosoma cruzi, Listeria monocytogenes, Leishmania major, helminths, Candida albicans, or Porphyromonas gingivalis, or diseases of toxoplasmosis or polymicrobic sepsis.

For example, the MDSC-related cancer may be a cancer with increased CD66c-positive MDSC, and includes solid cancer and hematologic cancer. The examples of the solid cancer include colon cancer, fibrosarcoma, thymoma, lung cancer, mesothelioma, lymphoma, prostate cancer, head and neck cancer, melanoma, stomach cancer, liver cancer, or breast cancer, or preferably colon cancer, stomach cancer, or liver cancer. The use of the prevention, inhibition, or treatment of cancer and cancer metastasis can, for example, inhibit cancer cell growth. Example of the hematopoietic malignancy includes acute myeloid leukemia, acute lymphoblastic leukemia, acute monocytic leukemia, Hodgkin's lymphoma, and non-Hodgkin's lymphoma.

The present invention relates to an antibody or antigen-binding fragment thereof binding to CD66c expressed in MDSC. CD66c (Cluster of Differentiation 66c) is also known as CEACAM 6 (carcinoembryonic antigen-related cell adhesion molecule 6) or NCA (non-specific cross-reacting glycoprotein antigen). It is known as an important protein associated with cell adhesion. CD66c may preferably be represented by the amino acid sequence of SEQ ID NO: 1 (Genbank Protein No. AAH05008), but is not limited thereto.

As used herein, the term, "antibody" means a substance produced by stimulation of an antigen in the immune system, and the kind thereof is not particularly limited. The antibody may be generated in a non-natural manner, for example, recombinantly or synthetically generated. The antibody may be an animal antibody (e. g., mouse antibody, etc.), a chimeric antibody, a humanized antibody or a human antibody. The antibody may be a monoclonal antibody or a polyclonal antibody.

The anti-CD66c antibody or antigen-binding fragment specifically binds to a specific epitope of CD66c described above, and can be selected from the group consisting of animal antibodies (e.g., mouse antibodies), chimeric antibodies, humanized antibodies, and antigen-binding fragments thereof. The animal antibody may be derived from an animal species other than human, for example, rat, mouse, goat,

guinea pig, donkey, rabbit, horse, llama, camel, bird (e.g., chicken, duck, etc.), but not limited thereto. Techniques for producing chimeric antibodies and/or humanized antibodies from such animal antibodies are well known in the art. The humanized antibody may be any suitable isotype such as IgG (IgG1, IgG2, IgG3, IgG4), IgM, IgA, IgD, IgE or any subclass, preferably IgG1 or IgG2 isotype, or more preferably de-fucosylated IgG1 or IgG2 isotype.

In addition, herein, an antibody can be understood to include an antigen-binding fragment of an antibody having antigen-binding ability, unless otherwise specified. In the present specification, the term, “complementarity determining regions (CDR)” refers to a region of antibody that imparts the binding specificity of antibody to an antigen among variable regions of the antibody. The antigen-binding fragment of the antibody described above may be an antibody fragment comprising at least one of the complementarity determining regions. The term, "CDR (complementarity determining region)" means an amino acid sequence of the hypervariable region of the heavy chain sand light chain of an immunoglobulin. Each of the heavy chain and light chain may comprise three CDRs (CDRH1, CDRH2, CDRH3 and CDRL1, CDRL2, CDRL3). The CDRs can provide key contact residues for the antibody to bind to an antigen or epitope. On the other hand, in the present disclosure, the terms "specifically bind" or "specifically recognize" means the same as those commonly known to those skilled in the art.

The term "antigen-binding fragment" refers to a fragment thereof for the entire structure of an immunoglobulin, and refers to a portion of a polypeptide including a portion to which an antigen can bind. For examples, the fragments may be scFv, (scFv)2, scFv-Fc, Fab, Fab' or F(ab')2, but not limited thereto.

The anti-CD66c antibody according to the present invention specifically recognizes and/or binds to CD66c, and the antibody includes a mouse antibody, chimeric antibody or humanized antibody. The chimeric antibody in the present invention is an antibody that the sequence of the variable region is derived from one species and the sequence of the constant region is derived from other species, for example, that the variable region is derived from mouse and the constant region is derived from human. The humanized antibody in the present invention is an antibody which has a low immunogenicity in human and an activity of non-human antibody. For example, it can be prepared by keeping non-human CDR region and substituting the rest of the region with human counterparts. For example, the literature is referenced: Morrison et al, Proc. Natl. Acad. ScL USA, 81:6851-6855(1984); Morrison and Oi, Adv.

Immunol., 44:65-92 (1988); Verhoeyen et al, Science, 239:1534-1536 (1988); Padlan, Molec. Immun., 28:489-498 (1991); Padlan, Molec. Immun., 31(3):169-217 (1994).

The antibody fragment in the present invention is not limited, as long as it recognizes specifically CD66c epitope and includes variable region of a light chain (V_L) and variable region of a heavy chain (V_H). It can be selected from a group consisting of Fab, Fab', F(ab')2, scFv, dsFv and CDR. Especially, scFv is an antibody fragment prepared as a single chain by connecting the variable region of a heavy chain (V_H) and variable region of a light chain (V_L) with a linker polypeptide.

The term "hinge region" is a region included in the heavy chain of an antibody, exists between the CH1 and CH2 regions, and refers to a region to provide flexibility of the antigen binding site in the antibody. For example, the hinge may be derived from a human antibody, and specifically, may be derived from IgA, IgE, or IgG, such as IgG1, IgG2, IgG3, or IgG4.

The anti-CD66c antibody may be a monoclonal antibody or a polyclonal antibody, such as a monoclonal antibody. Monoclonal antibodies can be prepared according to methods well known in the art. For example, it can be manufactured using a phage display technique.

Unlike mouse antibodies or chimeric antibodies, the humanized antibodies showed 10 times higher stability than chimeric 8F5 antibodies in terms of stability in addition to the different characteristic that significantly reduces the cause of immunogenicity when administered to humans. Specifically, at a high temperature, for example, 62° C, the antibody has a high stability because the fluorescence variability against the ANS reagent was less than 200%.

Under specific severe conditions, due to variations in antibody properties, chimeric 8F5 antibodies showed 1,406% of ANS reactivity change, whereas humanized antibodies showed relatively insignificant changes of about 114% and 133%, indicating that they were significantly stabilized protein.

The chimeric 8F5 and the humanized antibody increase the activation of T cells, which is also shown in increased activity of T cell caused by T cell activator and T cell activity conditions due to mixing of allogeneic dendritic cells and T cells of different people. This induction of T cell activation induces the death of cancer cells when co-cultured with cancer cells, and T cell activation under co-culture conditions with various cancer cells.

The antibody or fragment thereof according to the present invention has a tumor regression activity and a direct inhibitory effect on tumor cell lines. In the present

disclosure, the tumor regression includes inducing or promoting a decrease in the size of a tumor and/or inhibiting, stopping or reducing the growth of tumor cells. For example, the reduction in tumor size means that the tumor size obtained by administering the composition comprising the antibody or fragment thereof is 97% or less, 95% or less, 90% or less, 85% or less, 80% or less, and 75% or less, based on 100% before treatment of the composition comprising the antibody or fragment thereof of the present invention.

The antibody according to the present invention has antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and preferably ADCC characteristics.

The antibody or antigen-binding fragment thereof according to the present invention can improve or treat MDSC-related diseases by using a combination of natural killer cell or NK cell-derived cell therapy.

Specifically, the anti-CD66c antibody according to the present invention increases cancer cell killing ability by combination with natural killer cells, and thus, has an excellent effect of NK cells or NK cell therapeutic agents for effective removal of not only CEACAM6-positive cancer cells, but also CEACAM6-positive MDSCs.

In a specific experiment, as a result of measuring cell viability using the EZ-cytox enhanced cell viability kit (Daeil Lab), it was confirmed that the apoptosis effect by the combination with natural killer cells was higher in two types of cancer cell lines compared to the case of single treatment (Figs. 12a and 12b). According to the selective lysis result of MDSC caused by the anti-CD66c antibody of the present invention, and the combination therapy effect of anti-CD66c antibody and NK cells or NK cell therapeutics, the antibody could remove targets including CEACAM6-positive cancer cell and CEACAM6-positive MDSC. The anti-CD66c antibody according to the present invention shows ADCC against different target cells, such as MDSC and cancer cells, respectively. In the case of cancer patients in which two types of cells are actually increased together, the anti-CD66c antibody of the present invention can remove the two types of targets together, and shows increased efficacy of simultaneous removal of cancer cells and MDSC targets, in combination with NK cell therapeutics.

The antibody according to the present invention may remove partially or completely fucose as a sugar residue bound to the antibody. The fucose-removing antibody of the present invention has an apoptosis activity of MDSC, and in one embodiment, the antibody of the present invention has an apoptosis activity of MDSC as a low fucose form or afucose form compared to a fucose form of antibody, so as to

have high immunity enhancement. As used herein, “normal fucose” or “normal fucose content” refers to an antibody having a fucose content of at least 90% typically. The low fucose or afucose form of the antibody according to the present invention may be an antibody having a fucose content of about 10% or less, about 7% or less, or about 5% or less, for example, 0 to about 10%, 0 To about 7%, or 0 to about 5%.

Specifically, the antibody of the present invention can comprises the following complementarity determining regions (CDRs):

CDR-H1 comprising an amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 9,

CDR-H2 comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 10,

CDR-H3 comprising an amino acid sequence of SEQ ID NO: 3,

CDR-L1 comprising an amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 11 or SEQ ID NO: 12,

CDR-L2 comprising an amino acid sequence of SEQ ID NO: 5 and

CDR-L3 comprising an amino acid sequence of SEQ ID NO: 6 or SEQ ID NO: 13.

The heavy chain variable region of the antibody comprises at least one selected from the group consisting of framework sequence (V-FR1) including the amino acid sequence of SEQ ID NOs: 22, 23, 24, 25, 26 or 27, framework sequence (V-FR2) including the amino acid sequence of SEQ ID NOs: 32, 33, 34, 35, 36 or 37, framework sequence (V-FR3) including the amino acid sequence of SEQ ID NOs: 42, 43, 44, 45, 46 or 47, and framework sequence (V-FR4) including the amino acid sequence of SEQ ID NOs: 52, 53, 54, 55, 56 or 57.

The light chain variable region of the antibody comprises at least one selected from the group consisting of framework sequence (L-FR1) including the amino acid sequence of SEQ ID NOs: 28, 29, 30 or 31, framework sequence (L-FR2) including the amino acid sequence of SEQ ID NOs: 38, 39, 40 or 41, framework sequence (L-FR3) including the amino acid sequence of SEQ ID NOs: 48, 49, 50, or 51, and framework sequence (L-FR1) including the amino acid sequence of SEQ ID NOs: 58, 59, 60 or 61.

Te antibody comprises a heavy chain variable region including the amino acid sequence of SEQ ID NOs: 7, 14, 15, 16, 17 or 18, and a light chain variable region including the amino acid sequence of SEQ ID NOs: 8, 19, 20, or 21.

An example of a mouse antibody or a chimeric antibody according to the present invention can be an antibody or antigen-binding fragment thereof including at

least one selected from the group consisting of an amino acid sequence of VH CDR comprising the amino acid sequences of SEQ ID NOS: 1 to 3 and an amino acid sequence of VL CDR comprising amino acid sequences of SEQ ID NOs: 4 to 6. The CDRs and the variable regions of an example of the mouse antibody or chimeric antibody are summarized in Table 1 below.

Specifically, an example of the antibody of the present invention may include SEQ ID NO: 1 (CDR1), SEQ ID NO: 2 (CDR2) and SEQ ID NO: 3 (CDR3) as VH CDR and/or SEQ ID NO: 4 (CDR1), SEQ ID NO: 5 (CDR2), and SEQ ID NO: 6 (CDR3) as VL CDR.

The mouse antibody or chimeric antibody may comprise a VH region including the amino acid sequence of SEQ ID NO: 7 and a VL region including the amino acid sequence of SEQ ID NO: 8.

