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DESCRIPTION

TECHNICAL FIELD

[0001] The invention relates to the field of antibody engineering. In particular it relates to the field of therapeutic (human) antibodies for the treatment of diseases involving aberrant cells. More in particular it relates to bispecific antibodies for the treatment of tumors.

BACKGROUND OF THE INVENTION

[0002] In laboratories, bispecific antibodies have been widely used for the retargeting of immune effector cells to tumor cells. In this case, one binding site is directed against a tumor-associated antigen (TAA) and the second antigen against a trigger molecule on the effector cells, such as for example CD3 on T cells (Kontermann, MABS 2012 (4) 182-197; Chames and Baty, MABS 2009 (1) 539-547; Moore et al. Blood 2011 (117) 4542-4551). The first bispecific antibodies targeting CD3 and a tumor cell associated antigen were of rodent nature and were produced using hybrid hybridomas (Liu et al. 1985 PNAS 82: 8648, Staerz et al. 1986 PNAS 83:1453, Lanzavecchia et al. 1987, Eur.J.Imm. 17:105). In these hybrid hybridomas the reassortment of Ig heavy and light chains resulted in the production of bispecific functional antibody molecules within a much larger pool of monospecific and non-functional bispecific antibodies resulting from heavy and light chain mispairing. Because of their double specificity, these functional bispecific antibodies were able to bridge murine and human cytotoxic T lymphocytes (CTL) to target cells and trigger cytotoxic function resulting in the lysis of tumor cells displaying the relevant antigen. However, the CD3xTAA bispecific IgG mediated induction of tumor cell lysis by polyclonal resting human T cells could not be achieved unless co-stimulation was provided by added exogenous IL-2 or anti-CD28 mAb. This is exemplified by the hybrid rat IgG2b/mouse IgG1 CD3xCD19 bispecific molecule that was able to induce lysis of the CD19 positive REH B-ALL tumor cell line by resting human T lymphocytes only upon co-administration of IL-2 (Haagen et al. 1995 Blood 85:3208). Zeidler et al. demonstrated using a similar rat IgG2b/mouse IgG2a CD3xEpcam molecule that bispecific IgG-induced lysis of Epcam-positive tumor cells could be achieved in mixed cell cultures comprising both peripheral blood mononuclear cells (PBMC) and tumor cells without addition of exogenous IL2 (Zeidler et al. 1999 J. Immunol. 163:1246). The authors claimed that the 'third' arm of the antibody, the Fc region, is causing this effect through interaction with Fcγ receptor-positive accessory cells present within the PBMC fraction. In particular, the strong activation potential was correlated to the hybrid subclass combination mouse IgG2a/rat IgG2b that, in contrast to other reported combinations (e.g., mouse IgG2a/mouse IgG1 or rat IgG2b/mouse IgG1), not only binds but also activates Fcγ receptor-positive accessory cells. This so-called triomAb CD3xEpcam bispecific antibody, also known as catumaxomab, has been developed clinically and has been registered in Europe for palliative treatment of abdominal tumors of epithelial origin. While this bispecific antibody has clearly demonstrated clinical efficacy, its rodent nature induces antiproduct immune responses upon repetitive dosing and therefore prevents a widespread application of this format.

[0003] Alternative CD3xTAA formats have been explored to solve both the manufacturing issues and the immunogenicity problems associated with the hybrid rodent triomAb format. Such formats are often immunoglobulin-like molecules that deviate from full length human IgG molecules, and include molecules such as Dual-Affinity Re-Targeting (DART™) molecules that are developed by Macrogenics worldwide web at macrogenics.com/Platforms-DART.html, Bispecific T cell Engager (BiTE®) molecules that were developed by Micromet, now Amgen (Sheridan C, Nat Biotechnol. 2012 (30):300-1), Dual Variable Domain -immunoglobulin (DVD-Ig™) molecules that are developed by Abbott, and TandAb® RECRUIT molecules that are developed by Affimed world wide web at affimed.com/tandab-recruit. It was demonstrated for one of these formats that successful retargeting of peripheral blood lymphocytes to lyse CD19-positive tumor cells using a CD3xCD19 diabody required pre-activation of the peripheral blood T lymphocytes, now using anti-CD3 antibody plus human IL-2 (Kipriyanov et al. 1998 Int.J.Can. 77:763). Other formats, such as the bivalent single chain Fv CD3xTAA BiTE® format (Loffler et al. 2000 Blood 95:2098) do not require pre-activation of resting T cells and is able to induce antigen positive tumor cell lysis in vitro in an extremely efficient manner (Dreier et al. 2002 Int.J.Canc. 100:690). Additional studies using BiTE®s targeting different TAAs revealed that the potent efficacy of the BiTE® format was correlated to the antigen size and particularly to the distance of the epitope on the TAA to the tumor cell membrane (Bluemel et al. 2010 Cancer Immunol. Immunother. 59:1197). The effective formation of cytolytic T cell synapses was demonstrated for BiTE® molecules which is explained to form the structural basis for their potency (Offner et al. Molecular Immunology 2006 (43) 763-771) which is also believed to be linked to the small size of the BiTE® format. If size matters, this would suggest that larger molecules such as intact IgG would be too large to form effective cytolytic synapses. The CD3xCD19 BiTE®, blinatumomab, has demonstrated remarkable clinical efficacy in refractory non-Hodgkin lymphoma and acute lymphatic leukemia patients (Bargou et al. 2008 Science 321:974). Although the CD3xCD19 BiTE® displays very efficient tumor cell lysis at low levels in vitro, administration of this bispecific format to patients is associated with significant challenges. Due to their small size, BiTE®s are rapidly cleared from the circulation and dosing of patients thus requires continuous infusion. As the dosing regimen has an overall duration of more than 2 months, this treatment has a significant impact on the quality of life of the patients.

[0004] There thus remains a need for effective full length bispecific T cell engaging IgG molecules in eradicating aberrant cells that combine a long circulatory half-life upon intravenous administration without the need for continuous infusion without being immunogenic and with only limited side effects.

BRIEF DESCRIPTION OF THE FIGURES

[0005]

FIG. 1: CLEC12A and related sequences.

FIG. 2: T-cell activation by various antibodies: monoclonal bivalent CD3 IgG, bispecific CD3XCLEC12 IgG, bispecific CD3xisotype control IgG, monoclonal bivalent CLEC12A IgG, monoclonal bivalent isotype control IgG.

FIG. 3: Specific lysis of HL60 cells by CD3XCLEC12A bispecific IgG and control antibodies.

FIG. 4: Specific lysis of HL60 cells by CD3XCLEC12A bispecific IgG and control antibodies (E:T ratios).

FIG. 5: Specific lysis of HL60 cells with several CD3XCLEC12A bispecific IgG molecules consisting of various CLEC12A arms & fixed CD3 arm, and control antibodies.

FIG. 6: Specific lysis of HL60 cells with CD3XCLEC12A bispecific IgG in combination with Fc silencing (DM=Double mutant; TM= triple mutant; WT=wildtype, no Fc silencing).

FIG. 7: Fc silencing does not affect FcRn binding.

FIG. 8: CD3xCLEC12A bsAb target specific induction of T cell proliferation.

FIG. 9: CD8+ T cell compartment of AML patients compared to healthy donors.

FIG. 10: Specific CD3xCLEC12A DM-Fc induced T cell activation and HL60 tumor cell lysis by AML patient T cells.

FIG. 11: Specific lysis of AML blasts by autologous AML patient T cells.

FIG. 12: Specific monocyte lysis by patient T cells.

FIG. 13: Fc silencing significantly eliminates bystander cell cytokine release.

FIG. 14A: FACS staining anti-CD3 antibodies on HPB-ALL cells

FIG. 14B: Plate bound IgG, T cells labeled with CFSE, read out at day 5 by FACS

FIG 15:HL60 cytotoxicity assay

FIG 16: FACS staining anti-CLEC12A antibodies on HL60 cells

FIG. 17: HL60 cytotoxicity assay

FIG 18: FACS analysis

FIG 19: HL60 cytotoxicity assay

FIG 20: VH sequences of CD3-specific and CLEC12A-specific Fab arms. VL sequence of 012 common light chain. CDR sequences are bold and underlined.