The present invention relates to a pharmaceutical composition, a kit or a method of prevention or treatment of a MDSC-related disease and a symptom thereof, comprising a mouse antibody or chimeric antibody as an active ingredient.

The present invention also relates to a pharmaceutical composition for prevention or treatment of a MDSC-related disease and a symptom thereof, comprising a mouse antibody or chimeric antibody as an active ingredient, for example an anti-CD66c antibody or antigen-binding fragment thereof including CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3 of the antibody produced by a hybridoma cell deposited as an accession number of KCLRF-BP-00230. The hybridoma cell was deposited with the Korean Cell Line Research Foundation (KCLRF) as '8F5' on Feb. 22, 2010 and received an accession number of KCLRF-BP-00230, which has been described in detail in KR 10-1214177.

The present invention can prepare a humanized antibody by using the amino acid sequence of anti-CD66c antibody 8F5 in the mouse antibody or chimeric antibody and the framework sequences of human. On the basis of expression degree, aggregation, and cell binding degree, the humanized antibodies which are expressed normally, are less aggregated due to the instability of protein itself, and have ability to bind to target antigen-positive cells similar to the chimeric antibody. Specifically, the cell binding profile is similar to that of the chimeric antibody and is obtained by multiplying positive rate of antibody positivity (%gated) with the average fluorescence (mean), and then comparing with the chimeric antibody to select the candidate antibodies within the range of ± 20% (Example 2). Therefore, when CDR region sequences of the mouse antibody are inserted into the framework region of human antibody at the time of

preparing the humanized antibody, the binding ability of prepared antibody is rapidly decreased due to the change of the original protein structure. In consideration of the decreased the binding ability of prepared antibody, the selected humanized antibodies of the present invention are very excellent antibody.

Preferably, five kinds of recombinant humanized antibodies exhibiting a high binding affinity based on the cell binding ability when compared to the chimeric antibody are selected and subjected to binding assay for CD66c antigen and similar antigen to CD66 antigen by ELISA.

In addition, the humanized antibody according to the present invention exhibits excellent stability compared to the chimeric antibody, for example, an antibody having stability which is reflected as ANS reactivity variation of less than 200%. The ANS reactivity variation of less than 200% is regarded as a very small variation and the higher variation value than 200% can be interpreted as observing ANS reactivity due to the significant structural change of protein. Accordingly, the humanized antibody according to the present invention has similar antigen binding activity and cell binding ability to the chimeric antibody, and the increased physical stability of the antibody protein itself, which can be very excellent in terms of druggability of the therapeutic antibody.

The fluorescence variation of the antibody against the ANS reagent can be measured by dividing the difference between the fluorescence value measured at low temperature condition (e.g, 4°C.) and the fluorescence value measured at high temperature condition (e.g, 62 °C.), with the fluorescence value measured at low temperature conditions.

[Mathematic Equation]

Fluorescence variation = (fluorescence value measured at high temperature condition - fluorescence value measured at low temperature condition) / (fluorescence value measured at low temperature condition)

As a method for obtaining a specific fluorescence variation of antibody, the reactivity of ANS reagent was measured by a fluorescent reader after being left for 4 hours at a refrigeration condition (4 °C) and a temperature of 62 °C, and expressed as a fluorescence value, and the fluorescence variation can be obtained using the equation.

Examples of the humanized antibody according to the present invention may include one or more amino acid sequences selected from the group consisting of amino acid sequences that determine the CDRs of the heavy chain variable region or light chain variable region comprising the amino acid sequences of SEQ ID NOs: 9 to 13.

The examples of mouse antibody and the chimeric antibody ma include one or more amino acid sequences selected from the group consisting of amino acid sequences that determine the CDRs of the heavy chain variable region comprising the amino acid sequences of SEQ ID NOs: 1 to 3 or light chain variable region comprising the amino acid sequences of SEQ ID NOs: 4 to 6.

Specifically, an example of a humanized antibody includes an amino acid sequence determining the CDR1 of the VH region comprising the amino acid sequence of SEQ ID NO: 1 or 9, an amino acid sequence determining the CDR2 of the VH region comprising the amino acid sequence of SEQ ID NO: 2 or 10, and the CDR3 of the VH region comprising the amino acid sequence of SEQ ID NO: 3.

Examples of the humanized antibodies include an amino acid sequence determining the CDR1 of the VL region including the amino acid sequence of SEQ ID NO: 4, 11 or 12, an amino acid sequence determining the CDR2 of the VL region including the amino acid sequence of SEQ ID NO: 5, and an amino acid sequence determining the CDR3 of the VL region comprising the amino acid sequence of SEQ ID NO: 6 or 13.

Examples of the humanized antibody include a heavy chain variable region selected from the group consisting of the amino acid sequence of SEQ ID NO: 7 and SEQ ID NOs: 14 to 18 and a light chain variable region selected from the group consisting of the amino acid sequence of SEQ ID NO: 8 and SEQ ID Nos: 19-21, but does not include the antibody comprising SEQ ID NO: 7 and SEQ ID NO: 8.

The CDR sequences and variable region sequences according to an example of the humanized antibody are summarized in Table 1 below.

[Table 1]

Name	SEQUENCE	SEQ ID NO
8F5-chimeric V_H-CDR1	ASGYSFTDYTMN	1
8F5-chimeric V_H-CDR2	LINPFHGGTVSNQRFKV	2
8F5-chimeric V_H-CDR3	VRGDPVRHYYALAY	3
8F5-chimeric V_L-CDR1	GASENVYGTLN	4
8F5-chimeric V_L-CDR2	GATNLAD	5
8F5-chimeric V_L-CDR3	VATYYCQNVLSAPYT	6
8F5-chimeric V_H	EVQLQQSGPELVKPGASMKISCKASGYSF TDYTMNWVKQSHGKNLEWIGLINPFHGG TVSNQRFKVKATLTVDVSSNTAYMELLS LTSDDSAVYYCVRGDPVRHYYALAYWG QGTSVTVSS	7
8F5-chimeric V_L	DIQMTQSPASLSASVGETVTITCGASENV	8

	YGTLNWYQRKQGKSPQLLIYGATNLADG MSSRFSGSGSGRQYSLKISSLHPDDVATY YCQNVLSAPYTFGGGTKLEII	
8F5-human V_H-CDR1	ASGYSFTDYTMN	1
8F5-human V_H-CDR1	ASGYSFTDYTM**H**	9
8F5-human V_H-CDR2	INPFHGGTVSNQRFKV	2
8F5-human V_H-CDR2	LINPF**G**GST**SYA**Q**K**FK**G**	10
8F5-human V_H-CDR3	VRGDPVRHYYALAY	3
8F5-human V_L-CDR1	GASENVYGTLN	4
8F5-human V_L-CDR1	GASENVYGTL**A**	11
8F5-human V_L-CDR1	**R**ASENVYGTLN	12
8F5-human V_L-CDR2	GATNLAD	5
8F5-human V_L-CDR3	VATYYCQNVLSAPYT	6
8F5-human V_L-CDR3	**F**ATYYCQNVLSAPYT	13
8F5-human-VH5	QVQLVQSGAEVKKPGASVKISCKASGYS FTDYTMNWVRQAHGQNLEWIGLINPFHG GTVSNQRFKVKATLTVDVSTNTAYMELS RLRSDDTAVYYCVRGDPVRHYYALAYW GQGTLVTVSS	14
8F5-human-VH6	QVQLVQSGAEVKKPGASMKISCKASGYS FTDYTMNWVKQAPGQNLEWIGLINPFHG GTVSNQRFKVKATLTVDVSTNTAYMELS RLRSDDTAVYYCVRGDPVRHYYALAYW GQGTLVTVSS	15
8F5-human-VH7	QVQLVQSGAEVKKPGASMKISCKASGYS FTDYTMNWVRQAPGQGLEWIGLINPFHG GTVSNQRFKVKATLTVDVSTNTAYMELS RLRSDDTAVYYCVRGDPVRHYYALAYW GQGTLVTVSS	16
8F5-human-VH10	QVQLVQSGAEVKKPGASVKVSCKASGYS FTDYTMNWVKQAPGQNLEWIGLINPFHG GTVSNQRFKVKATMTVDVSTNTAYMEL SRLRSDDTAVYYCVRGDPVRHYYALAY WGQGTLVTVSS	17
8F5-human-VH11	QVQLVQSGAEVKKPGASVKISCKASGYS FTDYTMHWVKQAPGQNLEWIGLINPFGG STSYAQKFKGRVTMTRDTSTNTAYMELS RLRSDDTAVYYCVRGDPVRHYYALAYW GQGTLVTVSS	18
8F5-human-VK5	DIQMTQSPSTLSASVGDRVTITCGASENV YGTLAWYQRKPGKAPKLLIYGATNLADG VPSRFSGSGSGREYTLTISSLQPDDFATYY CQNVLSAPYTFGGGTKLEIK	19
8F5-human-VK7	DIQMTQSPSTLSASVGDRVTITCGASENV YGTLNWYQRKPGKAPKLLIYGATNLADG VPSRFSGSGSGTEYTLTISSLQPDDFATYY CQNVLSAPYTFGGGTKLEIK	20

8F5-human-VK8	DIQMTQSPSTLSASVGDRVTITCRASENV YGTLNWYQRKPGKAPKLLIYGATNLADG MPSRFSGSGSGTEYTLTISSLQPDDFATYY CQNVLSAPYTFGGGTKLEIK	21

The framework sequences of one example of a humanized antibody according to the present invention are shown in Tables 2 and 3 below, wherein said antibody may include at least one selected from the group consisting of frameworks 1 to 4 of the heavy chain variable region and frameworks 1 to 4 of the light chain variable region And may be an antibody comprising one or more frameworks.

Specifically, the amino acid sequence of framework 1 of in the heavy chain variable region may comprise SEQ ID NOS: 23 to 27, the amino acid sequence of framework 2 may comprise SEQ ID NOS: 32 to 37, and the amino acid sequence of framework 3 43 to 47, and the amino acid sequence of Framework 4 may include SEQ ID NOS: 53 to 57.

In the light chain variable region, the amino acid sequence of Framework 1 may comprise SEQ ID Nos: 29 to 31, the amino acid sequence of Framework 2 may comprise SEQ ID NOs: 39 to 41, and the amino acid sequence of Framework 3 may correspond to the amino acid sequence of SEQ ID NOs: 49-51, and the amino acid sequence of Framework 4 may comprise SEQ ID NOs: 59 to 61. The framework sequences according to examples of the humanized antibody are shown in the following table.

[Table 2]

Name	FR1	SEQ ID NO	FR2	SEQ ID NO
V_H- Chimeric	EVQLQQSGPELVKPGAS MKISCK	22	WVKQSHGKNLE WIG	32
VH5	**Q**VQL**V**QSG**A**E**VK**KPGA S**V**KISCK	23	WV**R**Q**A**HG**Q**NLE WIG	33
VH6	**Q**VQL**V**QSG**A**E**VK**KPGA SMKISCK	24	WVKQ**AP**G**Q**NLE WIG	34
VH7	**Q**VQL**V**QSG**A**E**VK**KPGA SMKISCK	25	WV**R**Q**AP**G**QG**LE WIG	35
VH10	**Q**VQL**V**QSG**A**E**VK**KPGA S**V**K**V**SCK	26	WVKQ**AP**G**Q**NLE WIG	36
VH11	**Q**VQL**V**QSG**A**E**VK**KPGA S**V**KISCK	27	WVKQ**AP**G**Q**NLE WIG	37

V_L-Chimeric	DIQMTQSPASLSASVGETVTITC	28	WYQRKQGKSPQLLIY	38
VK5	DIQMTQSP**ST**LSASVG**D****R**VTITC	29	WYQRK**P**GK**A**P**K**LLIY	39
VK7	DIQMTQSP**ST**LSASVG**D****R**VTITC	30	WYQRK**P**GK**A**P**K**LLIY	40
VK8	DIQMTQSP**ST**LSASVG**D****R**VTITC	31	WYQRK**P**GK**A**P**K**LLIY	41

[Table 3]

Name	FR1	SEQ ID NO	FR2	SEQ ID NO
V_H- Chimeric	NQRFKVKATLTVDVSSNTAYMELLSLTSDDSAVYYCVR	42	WGQGTSVTVSS	52
VH5	NQRFKVKATLTVDVS**T**NTAYMEL**SR**L**R**SDD**T**AVYYCVR	43	WGQGT**L**VTVSS	23
VH6	NQRFKVKATLTVDVS**T**NTAYMEL**SR**L**R**SDD**T**AVYYCVR	44	WGQGT**L**VTVSS	24
VH7	NQRFKVKATLTVDVS**T**NTAYMEL**SR**L**R**SDD**T**AVYYCVR	45	WGQGT**L**VTVSS	55
VH10	NQRFKVKAT**M**TVDVS**T**NTAYMEL**SR**L**R**SDD**T**AVYYCVR	46	WGQGT**L**VTVSS	56
VH11	**A**Q**K**FK**GRV**T**M**T**R**D**T**S**T**NTAYMEL**SR**L**R**SDD**T**AVYYCVR	47	WGQGT**L**VTVSS	57
V_L-Chimeric	GMSSRFSGSGSGRQYSLKISSLHPDD	48	FGGGTKLEII	58
VK5	G**VP**SRFSGSGSGRQYSLKISSLHPDD	49	FGGGTKLEI**K**	59
VK7	G**VP**SRFSGSGSG**TE**Y**T**L**T**ISS**LQ**PDD	50	FGGGTKLEI**K**	60
VK8	GM**P**SRFSGSGSG**TE**Y**T**L**T**ISS**LQ**PDD	51	FGGGTKLEI**K**	61

The humanized antibody may comprise a VH region selected from the group consisting of the amino acid sequences of SEQ ID NOs: 14 to 18 and a VL region selected from the group consisting of the amino acid sequences of SEQ ID NOs: 19 to 21. Specifically, the examples of the humanized antibody include an antibody (Vk8

+VH6) comprising a VH region including the amino acid sequence of SEQ ID NO: 15 and a VL region comprising the amino acid sequence of SEQ ID NO: 21, an antibody (Vk8 + VH11) comprising a VH region including an amino acid sequence of SEQ ID NO: 18 and a VL region comprising the amino acid sequence of SEQ ID NO: 21, an antibody (Vk5+VH7) comprising a VH region including the amino acid sequence of SEQ ID NO: 16, and a VL region comprising the amino acid sequence of SEQ ID NO: 19, an antibody (Vk7 + VH6) comprising a VH region including the amino acid sequence of SEQ ID NO: 17 and a VL region comprising the amino acid sequence of SEQ ID NO: 20, an antibody (Vk7 + VH10) comprising a VH region including the amino acid sequence of SEQ ID NO: 15, and a VL region comprising the amino acid sequence of SEQ ID NO: 20, an antibody (Vk7 + VH7) comprising a VH region comprising the amino acid sequence of SEQ ID NO: 16 and a VL region comprising the amino acid sequence of SEQ ID NO: 20, an antibody (Vk7 + VH5) comprising a VH region comprising the amino acid sequence of SEQ ID NO: 14 and a VL region comprising the amino acid sequence of SEQ ID NO: 20, and an antibody (Vk8 + VH7) comprising a VH region comprising the amino acid sequence of SEQ ID NO: 16 and a VL region comprising the amino acid sequence of SEQ ID NO: 21. Specific combinations and amino acid sequences of the antibodies are shown in Table 6 below. The preferred examples of antibody include an antibody (Vk8 +VH6) comprising a VH region including the amino acid sequence of SEQ ID NO: 15 and a VL region comprising the amino acid sequence of SEQ ID NO: 21, an antibody (Vk8 + VH11) comprising a VH region including an amino acid sequence of SEQ ID NO: 18 and a VL region comprising the amino acid sequence of SEQ ID NO: 21, an antibody (Vk5+VH7) comprising a VH region including the amino acid sequence of SEQ ID NO: 16, and a VL region comprising the amino acid sequence of SEQ ID NO: 19, an antibody (Vk7 + VH6) comprising a VH region including the amino acid sequence of SEQ ID NO: 17 and a VL region comprising the amino acid sequence of SEQ ID NO: 20, and an antibody (Vk7 + VH10) comprising a VH region including the amino acid sequence of SEQ ID NO: 15, and a VL region comprising the amino acid sequence of SEQ ID NO: 20.

The anti-CD66c antibody or fragment thereof may be coupled to various labeling agents, toxins, or anti-tumor drugs. It will be apparent to those skilled in the art that the antibody of the invention can be coupled to a labeling agent, a toxin, or an anti-tumor drug by a method well known in the art. Such coupling may be chemically

conducted on the site of attachment after expression of the antibody or antigen. Alternatively, the coupling product may be engineered into the antibody or antigen of the invention at the DNA level. Subsequently, the product may be expressed in a suitable host system as described herein below, and the expressed proteins are collected and, if necessary, renatured. The coupling may be performed via a linker that has been known in the art. In particular, various linkers that release a toxin or an anti-tumor drug under acidic or reductive conditions or upon exposure to specific proteases may be used with this technology. In some embodiments, it may be desirable that the linker is attached to the labeling agent, toxin, or anti-tumor drug via spacer arms in various lengths to reduce potential steric hindrance.

An antibody to an antigen-determining region of CD66c or a fragment thereof, may be produced using a typical method with a CD66c protein, an antigen-determining region of CD66c, a portion of CD66c containing an antigen-determining region of CD66c, or a cell expressing an antigen-determining region of CD66c serving as an antigen. For example, a method for producing an anti-CD66c antibody can be achieved through a method for producing a cell line producing an anti-CD66c antibody, comprising (a) injecting and immunizing an animal with a CD66c protein, an antigen-determining region of CD66c, a portion of CD66c containing an antigen-determining region of CD66c, or a cell expressing an antigen-determining region of CD66c, (b) obtaining splenocytes producing an antibody specific for CD66c, and (c) fusing the splenocytes with myeloma cells to give hybridoma cells and selecting a hybridoma cell producing an antibody to CD66c. The antibody can be isolated by culturing the cell line in vitro or by introducing the cell line in vivo. For example, the cell line may be intraperitoneally injected into mice, followed by isolating and purifying the antibody from the ascites. Isolation and purification of monoclonal antibodies may be achieved by subjecting the culture supernatant and ascites to ion exchange chromatography (DEAE or DE52) or affinity chromatography using an anti-immunoglobulin column or protein A column.

The antigen-determining region to which the antibody of the present invention binds exhibits MDSC-specific expression. Hence, the anti-CD66c antibody can not only be effectively used to detect MDSC, but can also exert cytotoxicity only on tumor cells when it carries a toxic substance.

Another embodiment provides a use of the anti- CD66c antibody according to the present invention as a marker for detection of MDSC, or specifically a use of

detecting MDSC, diagnosing MDSC-related diseases, or providing information on diagnosis of MDSC-related diseases, using the antibody or antigen-binding fragment thereof against CD66c.

For example, it provides a composition for detection of MDSC containing a substance that interacts with the antigen-determining region of the antibody by using the antibody or antigen-binding fragment thereof against CD66c. The interacting substance includes all substances being capable of interact with the antigen-determining region CD66c, and can be at least one selected from small molecular chemicals, antibodies, antigen-binding fragments of antibodies, aptamers, and the like.

The diagnostic composition of the present invention is useful in the detection of undesired expression or over-expression of CD66c in various cells, tissues or another suitable sample, by contacting a sample with an antibody of the present invention and determining the presence of a CD66c in the sample. Accordingly, the diagnostic composition of the invention may be available for assessing the onset or status of disease, as defined herein below. In particular, MDSC being capable of expressing CD66c can be targeted with the antibody of the present invention, or a fragment or derivative thereof. The cells which have bound the antibody of the present invention might be attacked by immune system functions such as the complement system or by cell-mediated cytotoxicity, and thus reduces the number of or completely eradicating the cells showing undesired expression or over-expression of CD66c.

As a specific example, a method or a composition for diagnosis MDSC-related diseases using the antibody or antigen-binding fragment for CD66c according to the present invention is provided.

In the case of diagnosing MDSC-related diseases, for example cancer, the antibody against CD66c or antigen-binding fragment thereof according to the present invention can be used for diagnosis and treatment by targeting MDSC infiltrated around cancer tissues regardless of the expression of CEACAM 6 antigen in cancer tissues or cancer cells. The antibody against CD66c according to the present invention not only binds to CD66c expressed in solid cancer cells, but also binds to CD66c expressed in MDSC, and thus, can detect the cancer by targeting the increased state of MDSC caused by cancer, even in cancers that do not express CD66c in solid cancer cells. Specifically, in cancer tissues of lung adenocarcinoma which is CEACAM6 positive in cancer cells, and lung squamous cell carcinoma, urinary bladder cancer, and melanoma malignancy which are CEACAM6 negative in cancer cells, the result of staining the cancer tissue confirmed that CEACAM 6-positive MDSC were in the non-tumor site of the cancer

tissue (FIG. 13).

Accordingly, the patients with cancer show the increased level of MDSCs regardless of the CEACAM 6 positivity in the surface of cancer cells, and thus, as shown in the result of Example 8, MDSC infiltrated into the cancer microenvironment can be detected and confirmed. When considered together with the results of Example 5.2 showing that MDSC can be selectively dissolved, this indicates that MDSC can be used as a target for diagnosis and treatment purposes regardless of the CEACAM6 positivity on cancer cells. Although the presence or absence of CEACAM6 expression on the surface of cancer cells may vary depending on the cancer type, MDSCs are increased in most cancer types regardless of CEACAM6 expression. Thus, the anti-CD66c antibody according to the present invention can target MDSC and can be used for diagnostic and therapeutic purpose in various applications.

In another embodiment, the antibody of the present invention, or a fragment or derivative thereof is coupled to a labeling agent. Such antibodies are particularly suitable for diagnostic applications.

The composition of the invention can be administered as an active agent alone or in combination with other agents.

A still further embodiment of the present invention relates to a method for detecting MDSC, which comprises (a) reacting the anti- CD66c antibody with a sample including MDSC, and (b) determining that the sample is MDSC if the sample is positive to the antibody. The sample may include, but is not limited to, lymphoid fluid, bone marrow, blood, and blood corpuscles. When used for screening MDSC, the anti-CD66c antibody may be conjugated with a label capable of indicating antigen-antibody reactivity. The label useful for this purpose may include a radioisotope, a fluorescent, a luminescent, a chromogen, and a dye.

Also, the anti- CD66c antibody of the present invention may be provided for a kit for diagnosing MDSC-related diseases. The diagnostic kit may comprise a means for detecting an antigen-antibody reaction in addition to the anti-CD66c antibody. The detecting means may be an agent useful for performing a technique selected from the group consisting of flow cytometry, immunohistochemical staining, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), enzyme immunoassay (EIA), fluorescence immunoassay (FIA), and luminescence immunoassay (LIA).

The therapeutic effect of the solid cancer are the effects of suppressing the cancer exacerbation including not only the growth inhibition (quantitative reduction) and apoptosis effect of cancer cells (especially cancer stem cells) or cancer tissues

including the same, as well as migration, invasion, metastasis, etc. In order to maximize the effect of the antibody according to the present invention, the antibody can be treated in combination with STING agonist or 5-Fu, which can be expected to obtain a higher effect in combination treatment.

As used herein the term “subject” or “patient” refers to a mammal, including a primate such as a human, a monkey, etc., and a rodent such as a mouse, a rat, etc., that is afflicted with, or has the potential to be afflicted with MDSC-related diseases or symptom and thus which is in need of alleviation, prevention, and/or treatment of the MDSC.

The administration of the antibody or its fragment according to the present invention may be conducted in any acceptable manner. For example, a therapeutic agent including the anti-CD66c antibody as an active ingredient is administered orally or parenterally, and preferably parenterally, to a subject, e.g., a human or an animal that has MDSC-related diseases. The therapeutic agent may include a pharmaceutically acceptable excipient, and the dose of the therapeutic agent may vary depending on the condition of the patient, and may range from, for example, 3 mg to 6,000 mg per day. The therapeutic agent may take such forms as liquids, powders, emulsions, suspensions or injections, but is not limited thereto.