SUMMARY OF THE INVENTION

[0006] The present invention is related to a fully human IgG bispecific full length antibody for the treatment of AML. One arm of the antibody binds an epitope on immune effector cells, CD3, whilst the other arm targets CLEC12A, a myeloid cell specific surface target that is expressed in 90-95% of de novo and relapsed AML patients. CLEC12A is expressed on AML leukemic stem cells, but not on normal haematopoietic cells. Unlike CD33, CLEC12A is not expressed on erythroid precursors or megakaryocytes, so the CD3xCLEC12A bispecific IgG1 antibody of the present invention should not induce platelet or red blood cell depletion. Experiments with bone marrow cell colonies have shown that depletion of CLEC12A+ cells in normal bone marrow does not affect the myeloid lineages that give rise to platelets and red blood cells. A CD3xCLEC12A bispecific IgG antibody according to the present invention in a preferred embodiment contains a modified Fc region so as to reduce non-specific immune activation resulting from engagement of T cells and FcγR expressing cells within PBMC. Based on data described for the triomAb bispecific antibody in the prior art it was highly doubted that a CD3xTAA bispecific IgG of a fully human IgG1 format would be able to induce lytic anti-tumor activity in resting peripheral blood lymphocytes without the need for pre-activation of T cells. In addition, the available data for the BiTE® format suggested that a full length IgG molecule would be too large to create effective cytolytic synapses between tumor cells and effector cells. Surprisingly, we demonstrated that a fully human CD3xCLEC12A bispecific full length IgG1 was able to induce very efficient T cell mediated lysis of CLEC12A-positive HL60 AML tumor cells in vitro. In fact, effective lysis was mediated by resting T lymphocytes purified from PBMC without the need of prior activation of the T cells. Furthermore, we demonstrated that this lytic activity is not necessarily dependent on interactions with FcγR present on HL60 cells as this lytic activity was not affected by the presence of excess human IgG when the assay was performed in human serum containing media. This is the first time that a full length human IgG1 bispecific T cell engager antibody exerts efficient tumor cell lysis without the need of pre-activation of T cells or the need of active FcγR interactions. Effective lysis is achieved despite the relatively large size of the IgG1 when compared to BiTE® molecules. Remarkably, when CH2/lower hinge mutations were introduced in the CD3xCLEC12A bispecific IgG1 molecule to further decrease Fc receptor interactions, this still resulted in efficient tumor cell lysis by immune effector cells. A bispecific human IgG1 T cell engager antibody has advantages over current IgG that make use of the hybrid subclass combination mouse IgG2a/rat IgG2b, since a human IgG1 will be less immunogenic and can thus be applied for repeated therapy. In addition, a full length bispecific human IgG1 T cell engager antibody has advantages over immunoglobulin/like molecules such as DART™, TandAb® or BiTE® as the full length human IgG1 is not rapidly cleared from the circulation and dosing of patients will thus not require continuous infusion, which is more beneficial to patients.

EMBODIMENTS

[0007] The invention provides a bispecific IgG antibody according to the claims, wherein said bispecific IgG antibody comprises one arm that specifically recognizes CLEC12A and a second arm that specifically recognizes an antigen on immune effector cells, CD3, capable of recruiting such cells to an aberrant cell expressing CLEC12A.

[0008] As used herein, the term "specifically recognizes CLEC12A" means that said arm has the capability of specifically recognizing CLEC12A, in the situation that CLEC12A is present in the vicinity of said antibody. Likewise, the term "specifically recognizes an antigen on immune effector cells" means that said arm has the capability of specifically recognizing said antigen when said antigen is present in the vicinity of said antibody. Such antigen recognition by an antibody is typically mediated through the complementarity regions of the antibody and the specific three-dimensional structure of both the antigen and the antibody arm allowing these two structures to bind together with precision (an interaction similar to a lock and key), as opposed to random, non-specific sticking of antibodies. As an antibody typically recognizes an epitope of an antigen, and as such epitope may be present in other compounds as well, antibodies according to the present invention that "specifically recognize CLEC12A", and "specifically recognize an antigen on immune effector cells" may recognize other compounds as well, if such other compounds contain the same kind of epitope. Hence, the terms "specifically recognizes CLEC12A", "specifically recognizes an antigen on immune effector cells" and "specifically recognizes CD3" do not exclude binding of the antibodies to other compounds that contain the same (kind of) epitope. Instead, cross-reactivity is allowed. An antibody according to the present invention is typically capable of binding CLEC12A and an antigen on immune effector cells, CD3, with a binding affinity of at least 1x10-5 M, as outlined in more detail below.

[0009] The term "antibody" as used herein means a proteinaceous molecule belonging to the immunoglobulin class of proteins, containing one or more domains that bind an epitope on an antigen, where such domains are derived from or share sequence homology with the variable region of an antibody. Antibodies for therapeutic use are preferably as close to natural antibodies of the subject to be treated as possible (for instance human antibodies for human subjects). Antibody binding can be expressed in terms of specificity and affinity. The specificity determines which antigen or epitope thereof is specifically bound by the binding domain. The affinity is a measure for the strength of binding to a particular antigen or epitope. Specific binding, or "specifically recognizing" is defined as binding with affinities (KD) of at least 1x10-5 M, more preferably 1x10-7 M, more preferably higher than 1x10-9M. Typically, antibodies for therapeutic applications have affinities of up to 1x10-10 M or even higher. Antibodies of the present invention are typically bispecific full length antibodies of the human IgG subclass. Preferably, the antibodies of the present invention are of the human IgG1 subclass.

[0010] The term 'full length IgG' according to the invention is defined as comprising an essentially complete IgG, which however does not necessarily have all functions of an intact IgG. For the avoidance of doubt, a full length IgG contains two heavy and two light chains. Each chain contains constant (C) and variable (V) regions, which can be broken down into domains designated CH1, CH2, CH3, VH, and CL, VL. An IgG antibody binds to antigen via the variable region domains contained in the Fab portion, and after binding can interact with molecules and cells of the immune system through the constant domains, mostly through the Fc portion. The terms 'variable region domain', 'variable region', 'variable domain', 'VH/VL pair', 'VH/VL', 'Fab portion', 'Fab arm', 'Fab' or 'arm' are used herein interchangeably. Full length antibodies according to the invention encompass IgG molecules wherein mutations may be present that provide desired characteristics. Such mutations should not be deletions of substantial portions of any of the regions. However, IgG molecules wherein one or several amino acid residues are deleted, without essentially altering the binding characteristics of the resulting IgG molecule, are embraced within the term "full length IgG". For instance, such IgG molecules can have one or more deletions of between 1 and 10 amino acid residues, preferably in non-CDR regions, wherein the deleted amino acids are not essential for the binding specificity of the IgG.

[0011] Full length IgG antibodies are preferred because of their favourable half life and the need to stay as close to fully autologous (human) molecules for reasons of immunogenicity. According to the invention, bispecific IgG antibodies are used. In a preferred embodiment, bispecific full length IgG1 antibodies are used. IgG1 is favoured based on its long circulatory half life in man. In order to prevent any immunogenicity in humans it is preferred that the bispecific IgG antibody according to the invention is a human IgG1. The term 'bispecific' (bs) means that one arm of the antibody binds to a first antigen whereas the second arm binds to a second antigen, wherein said first and second antigens are not identical. According to the present invention, said first and second antigens are in fact two different molecules that are located on two different cell types. The term 'one arm [of the antibody]' preferably means one Fab portion of the full length IgG antibody. Bispecific antibodies that mediate cytotoxicity by recruiting and activating endogenous immune cells are an emerging class of next-generation antibody therapeutics. This can be achieved by combining antigen binding specificities for target cells (i.e., tumor cells) and effector cells (i.e., T cells, NK cells, and macrophages) in one molecule (Cui et al. JBC 2012 (287) 28206-28214; Kontermann, MABS 2012 (4) 182-197; Chames and Baty, MABS 2009 (1) 539-547; Moore et al. Blood 2011 (117) 4542-4551; Loffler et al. 2000 Blood 95:2098; Zeidler et al. 1999 J. Immunol. 163:1246). According to the invention, bispecific antibodies are provided wherein one arm binds the CLEC12A antigen on aberrant (tumor) cells whereas the second arm binds an antigen on immune effector cells.