Further, the present invention provides a method for treating MDSC-related diseases, using at least one selected from among an antibody to an antigen-determining region of CD66c, a fragment of the antibody (F(ab')2, Fab, Fv, etc.), and a ligand to an antigen-determining region of CD66c. An antibody or a fragment thereof may be monoclonal or polyclonal, and may be derived from humans or animals. The anti-CD66c antibody or its fragment may further comprise the toxin described above. The toxin may be fused, coupled, conjugated or linked to the antibody using a well-known technique.

The pharmaceutical composition of the present invention may be administered as a single active agent or in combination with any other agents that are preferable for the treatment of the disease of interest. In addition, the antibody of the present invention may be used in conjunction with other anticancer therapies, such as chemotherapy, radiotherapy, cytotherapy, etc. The well-known various anticancer agents may be used in chemotherapy or cytotherapy.

【Effect of Invention】

The present invention provides to an immune-enhancing agent comprising an

antibody specifically binding to CD66c which is expressed in myeloid-derived suppressor cell (MDSC) or an antigen-binding fragment thereof, and a use of the immune-enhancing agent for prevention, improvement or treatment of MDSC-related diseases.

【Brief Description of Drawings】

Fig. 1 shows the result of cloning a gene of antibody from mouse 8F5 antibody and expressing it as a chimeric recombinant antibody and binding to the surface of CD66c antigen-positive A549 cells.

Figs. 2A to 2C show the results of HPLC analysis of eight kinds of recombinant humanized antibodies selected first among 96 kinds of recombinant humanized antibodies. The results are shown as left measured at OD 220 nm and right measured at OD 280 nm for each antibody. The result represent whether the aggregation of antibodies and the impurities derived from antibody such as fragments are or not.

Figs. 3a to 3e show similar degree of cell surface binding of recombinant humanized antibodies as compared to chimeric antibodies, as a result of confirming CD66c antigen positive cell surface binding of eight recombinant humanized antibodies firstly selected among 96 recombinant humanized antibodies

Figs. 4a and 4b show results of ELISA analysis for the binding ability of the five recombinant humanized antibodies selected from 96 recombinant humanized antibodies to the CD66c antigen. Fig. 4a shows the results for the CECACAM6 (CD66c) as an antigen and Fig. 4b shows the result for the CEACAM1 (CD66a) antigen.

Figs. 5a and 5b show results of evaluating the antibody stability at a severe temperature condition for five recombinant humanized antibodies selected from among 96 recombinant humanized antibodies.

Figs. 6a to 6d show the cell surface binding of CD66c antigen positive cell A549 of recombinant humanized antibodies expressed in CHO cell.

FIG. 7 is a schematic diagram for helping understanding of the MDSC analysis method and specifically a schematic diagram of the specific dot plot obtaining by designating only the monocyte and granulocyte regions excluding lymphocytes according to the size of the cells in dot-plot, selecting the groups with no or low expression of HLA-DR, and determining the group that is positive for CD11b and CD33 among the groups as MDSCs in the upper part, and the result of the positivity rate of DNP002 among the determined MDSC group in the lower part.

Fig. 8 is a representative result of analyzing the MDSC killing effect after

treatment with DNP002, showing that MDSCs was significantly reduced by afucoslyated DNP002. CD66b is expressed in granulocytic MDSC, but not monocytic MDSC, and is used for MDSC subtype classification. Most of MDSCs designated in Fig. 8 were CD66b-positive and could be classified as granulocytic MDSCs. Thus, the granulocytic MDSCs were significantly reduced by treatment with DNP002.

Fig. 9 is a result of analyzing the MDSC killing effect after treatment with DNP002 by showing a result of comparing the percentage of the decreased number of MDSCs due to the DNP002 treatment in all five patients compared to the control group, where P#1 on the horizontal axis in the graph means patient's whole blood #1, and the vertical axis represents a change in the relative MDSC viability % change.

Fig.10 shows a result of analyzing the MDSC killing effect using a flow cytometer after treating PBMCs isolated from blood of stomach cancer patient with DNP002 antibody.

Fig. 11a is a result of comparing the MDSC killing effect according to the isotype of DNP002, confirming that DNP002 in afucosylated IgG1 type induces MDSC killing in the blood most effectively.

Fig. 11b is a result of comparing the MDSC killing effect in five stomach patients according to the isotype of DNP002, by showing that DNP002 in afucosylated IgG1 type provides highest MDSC killing effect in the blood of five stomach patients from the result of comparing the percentage of the MDSC killing effect where P#1 on the horizontal axis in the graph means patient's whole blood #1, and the vertical axis represents a change in the relative MDSC viability % change.

Fig. 12a and Fig.12b are the results of the apoptosis effect analyzed under the conditions of DNP002 antibody alone, NK cells alone, and combination of DNP002 antibody and NK cells on stomach cancer cell line A549and pancreatic cancer cell line AsPC-1 which are positive for CEACAM6 as a target antigen.

Fig. 13 is a picture showing the presence of CEACAM6-positive MDSCs in non-tumor site of the cancer tissue by performing CEACAM6 immunostaining of cancer tissues of lung adenocarcinoma in which CEACAM6 is positive in cancer cells, and lung squamous cell carcinoma, urinary bladder cancer and skin cancer (Melanoma malignancy) in which CEACAM6 is negative in cancer cells themselves.

【Mode for the Invention】

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as

limiting the present invention.

Example 1: Preparation of anti-CD66c chimeric antibody

1.1. Gene sequence cloning ofanti-CD66c antibody

The 8F5 antibody gene was cloned using Mouse Ig-Primer Set (Millipore, Cat. #: 69831). The RNA isolated from the 8F5 hybridoma was PCR using the mouse Ig-primer set, inserted into a pGem-T vector (Promega, Cat. #: A3600), sequenced to confirm the DNA sequence, and the mouse antibody gene was identified through the IMGT site (www.imgt.org). The heavy chain variable region sequences and light chain variable region sequences of the analyzed 8F5 antibody are as follows.

[Table 4]

Name	sequence	SEQ ID NO
8F5-chimeric V_H-CDR1	ASGYSFTDYTMN	1
8F5-chimeric V_H-CDR2	LINPFHGGTVSNQRFKV	2
8F5-chimeric V_H-CDR3	VRGDPVRHYYALAY	3
8F5-chimeric V_L-CDR1	GASENVYGTLN	4
8F5-chimeric V_L-CDR2	GATNLAD	5
8F5-chimeric V_L-CDR3	VATYYCQNVLSAPYT	6
8F5-chimeric V_H	EVQLQQSGPELVKPGASMKISCKASGYSFTDYTMNWVKQSHGKNLEWIGLINPFHGGTVSNQRFKVKATLTVDVSSNTAYMELLSLTSDDSAVYYCVRGDPVRHYYALAYWGQGTSVTVSS	7
8F5-chimeric V_L	DIQMTQSPASLSASVGETVTITCGASENVYGTLNWYQRKQGKSPQLLIYGATNLADGMSSRFSGSGSGRQYSLKISSLHPDDVATYYCQNVLSAPYTFGGGTKLEII	8
8F5-chimeric V_H	Gaggtccagctgcaacagtctggacctgaactggtgaagcctggagcttcaatgaagatatcctgcaaggcttctggttactcattcactgactacaccatgaactgggtgaagcagagccatggaaagaaccttgagtggattggacttattaatcctttccatggtggtactgtctccaaccagaggttcaaggtcaaggccacattaactgtagacaagtcatccaacacagcctacatggagctcctcagtctgacatctgacgactctgcggtctattactgtgtaagaggtgacccggtccgccattactatgctttggcctactggggtcagggaacctcagtcaccgtctcctca	62
8F5-chimeric V_L	gacatccagatgactcagtctccagcttcactgtctgcatctgtgggagaaactgtcaccatcacatgtggagcaagtgagaatgtttacggtactttaaattggtatcagcggaaacagggaaaatctcctcagctcctgatctatggtgcaaccaacttggcagatggcatgtcatcgaggttcagtggcagtggttctggtagacagtattctc	63

1-2. Production of Chimeric antibody

Based on the amino acid sequence of the constructed anti-CD66c mouse antibody 8F5, an anti-CD66c chimeric antibody was prepared.

1-2-1. Plasmid production

For expressing the anti-CD66c chimeric antibody, a plasmid for heavy chain and a light chain expression plasmid were respectively prepared. POptiVEC (Invitrogen) vector was used as the light chain expression plasmid, and pcDNA3.3 (Invitrogen) vector was used as the heavy chain expression plasmid.

In order to express the variable region coding cDNA and the constant region coding cDNA of each antibody as a continuous amino acid sequence without additional amino acid insertion, the coding sequence of the cloned variable region and the known human IgG1 constant region (heavy chain) and the kappa constant region (light chain) coding sequences were synthesized (Bioneer). The synthesized heavy gene and light chain gene were cut with restriction enzymes Xho I and Sal I and the light chain gene fragment was ligated to the pOptiVec vector and the heavy chain gene fragment was ligated to the pcDNA3.3 vector, respectively, to construct a complete antibody expression plasmid (pcDNA3.3-anti-CD66c heavy chain expression plasmid and pOptiVEC-anti-CD66c light chain expression plasmid).

1-2-2. Transfection

The prepared pcDNA3.3-anti-CD66c heavy chain expression plasmid and pOptiVEC-anti-CD66c light chain expression plasmid were transfected into CHO cell-derived DG44 cells (Invitrogen).

Three days prior to transfection, DG44 cells in suspension were adapted to MEMS medium containing 5% FBS to convert them into adherent cells and to improve transfection efficiency. Transfection was performed on a 6-well plate using the ViaFect transfection regent (Promega, Cat. #: E4981). On the day before the transfection, DG44 cells adapted to the adhered state were prepared by subculturing at a concentration of 1 X 10^5 cells/well. The amount of DNA used for transfection was determined by using pcDNA3.3-anti-CD66c heavy chain expression plasmid and pOptiVEC-anti- CD66c light chain expression plasmids were used at an amount of 2ug and 1.5ug respectively at a ratio of 1.5: 1. Transfection was carried out for 48 hours. Flow cytometry was used to analyze the transfected cell population. As shown in Fig. 1, the expression of chimeric antibody was confirmed by A549 non-small cell lung cancer cell line. Fig. 1 shows the result of cloning an antibody gene from mouse 8F5 antibody and expressing it as a

recombinant chimeric antibody and binding to the surface of CD66c antigen-positive A549 cells.

Example 2: Preparation of Humanized Anti-CD66c Monoclonal Antibody

2.1 Selection of recombinant antibody sequence by in silico humanization

If the antigen binding affinity is equal or superior by maintaining the sequence of CDRs (CDRH1: ASGYSFTDYTMN) SEQ ID NO: 1, CDRH2: SEQ ID NO: 2 (LINPFHGGTVSNQRFKV); CDRH3: SEQ ID NO: 3 (VRGDPVRHYYALAY); CDRL1: SEQ ID NO: 4 (GASENVYGTL); CDRL2: SEQ ID NO: 5 (GATNLAD); CDR3: SEQ ID NO: 6 (VATYYCQNVLSAPYT) of the heavy chain and light chain of the mouse anti-CD66c antibody, 8F5 (heavy chain amino acid sequence: SEQ ID NO: 7, heavy chain encoding DNA: SEQ ID NO: 62; light chain amino acid sequence: SEQ ID NO: 8; light chain encoding DNA: SEQ ID NO: 63) as similar as possible the humanized antibody sequences recombined the framework region sequences with germline sequence encoding the human antibody gene were selected in silico method. The germline gene of human antibody used as a backbone of the recombinant humanized antibody sequence is most similar to the heavy chain and light chain of the mouse CD66c antibody 8F5, respectively, as shown in Table 5. The amino acid sequence and the nucleic acid sequence of the heavy chain variable region and the light chain variable region of the mouse CD66c antibody and the CDR sequences of the heavy chain variable region and the light chain variable region are shown in Table 6.

[Table 5]

Human Ab Germline	
Heavy chain	Light chain
IGHV1-69-2*01	IGKV1-27*01
IGHV1-2*02	IGKV1-5*01 Homo sapiens
IGHV1-46*01	IGKV1-39*01 Homo sapiens

Twelve (12) heavy chain variable regions and eight (8) light chain variable regions were selected as the humanized 8F5 antibody sequence selected using the human antibody germline gene sequence, as shown in Table 3. The amino acid sequences of the heavy chain variable region and the light chain variable region, CDR sequences, and the framework sequences of the selected humanized antibody are shown in Tables 6 to 8. The heavy chain variable region and the light chain variable region of the chimeric antibody and the humanized antibody are shown in Table 1. It is preferable

that the mouse antibody and the humanized antibody have the same amino acid sequences of heavy chain CDR3 and light chain CDR2. The bold and underlined parts in Table 6 are the CDR sequences of antibody. The bold and underlined parts in Table 7 indicate the modified amino acid.

[Table 6]

Antibody number	combination	name	Amino acid sequence
3043	Vk8+VH6	VH6	QVQLVQSGAEVKKPGASMKISCK**ASGYSFTDYTMN**WVKQAPGQNLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk8	DIQMTQSPSTLSASVGDRVTITC**RASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GMPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
3058	Vk8+VH11	VH11	QVQLVQSGAEVKKPGASVKISCK**ASGYSFTDYTM**HWVKQAPGQNLEWIG**LINPFGGSTSYAQKFKG**RVTMTRDTSTNTAYMELSRLRSDDTAVYYCVRG**DPVRHYYALAY**WGQGTLVTVSS
		Vk8	DIQMTQSPSTLSASVGDRVTITC**RASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GMPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
2938	Vk5+VH7	VH7	QVQLVQSGAEVKKPGASMKISCK**ASGYSFTDYTMN**WVRQAPGQGLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk5	DIQMTQSPSTLSASVGDRVTITC**GASENVYGTLA**WYQRKPGKAPKLLIY**GATNLAD**GVPSRFSGSGSGREYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
3007	Vk7+VH6	VH6	QVQLVQSGAEVKKPGASMKISCK**ASGYSFTDYTMN**WVKQAPGQNLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk7	DIQMTQSPSTLSASVGDRVTITC**GASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GVPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
3019	Vk7+VH10	VH10	QVQLVQSGAEVKKPGASVKVSCK**ASGYSFTDYTMN**WVKQAPGQNLEWIG**LINPFHGGTVSNQRFKV**KATMTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk7	DIQMTQSPSTLSASVGDRVTITC**GASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GVPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYTF**GGGTKLEIK
3010	Vk7+VH7	VH7	QVQLVQSGAEVKKPGASMKISCK**ASGYSFTDYTMN**WVRQAPGQGLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk7	DIQMTQSPSTLSASVGDRVTITC**GASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GVPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
3004	Vk7+VH5	VH5	QVQLVQSGAEVKKPGASVKISCK**ASGYSFTDYTMN**WVRQAHGQNLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk7	DIQMTQSPSTLSASVGDRVTITC**GASENVYGTLN**WYQRKPGKAPKLLIY**GATNLAD**GVPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPYT**FGGGTKLEIK
3046	Vk8+VH7	VH7	QVQLVQSGAEVKKPGASMKISCK**ASGYSFTDYTMN**WVRQAPGQGLEWIG**LINPFHGGTVSNQRFKV**KATLTVDVSTNTAYMELSRLRSDDTAVYYCVR**GDPVRHYYALAY**WGQGTLVTVSS
		Vk8	DIQMTQSPSTLSASVGDRVTITC**RASENVYGTLN**WYQRKPGKAPKLLI

			Y**GATNLAD**GMPSRFSGSGSGTEYTLTISSLQPDD**FATYYCQNVLSAPY T**FGGGTKLEIK

[Table 7]

Name	CDR1	SEQ ID NO	CDR2	SEQ ID NO	CDR3	SEQ ID NO
V_H- Chimeric	ASGYSFTDYT MN	1	LINPFHGGTVSNQRF KV	2	GDPVRHYYALA Y	3
VH5, 6, 7, 10	ASGYSFTDYT MN	1	LINPFHGGTVSNQRF KV	2	GDPVRHYYALA Y	3
VH11	ASGYSFTDYT M**H**	9	LINPF**G**GST**SYA**Q**K**F K**G**	10	GDPVRHYYALA Y	3
V_L-Chimeric	GASENVYGTL N	4	GATNLAD	5	VATYYCQNVLS APYT	6
VK5	GASENVYGTL **A**	11	GATNLAD	5	**F**ATYYCQNVLS APYT	13
VK7	GASENVYGTL N	4	GATNLAD	5	**F**ATYYCQNVLS APYT	13
VK8	**R**ASENVYGTL N	12	GATNLAD	5	**F**ATYYCQNVLS APYT	13

[Table 8]

Name	FR1	SEQ ID NO	FR2	SEQ ID NO	FR3	SEQ ID NO	FR4	SEQ ID NO
V_H-Chimeric	EVQLQQSGPEL VKPGASMKISC K	22	WVKQSHG KNLEWIG	32	NQRFKVKATLT VDVSSNTAYM ELLSLTSDDSA VYYCVR	42	WGQGTSV TVSS	52
VH5	**Q**VQL**V**QSG**A**E **VK**KPGAS**V**KIS CK	23	WV**R**Q**A**H G**Q**NLEWI G	33	NQRFKVKATLT VDVS**T**NTAYM EL**SR**L**R**SDD**T**A VYYCVR	43	WGQGT**L**V TVSS	53
VH6	**Q**VQL**V**QSG**A**E **VK**KPGASMKIS CK	24	WVKQ**AP**G **Q**NLEWIG	34	NQRFKVKATLT VDVS**T**NTAYM EL**SR**L**R**SDD**T**A VYYCVR	44	WGQGT**L**V TVSS	54
VH7	**Q**VQL**V**QSG**A**E **VK**KPGASMKIS CK	25	WV**R**Q**AP**G **QG**LEWIG	35	NQRFKVKATLT VDVS**T**NTAYM EL**SR**L**R**SDD**T**A VYYCVR	45	WGQGT**L**V TVSS	55
VH10	**Q**VQL**V**QSG**A**E **VK**KPGAS**V**K**V** SCK	26	WVKQ**AP**G **Q**NLEWIG	36	NQRFKVKAT**M** TVDVS**T**NTAY MEL**SR**L**R**SDD**T** AVYYCVR	46	WGQGT**L**V TVSS	56
VH11	**Q**VQL**V**QSG**A**E **VK**KPGAS**V**KIS CK	27	WVKQ**AP**G **Q**NLEWIG	**37**	**A**Q**K**FK**GRV**T**M** T**R**D**T**S**T**NTAY MEL**SR**L**R**SDD**T** AVYYCVR	47	WGQGT**L**V TVSS	57
V_L-	DIQMTQSPASL	28	WYQRKQG	38	GMSSRFSGSGS	48	FGGGTKL	58

Chimeric	SASVGETVTITC		KSPQLLIY		GRQYSLKISSLHPDD		EII	
VK5	DIQMTQSP**ST**LSASVG**DR**VTITC	29	WYQRK**P**GK**A**P**K**LLIY	39	G**VP**SRFSGSGSGRQYSLKISSLHPDD	49	FGGGTKLEI**K**	59
VK7	DIQMTQSP**ST**LSASVG**DR**VTITC	30	WYQRK**P**GK**A**P**K**LLIY	40	G**VP**SRFSGSGSG**TE**Y**T**L**T**ISS**L****Q**PDD	50	FGGGTKLEI**K**	60
VK8	DIQMTQSP**ST**LSASVG**DR**VTITC	31	WYQRK**P**GK**A**P**K**LLIY	41	GM**P**SRFSGSGSG**TE**Y**T**L**T**ISS**L****Q**PDD	51	FGGGTKLEI**K**	61

2.2 Expression and Analysis of Recombinant humanized antibodies

The sequences of selected antibody were expressed in 293 cells in the form of human IgG1 by connecting the human IgG1 heavy chain constant region and the kappa light chain constant region, respectively. Seven days after the transfection, the recombinant humanized antibody was purified using KanCap A resin (Kaneca).

The purified antibody was quantitated by measuring at OD 280 nm and SDS-PAGE was performed. The purity and the aggregation of the antibody were analyzed by analyzing with 280 nm and 220 nm by HPLC using Sepax Zenix-C SEC-300 size exclusion column (Sepax Technologies) (Figs. 2a to 2c)

2.3 Cell binding and antigen binding analysis of recombinant humanized antibodies

2-3-1 cell binding assay

Each expressed 96 recombinant humanized antibody was poured and reacted in a test tube containing the same amount (1 ug) of CD66c-positive A549 non-small-cell lung cancer cell line at 4℃ for 30 minutes, washed with PBS, and treated with FITC-conjugated goat anti-Huma IgG (DiNona Inc, Korea) was added and incubated at 4 °C for 15 minutes. After washing with PBS, the cells were analyzed with a flow cytometer (Stratedigm, S1000EXi) and the results are shown below.

Among the 96 recombinant humanized antibody candidates, eight were firstly selected based on the degree of expression, the presence of aggregation, and the degree of cell binding (Table 9 and Table 10, Figs. 3a to 3e). Tables 9 and 10 are the results of analysis of primary selected anti-CD66c humanized antibody and chimeric 8F5, and Table 10 shows the results of flow cytometer analysis.

[Table 9]

No.	#71	#86	#93	#43	#51	#45	#41	#74

Protein ID	3043	3058	2938	3007	3019	3010	3004	3046
H & L조합	Vk8+VH6	Vk8+VH11	Vk5+VH7	Vk7+VH6	Vk7+VH10	Vk7+VH7	Vk7+VH5	Vk8+VH7
Well	F11	H2	H9	D7	E3	D9	D5	G2
OD 280nm	3.54	3.86	3.32	4.01	4.03	3.5	3.56	3.54
Volume (ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Conc. (mg/ml)	2.52	2.71	2.36	2.85	2.86	2.49	2.53	2.52
Yield(mg)	0.66	0.71	0.61	0.74	0.74	0.65	0.66	0.66
extinction coefficient	102190	103680	102190	102190	102190	102190	102190	102190
MW(Da)	72750	72691	72616	72633	72521	72604	72669	7272

[Table 10]

No.	Protein ID	H & L combination	% gated	Mean	% (%gated x mean)
Chimeric antibody	**	**	73	619	100.0
#71	3043	Vk8+VH6	69	686	104.8
#86	3058	Vk8+VH11	72	645	102.8
#93	2938	Vk5+VH7	71	611	96.0
#43	3007	Vk7+VH6	71	565	88.8
#51	3019	Vk7+VH10	70	561	86.9
#45	3010	Vk7+VH7	70	554	85.8
#41	3004	Vk7+VH5	71	541	85.0
#74	3046	Vk8+VH7	71	523	82.2

Table 9 shows the degree of expression, the presence or absence of aggregation, and the degree of cell binding of 8 selected antibodies. Specifically, the expression levels and the molecular weights of the 8 selected antibodies were summarized. In addition, according to the results of flow cytometry in Table 10, it was confirmed that the eight recombinant humanized antibodies exhibited cell binding strengths of ± 20% which showed very similar cell binding strength to chimeric antibodies. As a result, the 8 antibody being normally expressed and having few aggregations formed due to the instability of the protein itself, and the similar binding affinity to the target antigen-

positive cells to the chimeric antibody were firstly selected from 96 humanized candidate antibodies

Some contents may be missed. In particular, the binding ability of cell lines seemed to be different in numerical values. However, as shown in Fig. 3, the actual cell binding profile was similar to that of chimeric antibody, and 8 kinds of humanized antibodies were determined by obtaining the numerical value with multiplying the antibody positivity (% gated) with the mean fluorescence (mean) and comparing it with the chimeric antibody, so as to select the humanized antibodies within ± 20%. In general, when the mouse antibody CDR region sequence is inserted into the framework region of the humanized antibody at the time of the production of the humanized antibody, the antibody binding affinity is sharply decreased due to the change of the original protein structure. In considering the general property of humanized antibody, very good humanized antibodies were be selected in the present invention.