[0012] The term 'CLEC12A' as used herein refers to C-type lectin domain family 12 member A, also known as C-type lectin-like molecule-1 (CLL-1), an antigen that is expressed on leukemic blast cells and on leukemic stem cells in acute myeloid leukemia (AML), including the CD34 negative or CD34 low expressing leukemic stem cells (side population) (A.B. Bakker et al. Cancer Res 2004, 64, p8443-50; Van Rhenen et al. 2007 Blood 110:2659; Moshaver et al. 2008 Stem Cells 26:3059). Expression of CLEC12A is otherwise restricted to the hematopoietic lineage, particularly to myeloid cells in peripheral blood and bone marrow, i.e., granulocytes, monocytes and dendritic cell precursors. More importantly, CLEC12A is absent on hematopoietic stem cells. This expression profile makes CLEC12A a particularly favorable target in AML. Alternative names for CLEC12A include dendritic cell-associated C-type lectin-2 (DCAL-2), myeloid inhibitory C-type lectin-like receptor (MICL) and killer cell lectin-like receptor subfamily L, member 1 (KLRL1) (Zhang W. et al. GenBankTM access.no: AF247788; A.S. Marshall, et al. J Biol Chem 2004, 279, p14792-802; GenBankTM access.no: AY498550; Y.Han et al. Blood 2004, 104, p2858-66; H.Floyd, et al. GenBankTM access.no: AY426759; C.H.Chen, et al. Blood 2006, 107, p1459-67). An alignment of these sequences is represented in FIG. 1. The full length form of CLEC12A comprises 275 amino acid residues, including an additional intracellular stretch of 10 amino acids which is absent in most other isoforms, and shows the strictly myeloid expression profile (surface expression and mRNA level). The term 'CLEC12A' means all variants that are referenced above.

[0013] The term 'aberrant cells' as used herein includes tumor cells, more specifically tumor cells of hematological origin including also pre-leukemic cells such as cells that cause myelodysplastic syndromes (MDS) and leukemic cells such as acute myeloid leukemia (AML) tumor cells or chronic myelogenous leukemia (CML) cells.

[0014] The term 'immune effector cell' or 'effector cell' as used herein refers to a cell within the natural repertoire of cells in the mammalian immune system which can be activated to affect the viability of a target cell. Immune effector cells include cells of the lymphoid lineage such as natural killer (NK) cells, T cells including cytotoxic T cells, or B cells, but also cells of the myeloid lineage can be regarded as immune effector cells, such as monocytes or macrophages, dendritic cells and neutrophilic granulocytes. Hence, said effector cell is preferably an NK cell, a T cell, a B cell, a monocyte, a macrophage, a dendritic cell or a neutrophilic granulocyte. According to the invention, recruitment of effector cells to aberrant cells means that immune effector cells are brought in close vicinity to the aberrant target cells such that the effector cells can directly kill, or indirectly initiate the killing of the aberrant cells that they are recruited to. In order to avoid non specific interactions it is preferred that the bispecific antibodies of the invention specifically recognize antigens on immune effector cells that are at least over-expressed by these immune effector cells compared to other cells in the body. Target antigens present on immune effector cells include CD3. Preferably, the antigen on immune effector cells is CD3 expressed on T cells. The most preferred antigen on an immune effector cell is the CD3ε chain. This antigen has been shown to be very effective in recruiting T cells to aberrant cells. Hence, a bispecific IgG antibody according to the present invention preferably contains one arm that specifically recognizes CD3ε.

[0015] Thus, the invention provides a bispecific full length IgG antibody according to the claims, wherein said bispecific antibody comprises one arm that specifically recognizes CLEC12A and a second arm that specifically recognizes a CD3 antigen on immune effector cells capable of recruiting such cells to an aberrant cell expressing CLEC12A, wherein said immune effector cells comprise T cells. The invention provides a bispecific IgG antibody according to the invention wherein said antigen on said immune effector cells is CD3, preferably human CD3ε.

[0016] The invention provides a bispecific IgG antibody according to the claims wherein both arms comprise a common light chain. The term 'common light chain' according to the invention refers to light chains which may be identical or have some amino acid sequence differences while retaining the binding specificity of the antibody. It is for instance possible within the scope of the definition of common light chains as used herein, to prepare or find light chains that are not identical but still functionally equivalent, e.g., by introducing and testing conservative amino acid changes, changes of amino acids in regions that do not or only partly contribute to binding specificity when paired with the heavy chain, and the like. The terms 'common light chain', 'common VL', 'single light chain', 'single VL', with or without the addition of the term 'rearranged' are all used herein interchangeably. The present invention uses as common light chain a human light chain that can combine with different heavy chains to form antibodies with functional antigen binding domains (WO2004/009618, WO2009/157771, Merchant et al. 1998, Nissim et al. 1994). Preferably, the common light chain has a germline sequence. A preferred germline sequence is a light chain variable region that is frequently used in the human repertoire and has superior ability to pair with many different VH regions, and has good thermodynamic stability, yield and solubility. A most preferred germline light chain is 012, preferably the rearranged germline human kappa light chain IgVκ1-39*01/IGJκ1*01 (nomenclature according to the IMGT database worldwide web at imgt.org) or fragment or a functional derivative thereof. The terms rearranged germline human kappa light chain IgVκ1-39*01/IGJκ1*01, IGKV1-39/IGKJ1, huVκ1-39 light chain or in short huVκ1-39 are used interchangeably throughout the application. Obviously, those of skill in the art will recognize that "common" also refers to functional equivalents of the light chain of which the amino acid sequence is not identical. Many variants of said light chain exist wherein mutations (deletions, substitutions, additions) are present that do not materially influence the formation of functional binding regions.

[0017] In a particularly preferred embodiment a bispecific IgG antibody according to the invention is provided wherein the arm that specifically recognizes CLEC12A comprises a heavy chain CDR1 sequence consisting of a sequence that is identical to SGYTFTSY and a heavy chain CDR2 sequence consisting of a sequence that is identical to IINPSGGS and a heavy chain CDR3 sequence consisting of a sequence that is identical to GTTGDWFD. The recited CDR sequences are the CDR sequences of Fab arm 4327 which, as shown in the Examples, has good CLEC12A binding properties. The heavy chain sequence of Fab arm 4327, hence the VH of CLEC12A-specific antibody 4327, is shown in Figure 20. In one preferred embodiment, a bispecific IgG antibody according to the invention comprises a variable heavy chain (VH) sequence that is identical to this VH of antibody 4327. Further provided is therefore a bispecific IgG antibody according to the invention, wherein the arm that specifically recognizes CLEC12A comprises a VH sequence consisting of a sequence that is identical to the sequence QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGS TSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCAKGTTGDWFDYWGQGTLV TVS. As shown in the Examples, bispecific antibodies according to the invention containing the above mentioned VH sequence, together with a VH sequence of a Fab arm recognizing CD3 (and together with a common light chain), have an excellent capacity of inducing T cell mediated lysis of CLEC12A-positive AML tumor cells.