2-3-2 Antigen binding assay

Among the eight selected recombinant humanized antibodies, five kinds of recombinant humanized antibodies exhibiting a high binding affinity as compared to that of the chimeric antibody were selected and analyzed for their binding affinity to CD66c antigen and similar CD66 antigens by ELISA, respectively.

[Table 11]

Protein ID	HC & LC combination
3043	Vk8+VH6
3058	Vk8+VH11
2938	Vk5+VH7
3007	Vk7+VH6
3019	Vk7+VH10

Antigen CD66c (CEACAM6; Sino Biological, Inc.) and CEACAM1 antigen (Sino Biological, Inc.) were coated on a 96-well plate at a rate of 100 ng per well and then blocked. The primary antibody was diluted 3-fold from 10 ug / ml and bound at 37 ° C for 1 hour. The primary antibody was diluted three times from 10ug/ml at initial concentration and was bound at 37 °C for 1 hour, and goat anti-Human Ig-HRP conjugate (Jackson ImmunoResearch) as a secondary antibody was diluted 1: 10,000 and incubated at 37 ° C for 30 minutes. The washing was carried out at three times between each step, and the TMB reaction was performed, stopped with 1N H_2SO_4

solution at the same amount of TMB solution (100ul) and then OD value was measured at 450nm.

As a result of the experiment, the binding affinities to CD66c antigen of the five kinds of recombinant humanized antibodies selected from among 96 kinds of recombinant humanized antibodies are shown in Fig. 4a, Table 12, Fig. 4b and Table 13. Fig. 4a and Table 12 show the binding capacity of the antibodies to CECACAM6 CD66c) antigen, and Fig. 4b and Table 13 are the results for the CEACAM1 (CD66a) antigen.

From the binding affinity of antibodies to the antigen in Fig. 4a, Table 12, Fig. 4b and Table 13, all antibodies to CECACAM6 showed a similar binding profile to the chimeric antibody to CEACAM6 and were divided into the groups that did not bind or bound weakly CEACAM1.

[Table 12]

Antibody concentration (ng/ml)	chi 8F5	hu 3043	hu 3058	hu 2938	hu 3007	hu 3019
10000.00	3.17	2.73	2.87	3.43	3.37	3.13
3333.33	3.41	2.95	3.18	3.27	3.24	3.26
1111.11	3.30	2.74	3.19	3.16	3.23	3.34
370.37	3.30	2.92	2.80	3.08	3.16	3.23
123.46	2.83	2.29	2.22	2.56	2.90	3.04
41.15	2.02	1.73	1.38	1.72	2.17	2.45
13.72	1.15	1.37	1.06	1.15	1.64	1.77
4.57	0.80	0.91	0.74	0.65	1.02	1.32
1.52	0.53	0.70	0.53	0.48	0.79	0.95
0.51	0.41	0.49	0.38	0.36	0.64	0.80
0.17	0.40	0.41	0.37	0.30	0.53	0.68

[Table 13]

Antibody concentration (ng/ml)	chi 8F5	hu 3043	hu 3058	hu 2938	hu 3007	hu 3019
10000.00	1.21	0.91	0.42	0.32	1.01	0.99
3333.33	1.24	1.22	0.31	0.27	1.15	1.07
1111.11	1.21	1.17	0.19	0.21	1.06	1.02
370.37	1.13	0.80	0.12	0.14	0.92	0.89
123.46	0.95	0.70	0.07	0.10	0.82	0.79

41.15	0.82	0.61	0.07	0.07	0.66	0.58
13.72	0.51	0.43	0.05	0.06	0.46	0.41
4.57	0.28	0.27	0.05	0.05	0.27	0.24
1.52	0.16	0.16	0.05	0.05	0.18	0.16
0.51	0.10	0.10	0.04	0.05	0.11	0.11
0.17	0.08	0.08	0.04	0.04	0.09	0.08

2.4 Stability analysis of recombinant humanized antibodies

The experiments were conducted to determine the stability of the antibodies by leaving the five recombinant humanized antibodies of Example 3.3 selected by the binding profile to antigen and cell under high temperature conditions.

The stability was determined by performing the binding experiments using 8-anilino-1-naphthalenesulfonic acid (ANS, Sigma). ANS is a compound that can detect the denaturation of proteins by measuring the change in fluorescence wavelength between the binding to and not binding to hydrophobic sites exposed when protein is denatured.

The recombinant humanized antibody was adjusted to a concentration of 0.2 mg /ml using PBS (phosphate buffered saline), and left at 50 °C for 4 hours as severe conditions. 0.2 μg/ml of ANS solution was mixed at 20 μl per 500 μl of the diluted solution of antibody to be analyzed, and analyzed after 5 minutes later with a fluorescent reader at 360 nm excitation and 460 nm emission conditions. In addition, the ANS reagent reaction was also measured at a temperature of 70 °C for additional 30 minutes.

Figs. 5a and 5b show the results of confirming the antibody stability of the five recombinant humanized antibodies shown in Table 11 under severe temperature conditions. That is, the reactivity of the ANS reagent was measured by fluorescence after leaving the antibody at 50 ° C for 4 hours under the sever condition, and further left at 70 °C for 30 minutes. As shown in the results of the experiment of Fig. 5a, most 5 kinds antibodies showed little ANS response when the antibodies were left at the temperature of 50 ° C for 4 hours, but the greatly increased fluorescence value of the antibodies when the antibodies were additional left at 70 °C for 30 minutes. Among them, the recombinant antibody having protein ID: 3058 exhibited the smallest increased fluorescence value, and thus showed the most stable property in the temperature change among the five recombinant humanized antibodies.

In order to measure the change rate of ANS reagent reactivity, ANS reagent

reactivity was analyzed with a fluorescent reader in the same manner as above after leaving the antibodies for 4 hours at a refrigeration condition (4 ± 2 °C.) and a temperature of 62 °C. The fluorescence value variability of the antibody against the ANS reagent can be determined by obtaining the difference between the fluorescence value measured at low temperature conditions (e.g., 4 °C.) and the fluorescence value measured at high temperature conditions (e.g., 62 °C.) and dividing with the fluorescence value measured at low temperature conditions.

[Mathematical Equation]

Fluorescence value variability = (fluorescence value measured at high temperature condition - fluorescence value measured at low temperature condition) / (fluorescence value measured at low temperature condition)

As shown in Fig. 5b, the reaction was allowed for 4 hours at a temperature of 62 ° C which was somewhat increased from temperature (50 ° C), and then the ANS reagent reactivity was confirmed. Five recombinant humanized antibodies and the chimeric antibodies showed little ANS response under refrigerated conditions, but increased with increasing temperature. However, Chimeric 8F5 antibody showed the reactivity of ANS reagent increased to 1,406% by keeping the temperature condition at 62 ℃ but the precipitates occurred to be unstable. However, the five humanized antibodies showed significantly lower variability of ANS reagent reactivity than chimeric antibodies, and no precipitates were produced. In particular, the humanized antibodies Protein ID 3019 and Protein ID 3058 had variability of ANS reagent reactivity of 114% and 133%, respectively, and thus were considered as most stable antibodies. The meaning of increased ANS reagent reactivity refers to the increased exposure of the hydrophobic amino acid placed inside the protein structure, which is responsible for the denaturation of the protein structure and the resulting protein aggregate, i.e., precipitate formation. The humanized antibody according to the present invention is considered to be stable antibody having the ANS reactivity variation of less than 200%. The ANS reactivity variation of less a change of less than 200% is considered to be very low, and over the value, a more significant change in protein structure is considered to be the observation of ANS reactivity. Accordingly, the humanized antibody according to the present invention has similar antigen binding and cell binding ability to chimeric antibody, and the increased physical stability of the antibody protein itself, such facts are a very excellent feature in the drugability for the therapeutic antibody.

2.5 CHO cell expression and analysis of recombinant humanized antibody

The five recombinant humanized antibodies selected in Example 2.3 were expressed in CHO cells used for expressing most therapeutic antibodies and analyzed. The light chain variable region DNA sequence and heavy chain variable region DNA sequence to construct the selected five recombinant humanized antibodies were performed by the codon optimization, synthesized, and ligated with the human IgG1 constant region gene by overlay PCR method. The product was cut with XhoI and EcoRI and ligated into the pcDNA3.4 vector (Life Technology). Table 11 shows the light chain and heavy chain combinations of humanized antibodies selected for CHO cell expression.

The DNA primer sequences used for PCR on the variable region and constant region gene are shown in Table 14 below.

[Table 14]

Primer name	use	Target gene	sequences	SEQ ID NO
8F01	1st frag forward	VH6, VH7, VH10, VH11	ATTACTCGAGGCCACC ATGAA	64
8F02	1st frag reverse	VH6, VH10, VH11	AGTTGAAGCGCTGCTC ACAGTCA	65
8F03	2nd frag forward	VH6, VH10, VH11	GTGAGCAGCGCTTCAA CTAAGGG	66
3E04	2nd frag reverse	VH6, VH7, VH10, VH11	AGTCGAATTCTCATTT CCCAGGAGAG	67
8F04	1st frag reverse	VH7	AGTTGAAGCAGAAGAC ACTGTCA	68
8F05	2nd frag forward	VH7	GTGTCTTCTGCTTCAAC TAAGGG	69
3E01	1st frag forward	Vk5, Vk7, Vk8	ATTACTCGAGGCCACC ATGAAGTGGG	70
8F06	1st frag reverse	Vk5, Vk7, Vk8	AACAGTCCGCTTGATC TCCAGCT	71
3EL02(2)	2nd frag forward	Vk5, Vk7, Vk8	GAGATCAAGCGGACTG TTGCTGC	72
3E08	2nd frag reverse	Vk5, Vk7, Vk8	ATTAGAATTCTCAGCA CTCGCCGCGG	73

The five (5) recombinant humanized antibodies were transiently transfected using the ExpiCHO (trademark) Expression System Kit (ThermoFisher, Cat. No. A29133), and the expressed antibodies were transfected with CD66c-positive A549 non-small lung cancer cell line and analyzed by flow cytometer, as shown in Fig. 6a to

6c. All five recombinant humanized antibodies showed similar binding affinities to chimeric antibodies. The measured fluorescence value of the flow cytometer was divided by the antibody expression amount of the CHO culture medium to obtain the relative the binding affinity of antibody to the antibody expression amount, as shown in Fig. 6d. Therefore, it was confirmed that the recombinant humanized antibody was properly expressed in CHO cells. The binding affinity of the antibody to the cell surface was determined as 100%, and the relative change was shown in Fig. 6d.

<Example 3> CHO cell expression and analysis of DNP002

DNP002, a humanized antibody against anti-CD66c was expressed and analyzed in CHO cells used to express most of the therapeutic. In order to test the difference in function according to the subtype of the DNP002 antibody, IgG1 type and IgG2 type antibodies were prepared.

After performing the codon optimization of DNA sequences of the light and heavy chain variable region for constructing humanized recombinant antibodies, they were synthesized and linked with constant region o human IgG1 or IgG2 by overlay PCR method, and XhoI and EcoRI gene fragment was cloned into pcDNA3.4 vector (Life Technology).

【Table 15】

classification	Amino acid sequence	cDNA sequence
Constant region of IgG1 heavy chain	ASTKGPSVFPLAPSSK	GCTTCAACTAAGGGACCAAGCGTATTCCCACTTGCTCCAT
	STSGGTAALGCLVKD	CTAGCAAGAGCACTAGCGGAGGAACAGCTGCTTTGGGGT
	YFPEPVTVSWNSGAL	GTTTGGTAAAGGATTACTTTCCCGAACCTGTTACCGTGAG
	TSGVHTFPAVLQSSGL	CTGGAACAGCGGGGCTTTGACAAGTGGCGTTCATACATTT
	YSLSSVVTVPSSSLGT	CCTGCCGTTTTGCAAAGCAGCGGCTTGTATAGCTTGAGCT
	QTYICNVNHKPSNTK	CTGTTGTTACCGTTCCAAGCTCATCTCTGGGCACACAAAC
	VDKKVEPKSCDKTHT	ATACATCTGCAACGTGAACCACAAGCCCTCAAACACCAA
	CPPCPAPELLGGPSVF	GGTGGACAAGAAGGTGGAGCCAAAGTCTTGCGACAAGAC
	LFPPKPKDTLMISRTP	CCACACCTGTCCACCTTGTCCAGCCCCTGAACTCCTGGGG
	EVTCVVVDVSHEDPE	GGCCCTTCAGTTTTTCTCTTTCCTCCTAAACCTAAAGATAC
	VKFNWYVDGVEVHN	ACTCATGATCAGTCGGACCCCTGAAGTTACCTGTGTGGTG
	AKTKPREEQYNSTYR	GTCGATGTGTCTCATGAAGATCCTGAAGTCAAGTTTAACT
	VVSVLTVLHQDWLN	GGTATGTGGACGGCGTGGAGGTGCATAATGCCAAGACCA
	GKEYKCKVSNKALPA	AGCCTCGGGAGGAGCAATATAATTCTACCTATCGCGTCGT
	PIEKTISKAKGQPREP	CTCTGTCCTCACCGTCCTGCATCAGGACTGGCTGAATGGC
	QVYTLPPSRDELTKN	AAAGAGTATAAGTGCAAAGTCAGTAACAAAGCCCTCCCC

	QVSLTCLVKGFYPSDI AVEWESNGQPENNY KTTPPVLDSDGSFFLY SKLTVDKSRWQQGN VFSCSVMHEALHNHY TQKSLSLSPGK **(SEQ ID NO: 74)**	GCCCCCATAGAGAAAACCATTAGTAAAGCCAAAGGGCAG CCCCGCGAGCCCCAGGTCTATACACTGCCCCCCAGTAGA GACGAGCTGACAAAGAATCAGGTGTCTCTGACATGCCTG GTGAAAGGCTTTTATCCCTCTGACATTGCCGTCGAGTGGG AGTCTAATGGGCAGCCCGAGAATAATTATAAGACAACAC CCCCCGTGCTGGACAGTGACGGCTCATTTTTCCTGTATTC AAAACTGACAGTGGACAAAAGTCGGTGGCAGCAGGGGA ATGTGTTTTCATGCAGTGTCATGCACGAGGCCCTCCACAA TCACTATACCCAGAAATCTCTGAGTCTCTCTCCTGGGAAA TGA **(SEQ ID NO: 75)**
Constant region of IgG2 heavy chain	ASTKGPSVFPLAPCSR STSESTAALGCLVKD YFPEPVTVSWNSGAL TSGVHTFPAVLQSSGL YSLSSVVTVPSSNFGT QTYTCNVDHKPSNTK VDKTVERKCCVECPP CPAPPVAGPSVFLFPP KPKDTLMISRTPEVTC VVVDVSHEDPEVQFN WYVDGVEVHNAKTK PREEQFNSTFRVVSVL TVVHQDWLNGKEYK CKVSNKGLPAPIEKTI SKTKGQPREPQVYTL PPSREEMTKNQVSLT CLVKGFYPSDIAVEW ESNGQPENNYKTTPP MLDSDGSFFLYSKLT VDKSRWQQGNVFSCS VMHEALHNHYTQKS LSLSPGK **(SEQ ID NO: 76)**	GCTTCCACCAAGGGCCCATCCGTGTTCCCTCTGGCCCCAT GTTCTAGGTCTACATCTGAGAGCACCGCCGCCCTCGGCTG TCTGGTGAAGGATTATTTCCCCGAGCCCGTGACCGTGTCT TGGAACAGCGGAGCCCTGACTAGCGGAGTGCACACCTTC CCAGCTGTGCTGCAGAGCTCCGGCCTGTACAGCCTCTCTT CTGTGGTGACCGTGCCCTCTAGCAACTTCGGAACACAGA CCTACACATGTAACGTGGATCACAAGCCTTCCAACACCA AGGTGGATAAGACCGTGGAGAGAAAGTGCTGTGTGGAGT GCCCTCCATGTCCTGCCCCACCTGTGGCTGGACCTTCTGT GTTTCTGTTCCCTCCAAAGCCAAAGGATACCCTGATGATC AGCAGAACTCCTGAGGTGACCTGTGTGGTGGTGGACGTG AGCCACGAGGATCCTGAGGTGCAGTTTAACTGGTACGTG GATGGCGTGGAGGTGCATAACGCTAAGACAAAGCCTAGG GAGGAGCAGTTTAACAGCACCTTCAGAGTGGTGAGCGTG CTGACCGTGGTGCACCAGGATTGGCTGAACGGCAAGGAG TATAAGTGTAAGGTGTCTAACAAGGGCCTGCCAGCCCCT ATTGAGAAGACCATCAGTAAGACCAAGGGACAGCCTAGG GAGCCTCAGGTGTACACCCTGCCTCCTTCCAGAGAGGAG ATGACAAAGAACCAGGTGAGCCTGACCTGTCTGGTGAAG GGCTTCTACCCTAGCGATATCGCCGTGGAGTGGGAGAGC AACGGCCAGCCTGAGAACAACTACAAGACCACCCCACCT ATGCTGGACAGCGATGGCTCTTTCTTCCTGTACTCTAAGC TGACCGTGGACAAGAGCAGATGGCAGCAGGGCAACGTGT TTTCTTGTTCTGTGATGCACGAGGCCCTGCACAACCACTA CACCCAGAAGTCTCTGTCTCTGTCTCCAGGCAAGTGA **(SEQ ID NO: 77)**
Constant region of light chain	RTVAAPSVFIFPPSDE QLKSGTASVVCLLNN FYPREAKVQWKVDN ALQSGNSQESVTEQD SKDSTYSLSSTLTLSK	CGGACTGTTGCTGCTCCATCTGTTTTTATATTTCCTCCCAG CGACGAGCAGCTGAAAAGCGGCACTGCCTCTGTGGTGTG TCTGCTGAATAATTTTTACCCCCGGGAAGCCAAAGTCCAG TGGAAGGTGGATAATGCCCTCCAGTCTGGGAACAGTCAG GAAAGTGTGACAGAACAGGATAGTAAGGACTCTACTTAT

	ADYEKHKVYACEVT HQGLSSPVTKSFNRGE C **(SEQ ID NO: 78)**	AGCCTCTCTTCTACACTGACTCTGTCAAAGGCCGACTATG AGAAGCATAAAGTGTATGCCTGCGAGGTGACACATCAGG GCCTGAGTTCACCCGTGACAAAATCTTTTAACCGCGGCGA GTGCTGA **(SEQ ID NO: 79)**

To prepare afucosylated DNP002 humanized antibody, when the DNP002 IgG1 type antibody was expressed, 2F-PF (2F-Peracetyl-Fucose; Merck, Cat#: 344827) was added to the culture medium at 50 uM and cultured, and then purified using Mabselect sure Protein A column (GE Healthcare Lifescience, Cat#:11003494). The purified antibody was dialyzed with phosphate buffered saline, and the absorbance at 280 nm was divided by the absorbance coefficient of 1.4 and converted into a concentration unit of "mg/mL", and then, it was used for the subsequent experiment.

Afucosylation was evaluated by relatively comparing the reactivity of Biotinylated Lens culinaris agglutinin (Vector laboratories, Cat#:B-1045) having a binding property to fucose. Fucose-conjugated IgG1 DNP002 reacted with Biotinylated Lens culinaris agglutinin and were used for TMB color development by SA-HRP (Jackson immunoresearch, Cat#:016-030-084). However, afucosylated DNP002 had relatively little color development (Table 16).

[Table 16]

Antibody concentration (ng/ml)	DNP002 humanized antibody	afucosylated DNP002 humanized antibody
10000	1.452	0.229
3333	1.254	0.210
1111	0.993	0.164
370	0.283	0.115
123	0.116	0.076
41	0.080	0.083
14	0.095	0.059
5	0.094	0.070

<Example 4> Investigation of reactivity of DNP002 antibody to MDSC

The reactivity of DNP002 antibody against MDSC was evaluated by flow cytometric analysis.

Specifically, after preparing blood from stomach cancer patients, DNP002 with bound with APC and the antibodies (anti-HLA-DR-FITC, CD11b-PE, CD33-PE antibodies) against the labeled antigens of MDSC with different fluorescence together

were added to 100 uL of whole blood and reacted at 4°C for 20 minutes. The product was added with 5 ml of red blood cell (RBC) lysis buffer of 1X RBC Lysis Buffer (ThermoFisher, Cat#:00-4333-57), reacted at room temperature for 30 minutes, centrifuged to remove the decomposed RBC, washed again with PBS, and performed by flow cytometry. The staining intensity was measured as a log of fluorescence intensity and expressed in units of tens.

In the analysis of the results, after only monocytes and granulocyte regions except lymphocyte were designated according to the cell size in the dot plot, the groups with no or low HLA-DR expression were selected, and the groups being positive for CD11b and CD33 were selected from that groups and designated as MDSC. The positive rate of DNP002 in the designated MDSC group was confirmed (Fig. 7).

Specifically, from left to right direction in the upper graphs in FIG. 7, 1) gated only monocytes and granulocytes in FSC and SSC dot plots with excluding lymphocytes. (FSC: forward scatter, a variable indicating the size of cells to be analyzed, SSC: side scatter, a variable indicating the granularity of the cells to be analyzed and the degree of granule existing in the cells), 2) gated HLA-DR Low or (-) groups, and 3) MDSC being positive to CD11b and CD33among the 1st and 2nd groups. DNP002 positive MDSCs are 90.9% (upper right site in the right dot plot of Fig. 7), and DNP002 negative MDSCs are 4.4% (upper left site in the right dot plot of Fig. 7). MDSC is divided into subtypes of a monocytic MDCS and a granulocytic MDSC. The granulocytic MDSC expresses CD66b, but the monocytic MDCS does not express CD66b, so whether or not CD66b is expressed can be usefully used for discriminating MDSC subtypes.

Fig. 7 is a result of proving the method of defining MDSC (excluding lymphocytes, HLA-DR low/(-), CD11b+, CD33+) and the reactivity of DNP002 (anti-CD66c) specific to MDSC. Because DNP002 binds to MDSC, DNP002 can be used as a method for specifying MDSC (Fig. 7), and DNP002 can cause ADCC effects to remove MDSC (Fig. 8). In the following test results, the MDSC bound with DNP002 was 90.9%.

Fig. 8 shows a phenomenon in which CD66b-positive granulocytic MDSCs were killed by DNP002 treatment and descried in their ratio. In Fig. 8, the upper dot plot (control group) represents a sample before treatment with DNP002, and the bottom dot plot (DNP002) is the result of decreased MDSC after treatment with DNP002. CD66b is a marker that can differentiate between monocytic MDSC and granulocytic MDSC. In the following test results, most of the MDSCs were CD66b-positive

granulocytic MDSCs, and granulocytic MDSCs were effectively killed by DNP002.

As a result of analyzing the blood of 19 stomach cancer patients, the positive rate of DNP002 on MDSC in all PBMCs was 34.3 ~ 76.7%, and the average positive rate was 55.1%. Table 17 below is an analysis result of the reactivity of DNP002 antibody to MDSC with samples of 19 stomach cancer patients.