[0018] In a further preferred embodiment a bispecific IgG antibody according to the invention is provided wherein the arm that specifically recognizes CLEC12A comprises a heavy chain CDR1 sequence consisting of a sequence that is identical to SGYTFTSY and a heavy chain CDR2 sequence consisting of a sequence that is identical to IINPSGGS and a heavy chain CDR3 sequence consisting of a sequence that is identical to GNYGDEFDY. The recited CDR sequences are the CDR sequences of the VH region of antibody 4331 which, as shown in the Examples, has good CLEC12A binding properties. The VH sequence of Fab arm 4331 is also shown in Figure 20. In one preferred embodiment, a bispecific IgG antibody according to the invention comprises a VH sequence that is identical to this VH of Fab arm 4331. Further provided is therefore a bispecific IgG antibody according to the invention, wherein the arm that specifically recognizes CLEC12A comprises a VH sequence consisting of a sequence that is identical to the sequence EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGS TSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGNYGDEFDYWGQGTLV TVSS. As shown in the Examples, bispecific antibodies according to the invention containing the VH sequence of Fab arm 4331, together with a VH sequence of a Fab arm recognizing CD3, (together with a common light chain) have an excellent capacity of inducing T cell mediated lysis of CLEC12A-positive AML tumor cells.

[0019] In a further preferred embodiment a bispecific IgG antibody according to the invention is provided wherein the second arm specifically recognizes CD3 and comprises a heavy chain CDR1 sequence consisting of the sequence SYGMH and a heavy chain CDR2 sequence consisting of the sequence IIWYSGSKKNYADSVKG and a heavy chain CDR3 sequence consisting of the sequence GTGYNWFDP. Preferably, said CD3-specific arm comprises a VH sequence consisting of the sequence QVQLVESGGGVVQPGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAIIWYSGSK KNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWGQGTLV TVSS. The recited CDR sequences and VH sequence are the sequences of antibody 3896. These sequences are also depicted in Figure 20. A heavy chain comprising these CD3-specific CDR sequences and/or the recited VH sequence of Fab arm 3896 is preferred for a bispecific IgG antibody according to the invention, because these sequences provide the bispecific antibody with an optimal affinity for CD3-expressing immune cells, while simultaneously allowing sufficient binding to CLEC12A-positive AML tumor cells. Without wishing to be bound to theory, it is thought that the overall effect of a bispecific antibody is determined by the combined effect of the affinity of one arm for antigen 1 and the affinity of the other arm for antigen 2. For an antibody of the present invention, having a specificity for CLEC12A and an antigen on immune effector cells, CD3, an optimized timing of binding to CD3-positive immune cells and CLEC12A-expressing tumor cells is preferred in order to efficiently induce T cell mediated lysis of CLEC12A-positive tumor cells. It is hypothesized that the balance between affinities of a CLEC12A/CD3 bispecific antibody is of utmost importance. It is thought that a significantly higher affinity for CD3 versus a much lower affinity for CLEC12A (i.e., a too high affinity for CD3) will result in a situation wherein the antibodies would primarily bind CD3 expressing T cells. Such 'bispecific antibody-loaded' T-cells may either internalize their CD3 or may invade the tissues thereby leaving the circulation before they have even encountered a CLEC12A-positve tumor cell. This would diminish the therapeutic effect of the bispecific antibody.

[0020] In a more favorable mode of action, CLEC12A-positive tumor cells are first bound by one or more bispecific antibodies according to the invention, where after T cells are attracted by the free CD3 arm of the bispecific antibody and subsequent T cell activation takes place. Alternatively, CD3 positive T cells and CLEC12A-positve tumor cells are bound essentially simultaneously by the bispecific antibody. Hence, the affinities for both CLEC12A and for an antigen on immune effector cells, CD3, are preferably chosen or modulated such that the right balance is achieved, i.e. that the resulting bispecific antibodies will either bind CLEC12A and CD3 essentially simultaneously or that the bispecific antibodies have a tendency to bind CLEC12A-positive tumor cells to a sufficient extent, where after T cell activation takes place and the tumor cells are lysed. According to the present invention, such excellent balance between the binding affinities for CD3 and CLEC12A is preferably achieved by combining a VH having the CDR sequences (or whole VH sequence) of Fab arm 3896 (which is specific for CD3) with a VH having the CDR sequences (or whole VH sequence) of either Fab arms 4327 or 4331 (which are specific for CLEC12A). Such resulting bispecific antibodies display a favorable balance between the binding affinities for CD3 and CLEC12A, so that T cells and CLEC12A-positive AML tumor cells are efficiently brought together, and T cell mediated lysis of CLEC12A-positive AML tumor cells is optimally induced.

[0021] As described herein before, a bispecific IgG antibody according to the claims is provided wherein both arms comprise a common light chain variable domain. A particularly preferred common light chain is the human rearranged kappa light chain IgVκ1-39*01/IgJκ1*01, also named 012. The nucleotide and amino acid sequence of the 012 VL are also depicted in Figure 20. The CDR sequences are bold and underlined. A bispecific antibody according to the invention containing a common light chain that at least comprises the CDR sequences of 012 is therefore provided. One aspect of the invention therefore provides a bispecific IgG antibody according to the invention, wherein the first and the second arms further comprise a light chain CDR1 sequence consisting of a sequence that is identical to RASQSISSYLN and a light chain CDR2 sequence consisting of a sequence that is identical to AASSLQS and a light chain CDR3 sequence consisting of a sequence that is identical to QQSYSTPPT. A bispecific IgG antibody according to the invention comprises a VL sequence that is identical to the 012 VL chain. Further provided is therefore a bispecific IgG antibody according to the invention, wherein first and the second arms comprise a VL sequence consisting of a sequence that is identical to the sequence
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPTFGQGTKVEIK.

[0022] A bispecific full length IgG antibody according to the invention by definition has two different antigen binding sites but the Fc region of the IgG also comprises a third binding site for an Fc receptor. If a cell carries both an Fc receptor and one of the targets of the bispecific antibody, cross-linking of the Fc receptor and said target on the surface of said cell may occur, which may lead to undesired effects. In a preferred embodiment the invention provides a bispecific full length IgG antibody according to the invention, wherein said bispecific IgG antibody has mutated lower hinge and/or CH2 domains such that interaction of said bispecific IgG antibody with Fc gamma (Fcγ) receptors is significantly reduced. As used herein, the term "such that interaction of said bispecific IgG antibody with Fc gamma receptors is significantly reduced" means that the capability of said bispecific IgG antibody of interacting with Fc gamma receptors, if such Fc gamma receptors are present in the vicinity of said antibody, is reduced. Thus, according to the invention a region of the antibody, preferably the lower hinge and/or the CH2 domain of the antibody is mutated (typically by expressing a mutated nucleic acid sequence encoding it) whereby the ability to interact with an Fc receptor is diminished. It is preferred that the interaction with the Fc receptor is essentially abolished. Amino acid residues in human IgG1 that are involved in binding to Fcγ receptors have been mapped previously. In addition to residues which, when altered, improved binding only to specific receptors or simultaneously improved binding to one type of receptor and reduced binding to another type, several residues were found that abrogated binding to one or more of the receptors (Shields RL et al. JBC 2001 (276) 6591-6604; Armour et al. Mol. Immunol. 2003 (40) 585-593). In a further preferred embodiment, said mutated lower hinge and/or CH2 domains comprise at least one substitution at amino acids positions 235 and/or 236 (numbering according to Kabat). Preferably, both amino acids positions 235 and 236 are substituted. It is shown in the examples that substitutions at these sites are capable of essentially preventing the interaction between the bispecific antibody and the Fc receptor present on the tumor cells. In particular it is shown that substitutions L235G and/or G236R are very suitable for that purpose. A bispecific IgG antibody according to the invention, wherein said mutated CH2 and/or lower hinge domains comprise substitution L235G and/or G236R, is therefore also provided herein. Preferably, both L235G and G236R are substituted. Alternatively, a person skilled in the art may introduce lower hinge and/or the CH2 domain mutations that comprise the substitutions 234F, 235E and/or 331S (Oganesyan et al. Biol. Crystall. 2008(D64)700). Preferably, all three substitutions are introduced in this alternative.

[0023] In our US provisional application 61/635,935, which has been followed up by US regular application No. 13/866,747 and PCT application No. PCT/NL2013/050294, we disclose methods and means for producing bispecific antibodies from a single cell, whereby means are provided that favor the formation of bispecific antibodies over the formation of monospecific antibodies. These methods can also be favorably employed in the present invention. Thus the invention provides a method according to the claims for producing a bispecific full length IgG antibody according to the invention from a single cell. Said first and second nucleic acid molecules may be part of the same vector or gene delivery vehicle and may be integrated at the same site of the host cell's genome. Alternatively, said first and second nucleic acid molecules are separately provided to said cell.

[0024] A preferred embodiment provides a method for producing a full length bispecific IgG antibody according to the invention from a single cell, wherein said bispecific IgG antibody comprises two CH3 domains that are capable of forming an interface, said method comprising providing:

- a cell having a) a first nucleic acid sequence encoding said IgG heavy chain that specifically recognizes CLEC12A and that contains a 1st CH3 domain, and b) a second nucleic acid sequence encoding said IgG heavy chain that specifically recognizes an antigen on immune effector cells, CD3, and that contains a 2nd CH3 domain, wherein said nucleic acid sequences are provided with means for preferential pairing of said 1st and 2nd CH3 domains, said method further comprising the step of culturing said cell and allowing for expression of said two nucleic acid sequences and harvesting said bispecific IgG antibody from the culture. Said cell also has a third nucleic acid sequence encoding a common light chain according to the claims. A preferred common light chain is 012, preferably the rearranged germline human kappa light chain IgVκ1-39*01/IGJκ1*01, as described above. The preferred mutations to produce essentially only bispecific full length IgG molecules are the amino acid substitutions L351K and T366K (numbering according to Kabat) in the first CH3 domain and the amino acid substitutions L351D and L368E in the second CH3 domain, or vice versa. Further provided is therefore a method according to the invention for producing a bispecific IgG1 antibody, wherein said first CH3 domain comprises the amino acid substitutions L351K and T366K (numbering according to Kabat) and wherein said second CH3 domain comprises the amino acid substitutions L351D and L368E, said method further comprising the step of culturing said cell and allowing for expression of said nucleic acid sequences and harvesting said bispecific antibody from the culture. Also provided is a method according to the invention for producing a bispecific IgG1 antibody, wherein said first CH3 domain comprises the amino acid substitutions L351D and L368E (numbering according to Kabat) and wherein said second CH3 domain comprises the amino acid substitutions L351K and T366K, said method further comprising the step of culturing said cell and allowing for expression of said nucleic acid sequences and harvesting said bispecific antibody from the culture. Antibodies that can be produced by these methods are also part of the present invention.

[0025] The invention further provides a pharmaceutical composition comprising a bispecific IgG antibody according to the invention and a pharmaceutically acceptable carrier. As used herein, such 'pharmaceutically acceptable carrier' includes any and all solvents, salts, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Depending on the route of administration (e.g., intravenously, subcutaneously, intra-articularly and the like) the active compound may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

[0026] The antibodies and pharmaceutical compositions according to the invention find their use in the treatment of various leukemias and pre-leukemic diseases of myeloid origin but also B cell lymphomas. Diseases that can be treated according to the invention include myeloid leukemias or pre-leukemic diseases such as AML, MDS and CML and Hodgkin's lymphomas and most non-Hodgkin's lymphomas. Thus the invention provides a bispecific full length IgG antibody according to the invention for use as a pharmaceutical in the treatment of myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML) or preferably acute myeloid leukemia (AML). Also contemplated is a use of a bispecific IgG antibody according to the invention in the preparation of a medicament for the treatment or prevention of myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML) or preferably acute myeloid leukemia (AML).

[0027] The amount of antibody according to the invention to be administered to a patient is typically in the therapeutic window, meaning that a sufficient quantity is used for obtaining a therapeutic effect, while the amount does not exceed a threshold value leading to an unacceptable extent of side-effects. The lower the amount of antibody needed for obtaining a desired therapeutic effect, the larger the therapeutic window will typically be. An antibody according to the invention exerting sufficient therapeutic effects at low dosage is, therefore, preferred.

[0028] Approximately 30.000 patients are diagnosed each year with acute myeloid leukemia (AML) in Europe and US. The majority of these patients are 60 years of age or older. Older age is a major negative determinant of outcome in AML and long-term survival (at 5 years) of intensively treated older AML patients is approximately 10%. In almost all patients that have achieved remission upon induction chemotherapy, disease progression is observed within 3 years. Current post-remission treatment has shown limited, if any, value in older patients with AML. Therefore, a significant load of residual resistant leukemia remains, and the surviving subpopulation of drug-resistant leukemic cells rapidly generates recurrence. Novel types of drugs with entirely different modes of action are needed to target these chemotherapy non-responsive AML tumour cells in efforts to induce and sustain complete remissions. Although complete remission (CR) can be achieved with a number of intensive chemotherapy combinations in more than 50% of elderly AML patients and around 80% in younger patients, advancements of response or survival have remained a major investigational challenge. In a recently published network meta-analysis of 65 randomized clinical trials (15.110 patients) in older patients with AML most of the amended investigational induction regimens have similar or even worse efficacy profiles as compared to the conventional 3+7 induction regimen with daunorubicin and cytarabine. This standard treatment of AML is associated with high morbidity and even mortality. The majority of the patients in CR relapse due to remaining leukemic stem cells after chemotherapy. Further dose intensification is limited due to unacceptable toxicity. An urgent need for new treatment modalities preferably with less toxicity is thus emerging especially in elderly patients with AML.

[0029] Treatment of chemotherapy unresponsive AML could be achieved by engaging T cells from the patient's own immune system and AML tumour cells using a bispecific antibody. In this manner, the patients' immune system is strengthened and retargeted to attack and eradicate the AML tumour cells. The present invention provides CD3xCLEC12A bispecific IgG antibodies that efficiently induce AML tumour cell lysis. CD3xCLEC12A bispecific antibodies thus are a targeted therapy with fewer side effects that specifically eradicates leukemic stem cells in order to improve the prognosis of AML patients. Because CLEC12A is expressed on leukemic stem cells (LSC) and not on normal haematopoietic stem cells, therapy directed against this antigen (as has been shown in vitro) will eradicate the LSC while sparing the normal stem cell. It most probably will have the greatest impact in situations of Minimal Residual Disease (MRD). The expectancy is that relapse percentage will drop due to the eradication of the MRD. So the impact for the AML patient of this new treatment modality would be a less toxic treatment with a lesser percentage of relapse resulting in an improvement of outcome associated with a better quality of life. These full length IgG bispecific antibodies are clinically evaluated in relapsed AML patients. The clinical efficacy is analyzed using AML blast reduction in the bone marrow as an objective response criterion. An efficacious bispecific IgG for AML provides a novel therapeutic option for a large patient segment for which there is currently no treatment available. In addition to providing a means to achieve durable remissions, this treatment option also has a curative potential for AML when applied during remission.