[Table 17]

Blood #	**DNP002^{+}MDSC of total PBMC (%)**	
GC patient #1	49.8	55.1
GC patient #2	50.5	
GC patient #3	68.6	
GC patient #4	61.8	
GC patient #5	48.8	
GC patient #6	70.5	
GC patient #7	45.6	
GC patient #8	36.0	
GC patient #9	51.4	
GC patient #10	34.3	
GC patient #11	49.8	
GC patient #12	53.5	
GC patient #13	63.7	
GC patient #14	72.0	
GC patient #15	43.5	
GC patient #16	67.4	
GC patient #17	76.7	
GC patient #18	42.5	
GC patient #19	60.4	

<Example 5> lysis effect of DNP002 on MDSC

5.1. Lysis effect of DNP002 on MDSC in whole blood

In order to check the MDSC killing effect by DNP002, erythrocyte lysis buffer of 1X RBC Lysis Buffer (ThermoFisher, Cat#:00-4333-57) was added to the blood of 5 stomach cancer patients to dissolve red blood cells, and then the product was poured 1 X 10^5 per a well of 12-well plate. DNP002 antibody was added to each well at a concentration of 10 ug/mL and incubated in an incubator at 37°C for one day. After incubation, the cells were washed with PBS, and reacted with antibodies (anti-HLA-DR, CD11b, CD33 antibodies) against the MDSC-labeled antigens with different

fluorescence at 4°C for 20 minutes. After washing with PBS, the flow cytometry was performed. The staining intensity was measured as a log of fluorescence intensity and expressed in units of tens.

As a result of the above experiment, FIG. 8 shows a representative result in which the DNP002 antibody effectively induces apoptosis of MDSC in the blood, and the MDSC ratio is significantly reduced compared to pre-treatment with the DNP002 antibody. Fig. 9 is a diagram illustrating the same test as in FIG. 8 for the blood samples of five (5) stomach cancer patients. The open bar means the ratio of MDSC before DNP002 treatment, and the closed bar means the relative ratio of MDSC after DNP002 treatment. After DNP002 treatment, it can be seen that the MDSC ratio has been significantly reduced in all 5 patient samples.

5.2. Lysis effect of DNP002 on MDSC in PBMC

In order to more clearly clarify the MDSC-targeted killing ability of the DNP002 antibody, MDSC was only obtained with excluding mature neutrophils, and the MDSC killing effect of the DNP002 antibody was tested without the effect of neutrophils.

Specifically, only the PBMC layer containing MDSC was separated from the blood of two stomach cancer patients using Ficoll-Paque PLUS (Ge healthcare, Cat#:17-1440-02) solution. The density gradient separation of blood cells through the Ficoll solution effectively excludes mature neutrophils, so that MDSC killing effects can be analyzed more accurately. The prepared PBMC were dispensed at 1×10^5 per well into a 12 well plate, and DNP002 antibody was added to each well at a concentration of 10μg/mL, followed by incubation at 37°C for 48 hours. At this time, the MDSC killing ability was compared using Nivolumab (Bristol-Myers Squibb) of an antibody against PD-1, as a control. After culture, the cells were washed with PBS, and the increase or decrease of the MDSC group was analyzed through flow cytometry (FIG. 10).

As a result of the flow cytometry, the group treated with DNP002 showed an average of about 49% apoptosis compared to the group with no treatment of DNP002. On the other hand, Nivolumab used as a control showed only about 24% MDSC killing effect. The MDSC killing effect was the same in both MDSCs isolated from two patients. Therefore, this experiment confirms that the effect of killing MDSC by the DNP002 antibody is significant.

The lysis effect of MDSC by DNP002 was confirmed for whole blood (Example 5.1) and PBMC (peripheral blood mononuclear cells; Example 5.2),

respectively. In whole blood and PBMC, NK cells of the patient who can induce ADCC are included, and can lyse CEACAM6-positive cells with ADCC via DNP002. However, in whole blood, neutrophils positive for the CEACAM6 target antigen and MDSC are mixed, and it is difficult to say that only MDSs are selectively lysed. In order to clarify the selective lysis of MDSC by DNP002, an additional experiment was performed on PBMCs in which neutrophils are removed by layer separation with centrifugation (Example 5.2). Accordingly, it was confirmed that the MDSC lysis by DNP002 was evident.

<Example 6> Lysis effect of different antibody isotypes on MDSC

In order to test the ability of the DNP002 antibody to kill the MDSC target, three types of DNP002 antibodies were prepared. The antibodies differ in the affinity to FcrRIII (CD16) expressed on NK cells depending on the isotypes of antibodies, and antibody-dependent cell-mediated cytotoxicity (ADCC) increases in proportional to the affinity. The IgG2 isotype had a very low affinity to FcrRIII and did not have ADCC efficacy, whereas the IgG1 isotype had high affinity for FcrRIII and had excellent ADCC efficacy. It has been reported that the ADCC efficacy of an antibody depends not only on the isotype but also on the sugar chain structure linked to the 297th asparagine of the antibody. In particular, when there is no fucose in the sugar chain, ADCC efficacy increases (Shitara K., et al, J Immunol Methods. 2005 Nov 30;306(1-2) IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides).

In vitro tests were performed to test the ability to kill MDSC targets depending on the isotype and the afucosylation of the DNP002 antibody. RBC lysis buffer of 1X RBC Lysis Buffer (ThermoFisher, Cat#:00-4333-57) was added to the blood of five stomach cancer patients to lyse RBC, and then the product was poured at 1 X 10^5 per well into a 12-well plate. Three kinds of antibodies such as DNP002 IgG1 type, DNP002 IgG2 type, and aflucosylated IgG1 type were added to each well at a concentration of 10 ug/mL and incubated in an incubator at 37°C for one day. After incubation, the cells were washed with PBS, and reacted with antibodies (anti-HLA-DR, CD11b, CD33 antibodies) against the MDSC-labeled antigens with different fluorescence at 4°C for 20 minutes. After washing with PBS, the flow cytometry was performed. The staining intensity was measured as a log of fluorescence intensity and expressed in units of tens.

It was observed that the MDSC killing effect was increased in the order of IgG2,

IgG1, and afucosylated IgG1 type in all five stomach cancer patients as test subjects (FIGS. 11a and 11b). The difference in MDSC killing effect according to the isotype and the afucosylation degree (the fucose content is less than 10%) is considered by the difference in the affinity of the antibodies to FcrRIII, and the killing effect of MDSC can be understood as the ADCC effect by NK cells.

As shown in FIG. 11a, it is a representative result confirming the DNP002 formulation type capable of effectively removing MDSC. Compared to the upper part (control), a large portion of MDSC remained in DNP002 IgG2, but the MDSC ratio was significantly reduced in DNP002 IgG1. This test confirmed that the killing effect of MDSC by DNP002 IgG1 was significant high, but that by DNP002 IgG2 was not significant. Since the ADCC effect of the IgG2 isotype was absent or very low compared to the IgG1 isotype, the MDSC killing effect by DNP002 IgG1 was inferred by ADCC.

As shown in FIG. 11b, the same test as in FIG. 9 is tested and plotted in blood samples of 5 stomach cancer patients, and the horizontal axis of the graph represents each of the 5 stomach cancer patients (P#1, P#2, P#3. , P#4, P#5). The MDSC killing effects were observed in the order of Control, IgG2, IgG1, and IgG1-afucosylated. The IgG2 isotype has no or insignificant MDSC killing effect, because it does not have ADCC function, but the MDSC killing effect is excellent in IgG1 having ADCC function and IgG1-afucosylated having enhanced ADCC function by defucosylation.

< Example 7> Cancer cell killing effect by combined use of DNP002 and natural killer cells

In vitro tests were performed to test the combined effect of the DNP002 antibody and natural killer (NK) cells. After separating PBMC from three normal blood using Ficoll-Paque PLUS (Ge healthcare, Cat#:17-1440-02) solution, only CD56-positive natural killer cells were isolated by using CD56 micro bead (Miltenyi Biotec, Cat#:130-050-401). In FIGs. 12a and 12b, the horizontal axis represents three blood donors, which are the origins of the natural killer cells, respectively.

The stomach cancer cell line A549 and the pancreatic cancer cell line AsPC-1 which were positive for CEACAM6 of a target antigen of DNP002, were dispensed in a 96-well plate at $1x10^4$ per well, dispensed with the previously isolated natural killer cells at $2x10^5$ per well, treated withDNP002 antibody at 10μg/mL, and then was incubated at 37°C for 6 hours.

As a result of measuring cell viability using the EZ-cytox enhanced cell

viability kit (Daeil Lab), it was confirmed that the apoptosis effect by the combination of DNP002 antibody and natural killer cells in both cancer cell lines increased compared to single treatment (Fig. 12a and Fig. 12b).

This indicates that the cancer cell killing ability of DNP002 was significantly amplified by the combination with natural killing cells. Through this, it is indicated that combination treatment with NK cells or NK cell therapeutic agents can be excellent for effective removal of CEACAM6-positive MDSCs as well as CEACAM6-positive cancer cells.

The selective lysis of MDSC by DNP002 in Example 5.2 and the combined effect with NK cells or NK cell therapeutic agents in Example 7 confirm that both CEACAM6-positive cancer cells and CEACAM6-positive MDSCs can be eliminated as the targets. Although Example 5.2 and Example 7 showed ADCC for different target cells as MDSC and cancer cells, respectively, in the case of cancer patients in which two types of cells are actually increased together, DNP002 can simultaneously remove both types of targets, and can be used in combination with NK cell therapeutic agent, in order to double the efficacy of simultaneous removal of cancer cells and MDSCs targets.

<Example 8> Detection of MDSC in cancer microenvironment of CEACAM6-negative patient

Since CEACAM6 antigen is expressed not only in cancer cells but also in MDSC, it is possible to detect not only cancer cells but also MDSC using the DNP002 antibody. To test this, MDSC but not cancer cells were detected in cancer patient tissues with positive or negative CEACAM6 antigens, by immunohistochemistry (Immunohistochemistry).

Immunohistochemical staining was performed in the following manner. The tissue was deparaffinized in xylene for 10 minutes at 3 times, 100% alcohol for 10 minutes at 2 times, 80% alcohol for 5 minutes, and 70% alcohol for 3 minutes, and then washed with 3rd distilled water. Then, Peroxidase blocking was carried out by immersing in 0.03% H2O2 for 10 minutes at room temperature, and washed with 3rd distilled water. Immediately, the slide was put in 1X citrate buffer (Citrate buffer, pH 6.0), heated in a boiling tap water for 20 minutes, cool it slowly, wash it with 3rd distilled water, and washed it once again with 1X PBS. The monoclonal antibody of DNP002, 8F5 antibody was reacted on the region of tissue at room temperature for 90 ± 5 minutes at 150 ul (10 ug/ml) per slide. After the reaction, the slide was washed with 1X PBS for 5 minutes each at 4 times. The secondary antibody was reacted with 100 ul

per slide for 20 minutes at room temperature, and after the reaction, washed 4 times with 1X PBST for 5 minutes each. DAB Chromogen developed 100 ul per slide at room temperature for 3 minutes and the slide was washed with tap water for 10 minutes. Mayer's Hematoxylin was counter-stained at room temperature for 3 minutes at 100 ul per slide and washed under running tap water for 10 minutes. After dehydration, the slide was mounted.

As results of CEACAM6 immunostaining on lung adenocarcinoma which was CEACAM6 positive in cancer cells, and lung squamous cell carcinoma, urinary bladder cancer, and melanoma malignancy which were CEACAM6 negative in cancer cells themselves, it was confirmed that there was CEACAM6-positive MDSC in the non-tumor site of the cancer tissue (Fig. 13). This indicates that, regardless of the expression of CEACAM6 antigen in cancer tissues or cancer cells themselves, MDSCs infiltrated around cancer tissues can be used as a target for diagnosis and treatment.

Regardless of the degree of CEACAM6 on the cell surface of cancer cells, MDSC tended to increase in cancer patients, which could detect and confirm MDSCs infiltrating the tumor microenvironment as in Example 8. This indicates that MDSC can be used as a target for diagnosis and treatment purposes regardless of the positivity of CEACAM6 on cancer cells, when considered together with the result of Example 5.2 showing the selective lysis of MDSC. The presence or absence of CEACAM6 expression on the cell surface of cancer cells may differ depending on the cancer type, but regardless of this, MDSC is increased in most cancer types. Accordingly, it indicates that DNP002 can be used for diagnostic and therapeutic purposes in a most cancers by targeting MDSC.