EXAMPLES

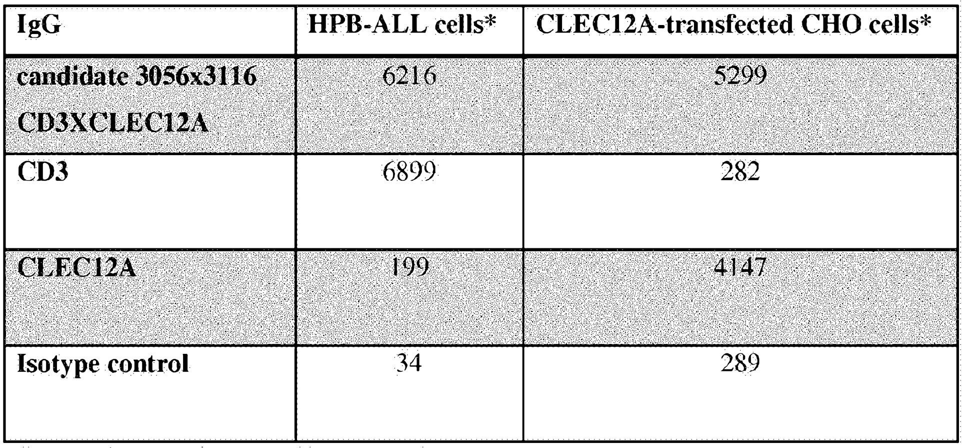
Example 1: Generation and functional characterization of a candidate CD3xCLEC12 bispecific IgG1

[0030] To validate the concept of targeting an immune effector cell to an aberrant cell with a bispecific full length IgG, a candidate CD3XCLEC12A bispecific IgG1 was generated for which the CD3 and CLEC12A Fab arms were derived from antibodies previously described. In the CD3 Fab arm, the VH region from anti-CD3 antibody 15C3, one of the CD3-specific antibodies as disclosed in WO2005/118635, was used and this VH is referred to as '3056'. In the CLEC12A Fab arm, the VH region from scFv SC02-357, one of the CLEC12A-specific antibodies as disclosed in WO2005/000894, was used (hereafter named 'CLEC12A benchmark [Fab arm or antibody]'; alternatively this VH is referred to as '3116'). The nucleotide and amino acid sequences of the VH of the CD3 arm (3056), as well as the nucleotide and amino acid sequences of the VH of the CLEC12A arm (3116) of this candidate molecule, which is referred to as candidate 3056x3116, are provided in Figure 20. The nucleotide and amino acid sequences of the common VL (huVκ1-39; 012) are also provided in Figure 20.

[0031] The respective VH regions were cloned into expression vectors using methods known in the art for production of bispecific IgG1 (Gunasekaran et al. JBC 2010 (285) 19637-19646; WO2009/089004), in conjunction with the rearranged human IGKV1-39/IGKJ1 (huVκ1-39) light chain. The huVκ1-39 was previously shown to be able to pair with more than one heavy chain thereby giving rise to antibodies with diverse specificities, which facilitates the generation of bispecific molecules (De Wildt RM et al. J. Mol. Biol. 1999 (285) 895-901; De Kruif et al. J. Mol. Biol. 2009 (387) 548-58; WO2009/157771).

[0032] First, the binding of the candidate 3056x3116 CD3xCLEC12A bispecific IgG1 to CD3ε on HPB-ALL cells was demonstrated by flow cytometry, which was performed according to standard procedures known in the art (Table 1). Binding to cell-expressed CD3ε is confirmed using CHO cell transfected with CD3δ/ε or CD3γ/ε. The binding of the candidate 3056x3116 bispecific IgG1 to CLEC12A was determined using CHO cells transfected with a CLEC12A expression construct; CD3 monospecific antibody (3056x3056) and CLEC12A monospecific antibody (3116x3116), as well as an irrelevant IgG1 isotype control mAb were taken as control.

Table 1: Binding to cell-expressed CD3 and CLEC12A by flow cytometry.



IgG	**HPB-ALL cells***	**CLEC12A-transfected CHO cells***
candidate 3056x3116 CD3XCLEC12A	6216	5299
CD3	6899	282
CLEC12A	199	4147
Isotype control	34	289

*Results are given as the mean fluorescent intensity.

[0033] Affinity measurements of the candidate 3056x3116 bispecific IgG1 for CD3δ/ε and the extracellular domain of CLEC12A are determined by surface plasmon resonance (BIAcore). Briefly, purified recombinant antigens are covalently coupled to the surface of a CM5 sensor chip using free amine chemistry: antigens are diluted in a kAc buffer to 10µg/ml and coupled to a surface that is activated with NHS/EDC (according to the manufacturer's recommendations). To determine the affinities of the Fab arms present in bispecific antibodies, these are serially diluted to 100, 50, 20, 10, 1 and 0.1 nM in Hepes buffered saline (HBS) and flowed over the antigen-coupled surface of the CM5 sensor chip at a high (30µl/min) flow rate (to prevent re-binding). Flow cell 1 (FC1) is used as a control (blanc) surface and the responses (sensor grams) resulting from this surface are subtracted from the responses measured on other flow cells (FC). FC2 and FC3 are used for the two different antigens recognized by the bispecific antibody, to be able to measure the affinities of both Fab arms in a single kinetic run over all three surfaces. As the concentration of antibody does not significantly change when it is flowed over an antigen-coupled surface, the on-rates (that are concentration-dependent) of bispecific antibodies are simultaneously measured on the two different antigens they recognize. Sensorgrams of the association and dissociation phases of the different bispecific proteins are thus obtained. Using the BIA evaluation software and curve-fitting employing a 1:1 interaction model (for monovalent interaction), the affinities of the Fab arms are determined. In case the binding of the bispecific protein to the antigen-coated surface of the sensor chip is compromised (i.e., when very little protein binds, resulting in low responses and/or very fast off-rates), the setup of the experiment is reversed: the bispecific antibody is covalently coupled to the surface of the sensor chip using free amine chemistry and recombinant purified antigen is flowed over the surface at a high (30µl/min) flow rate to measure the affinity of the Fab arm directed to that antigen.

[0034] Next, the functionality of the candidate 3056x3116 CD3xCLEC12A bispecific Ig was tested. First, the T-cell stimulatory capacity was investigated with healthy donor resting T-cells. Briefly, peripheral blood was obtained from healthy donors after informed consent. T-cells were isolated by standard density gradient isolation to enrich for peripheral blood mononuclear cells (PBMC), followed by negative selection using magnetic beads (pan T-cell kit, Miltenyi Biotec, cat.no.130-091-155). Using this purification strategy, T-cells were so-called 'untouched' (i.e., not stained by antibodies, so-called 'resting T cells') to limit the possibility of pre-activation. Purified resting T-cells were subsequently incubated with cells from the leukemia-derived HL60 cell line in 10% fetal bovine serum (FBS) or 10% normal human serum (HS) at an effector: target cell ratio of 10:1 for two days. Results were expressed as the percentage of CD69-positive or CD25-positive cells within the CD4-positive or CD8-positive T-cell population.

[0035] Both the bivalent CD3 IgG and the CD3XCLEC12A bispecific IgG efficiently induced upregulation of the T-cell activation markers CD69 and CD25 on CD4-positive and CD8-positive T-cells (FIG. 2). In the presence of FBS which did not block Fc receptors present on HL60 cells (Liesveld et al. 1988, J. Immunol. 140(5), pages 1527-1533), also the control bispecific molecule CD3Xisotype control IgG was shown to induce T-cell activation. This effect was diminished in the presence of HS, suggesting that the observed T-cell activation by monovalent CD3 binding of the CD3Xisotype control IgG was dependent on Fc cross-linking. However, T-cell activation induced by the candidate 3056x3116 CD3xCLEC12A bispecific IgG was only partially dependent on Fc-interactions, as the potency to upregulate CD69 and CD25 was largely maintained in the presence of HS (FIG. 2). This indicated that the intrinsic potency of monovalent CD3 binding was sufficient to activate T-cells when the binding molecule bridged to the CLEC12A antigen on the HL60 target cells following binding with the other Fab arm.

[0036] To investigate whether the extent of T-cell activation by the candidate 3056x3116 CD3XCLEC12A bispecific IgG is sufficient to induce target cell lysis, the HL60 cells in this assay were labeled with carboxyfluorescein diacetate succimidyl ester (CFSE) and cocultured with T-cells at various effector:target cell ratios. After one, two or three days, surviving CFSE-positive HL60 cells were quantified by flow cytometry. Results were expressed as the percentage of specific lysis related to PBS.

[0037] As expected, CD3 monospecific bivalent IgG induced resting T-cell mediated killing of HL60 cells (FIG. 3). Surprisingly, CD3XCLEC12A bispecific monovalent IgG and the control CD3Xisotype control also induced resting T-cell mediated killing of HL60 cells. These effects were most prominent when the assay was performed in the absence of excess of human IgG, i.e., when the Fc receptors on the HL60 target cells were not blocked (FBS condition; FIG. 3). Surprisingly, even in the presence of excess human IgG (10% HS condition) the CD3XCLEC12A bispecific IgG was very efficient in killing HL60 cells indicating that the induction of HL60 lysis is not dependent on Fcγ receptor interactions. On day 3 also HL60 lysis induced by the CD3Xisotype control was observed, probably due to incomplete Fc-gamma receptor blockade upon extended incubation periods. HL60 target cell killing varied with different effector:target cell ratios (FIG 4).

[0038] In conclusion, this example demonstrates that a CD3xCLEC12A bispecific molecule is a potent inducer of T-cell mediated tumor cell lysis and confirms our hypothesis that T cell engagement for effective killing of aberrant cells can be mediated by a CD3xCLEC12A full length IgG1 bispecific antibody. Surprisingly, the activity induced by the CD3XCLEC12A bispecific IgG is not dependent on Fcγ receptor interactions. To extend the panel of CD3XCLEC12A bispecific full length IgG in order to arrive at a final clinical candidate, panels of CD3 Fab arms and CLEC12A Fab arms are generated. Validation of specificity and functionality of CD3 and CLEC12A Fab arms is done by fixing the other arm using the respective Fab from the candidate 3056x3116 CD3XCLEC12A bispecific IgG shown in the current example.

Example 2: Generation and characterization of CD3 Fab arms for CD3xCLEC12 bsAb

[0039] Example 1 showed that CD3xCLEC12A bispecific molecules can be potent inducers of T-cell mediated tumor cell lysis. Therefore, to generate more extensive panels of such bispecific molecules separate panels of CD3 binders as well as CLEC12A binders were generated.

[0040] For generation of a panel of CD3 binders, CD3ε-specific VH regions are generated by immunization of mice transgenic for the huVκ1-39 light chain (WO2009/157771) and for a human heavy chain (HC) minilocus with CD3ε in various formats: (1) isolated CD3δ/ε or CD3γ/ε that may be fused of coupled to a carrier molecule (such as human IgG-Fc or a His-tag) as known in the art with or without adjuvant, (2) cells expressing CD3δ/ε or CD3γ/ε, or (3) DNA construct encoding CD3δ/ε or CD3γ/ε, or a combination of these strategies. From immunized mice displaying a sufficient antigen-specific titer as determined by ELISA and/or flow cytometry, spleens and/or lymph nodes are harvested from which Fab phage libraries are generated. Alternatively, VH region sequences are derived directly from spleen and lymph node material by deep sequencing (co-pending US provisional application 61/539,116).

[0041] Antigen-specific Fab arms are selected from phage libraries from immunized mice or from synthetic phage display libraries which contain the VL region of the huVκ1-39 light chain and a collection of human VH regions. For generation of synthetic libraries, randomized CDR3 primers were used as described in De Kruif et al. 1995, J Mol Biol 248(1), pages 97-105. Bacteriophages from these libraries are selected in multiple rounds for binding to isolated CD3δ/ε protein that may be coupled to a carrier molecule (see above) or to cells expressing CD3ε such as HPB-ALL or cells transfected to express CD3δ/ε or CD3γ/ε, or a combination of these strategies. Non-binding phages are removed and binding phages are eluted with an acidic buffer or, to direct the selected Fab repertoire to a desired specificity, with antibodies against a specific epitope, for example with antibodies that are cross-reactive to cynomolgous CD3ε. These phages are then transfected into competent bacteria which were grown under selection pressure for phage-containing bacteria. After picking a number of surviving bacterial colonies, phages are rescued and submitted to the next selection round.

[0042] After completing selection, the remaining phages are screened for binding to cell-expressed antigen by flow cytometry and to isolated antigen by ELISA. As a positive control for binding, benchmark CD3 antibodies are used such are known in the art, e.g., OKT-3. Nucleotide material from essentially all phages that showed specific binding to antigen-expressing cells is submitted to colony PCR to amplify the VH regions and sequence PCR to determine the VH region sequence. The resulting sequences are clustered based on uniqueness of their HCDR3. For sequences derived from immunized mice, in which (limited) somatic hypermutation can occur, VH sequences are further grouped based on the likelihood of a unique VDJ (i.e., if HCDR3 in different clusters contain <2 amino acids difference, they are considered part of the same cluster and are grouped together). From each cluster, one or a few VH regions per cluster are selected for cloning into vectors for expression in a IgG monospecific bivalent format in conjunction with the huVκ1-39 light chain. VH regions for which specific binding to isolated antigen and cell-expressed antigen is confirmed are subsequently cloned in vectors for expression in a CD3XCLEC12A bispecific format. These are then characterized to select a candidate with therapeutic potential (see following examples).

Example 3: Generation and characterization of CLEC12 Fab arms for CD3xCLEC12 bsAb

[0043] As it was demonstrated in Example 1 that CD3xCLEC12A bispecific molecules have the potency to induce T-cell mediated tumor cell lysis, we next wished to establish more extensive panels of such bispecific molecules. In addition to the panel of CD3 binders as described in Example 2 we also generated a panel of CLEC12A binders.

[0044] Briefly, CLEC12A-specific Fab arms were selected from Fab synthetic phage display libraries which contained the rearranged human IGKV1-39/IGKJ1 VL region and a collection of human VH regions (De Kruif et al. Biotechnol Bioeng. 2010 (106)741-50). Bacteriophages from these banks were selected in two rounds for binding to CLEC12A. This was done by incubation with the extracellular domain of CLEC12A (amino acids 75 to 275) coupled to a His-tag (Sino Biological, cat.no. 11896-H07H) which was coated to a surface. Non-binding phages were removed, binding phages were chemically eluted, and used to infect bacteria which were grown under selection pressure for phage-containing bacteria. After picking a number of surviving bacterial colonies, phages were rescued and submitted to the next round of selection and propagation.

[0045] After completing selection, the remaining phages were screened for binding to CLEC12A expressed on the tumor cell line HL60 by flow cytometry. As a positive control for binding, the CLEC12A benchmark antibody was used. Nucleotide material from essentially all phages that showed specific binding to CLEC12A-expressing cells was submitted to colony PCR to amplify the VH regions and sequence PCR to determine the VH region sequence. The resulting sequences were clustered based on uniqueness of their HCDR3. The VH regions from each unique HCDR3 cluster were cloned into vectors for expression in IgG monospecific or bispecific formats in conjunction with the rearranged human IGKV1-39/IGKJ1 LC.

[0046] Three selected CLEC12A binding molecules with a unique HCDR3 sequence showed the desired profile in IgG format, which comprised the following characteristics (Table 2 and data not shown):

[0047] Specific binding to isolated extracellular domain of CLEC12A.

[0048] Specific binding to CLEC12A expressed on a tumor cell line.

[0049] Confirmation of myeloid lineage-specific expression pattern on human PBMC.

Table 2: Characterization of CLEC12A Fab arms.

Fab	**CDR3 length**	**Binding to coated CLEC12A***	**Binding to CLEC12A-expressing cells****	**Competition for epitope with CLEC12A benchmark*****
CLEC12A benchmark	9	1.422	1467	-
3918	10	1.253	899	Yes
4327	9	1.307	1559	No
4331	9	1.328	1106	Yes

* Tested in ELISA, extracellular domain of CLEC12A (Sino Biological) coated at 2 μg/ml, results given as optical density (background signal isotype control: 0.127).

** Tested by flow cytometry on HL60 cells with optimized IgG concentration, results given as mean fluorescent intensity (background signal isotype control: 116).

*** Tested in ELISA with Fab format, against bench mark IgG at 20 μg/ml.

Example 4: Selection of functional CLEC12 Fab arm for CD3xCLEC12 bsAb

[0050] The selected CLEC12A Fab arms as described in Example 3 were subsequently expressed in bispecific IgG format with a new CD3 Fab arm as a fixed arm. This new CD3 Fab arm, referred to as '3896 CD3 IgG' or '3896' in short, also uses the huVκ1-39 light chain. The nucleotide and amino acid sequences of this CD3-specific VH candidate 3896 are depicted in Figure 20. Hence, various bispecific CD3XCLEC12A molecules were expressed that all had the same 3896 anti-CD3 arm but that differed in the CLEC12A arm (either the CLEC12A benchmark arm, or any one of the candidate CLEC12A Fab arms 4327, 4331 or 3918). These CD3XCLEC12A bispecific molecules were then functionally tested in a target cell lysis assay as described in Example 1. Results were expressed as the percentage of specific lysis related to the isotype control. All candidate CLEC12A Fab arms showed a dose-dependent specific lysis of HL60 target cells in the bispecific format, with kinetics that were similar to or better than when the CLEC12A benchmark Fab arm used (FIG. 5).

[0051] Also, the CD3xisotype control bsAb showed a dose-dependent target cell lysis, although 1 log higher concentrations were required for the same extent of specific lysis. Despite the presence of excess human IgG via addition of HS, killing activity of this monovalent CD3 IgG was still apparent, probably by Fc-mediated cross-linking. As will be clear from Example 7, this target non-specific lysis can indeed be fully abrogated via silencing Fc receptor interaction by CH2 engineering.

Example 5: efficacy of CD3xCLEC12 product candidates using AML T cells and/or AML tumor cells

[0052] Examples 1 and 4 demonstrated the potency of CD3XCLEC12A bispecific IgG using either CD3 Fab arm 3056 or 3896 and using the CLEC12A Fab arm candidates 4327, 4331 or 3918 or the CLEC12A benchmark Fab arm 3116 in inducing HL60 target cell lysis mediated by healthy donor resting T-cells. In the current example, it is investigated whether T-cells derived from patients with AML, one of the primary indications for therapeutic application of a CD3XCLEC12A bispecific drug, can be stimulated to kill tumor targets upon stimulation with a CD3XCLEC12A bispecific full length IgG. Next, it is determined whether patient-derived T-cells can kill autologous AML tumor cell blasts upon stimulation with a CD3XCLEC12A bispecific full length IgG.

[0053] T-cells are isolated from peripheral blood of AML patients according to procedures as described in Example 1. Purified patient-derived T-cells are then incubated with CFSE-labeled HL60 cells and monitored for cell lysis as described in Example 1.

[0054] In addition, the T-cell mediated target cell lysis assay is performed with AML tumor blasts isolated from the same patient (Norde et al. Blood 2009 (113)2312). Isolated blasts are then labeled with CFSE and cocultured with autologous patient-derived T-cells in the presence of the cytokine mixture as described in Norde et al. and in the presence of the CD3XCLEC12A bispecific IgG or controls. Target cell lysis is monitored as described in Example 1.

Example 6: Cytokine release by T cells after contact with CD3XCLEC12A bispecific IgG

[0055] Using T-cell stimulatory biologicals, overstimulation of T-cells is a serious risk as this may lead to cytokine release syndrome (Suntharalingam et al. 2006, New England J Med 355(10), pages 1018-1028; Chatenoud et al. 1990, Transplantation 49(4), pages 697-702). To investigate the extent of T-cell stimulation induced by CD3XCLEC12A bispecific IgG, the induction of T-cell cytokines was studied in a coculture of T-cells and Fc receptor-expressing target cells.

[0056] Briefly, healthy donor resting T-cells were cocultured with HL60 target cells in the presence of the candidate 3056x3116 CD3XCLEC12A bispecific IgG (1 µg/ml) or control IgG as described in Example 1. After two days, the supernatant was sampled and cytokine production levels were determined in a Luminex assay as known in the art using the human Cytokine Human 10-Plex Panel (Invitrogen, cat.no.LHC0001). This panel covers the ten major Th1 and Th2 cytokines.

[0057] As expected, the CD3 monospecific bivalent IgG induced strong production of IFNγ, TNFα and IL-2 (Table 3), which are considered to mainly drive cytokine release syndrome. In addition, production of IL-4, IL-6, IL-8 and IL-10 was increased by incubation with CD3 IgG. In contrast, the CD3XCLEC12A bispecific IgG only induced IL-8 production to a similar level as CD3 IgG; the other cytokines were not significantly induced by the bispecific IgG. GM-CSF was below the detection limit in any condition.

Table 3: antibody induced cytokine release by T cells.

Cytokine	**CD3 IgG**	**CD3XCLEC12A IgG**	**CD3Xisotype control**
IFNγ	484.3 ± 155.0	13.5 ± 19.1	0.0 ± 0.0
TNFα	85.8 ± 23.1	14.5 ± 1.4	4.6 ± 1.1
IL-2	285.6 ± 325.5	3.4 ± 0.8	1.7 ± 0.6
IL-4	23.6 ± 3.7	10.2 ± 0.2	7.3 ± 1.3
IL-6	9.0 ± 1.8	3.7 ± 0.3	2.3 ± 0.0
IL-8	1567.8 ± 5.2	1280.1 ± 118.4	359.6 ± 183.6
IL-10	531.5 ± 224.0	21.1 ± 3.0	3.7 ± 4.0
IL-1β	4.4 ± 0.4	3.3 ± 0.1	2.5 ± 0.1
IL-5	2.1 ± 0.2	0.7 ± 0.0	0.6 ± 0.1

Results are given as the average concentration of cytokine in pg/ml of two donor ± standard deviation.

[0058] The data shown here suggest a favorable therapeutic profile for the different CD3XCLEC12A bispecific IgG molecules, as they potently induce target cell lysis (Examples 1 and 4) without triggering T-cells to secrete potentially harmful amounts of pro-inflammatory cytokines as observed with CD3 IgG.

Example 7: Effect of Fc silencing on *in vitr*o efficacy of CD3XCLEC12A bsAb

[0059] The dose-dependent target cell lysis by the CD3Xisotype control bsAb shown in Example 4 was suggested to be due to interaction of the bsAb Fc part with Fc receptors on HL60 target cells. As such target non-specific cell lysis may als occur *in vivo,* either by interaction with Fc receptors on target cells or on bystander cells such as NK cells, engineering of the CH2/lower hinge region was employed to induce silencing of Fc-mediated activity of the bsAb.

[0060] For this, two Fc mutation strategies were examined, using either a 235G 236R double mutation (DM; DM-Fc) or a 234F 235E 331S triple mutation (TM; TM-Fc). CD3XCLEC12A bsAbs (3056x3116) with either a DM-Fc or a TM-Fc were generated and confirmed to bind CLEC12A-expressing cells by flow cytometry with the same intensity as the bsAb with wild type Fc (data not shown). Next, these bsAbs and the wild type, DM-Fc and TM-Fc versions of the CD3Xisotype control bsAb were tested in the HL60 target cell lysis assay (see Examples 1 and 4). Results were expressed as the percentage of specific lysis related to the isotype control.

[0061] Fc silencing either by the DM or by the TM had no or only a minor influence on the extent of HL60 cell specific lysis induced by CD3XCLEC12A bsAb (FIG. 6). For the CD3Xisotype control bsAb, however, the potency to induce lysis of HL60 cells was significantly reduced with the TM and even further with the DM.

[0062] This demonstrates that Fc silencing by CH2/lower hinge engineering further contributes to target-specific killing of aberrant cells by creating a bispecific CD3xCLEC12A IgG1 format that efficiently and specifically recruits effector cells, and diminishes the potential non-specific immune activation mediated by normal Fcγ receptor expressing accessory cells.

Example 8: Effect of Fc-silencing on binding to FcRn, CD16, CD32, CD64 and C1q

[0063] Binding of the candidate 3056x3116 CD3XCLEC12A bsAb with WT Fc or with silenced DM-Fc or a TM-Fc to human FcRn was determined by Bio-Layer Interferometry (BLI, Octet QK, ForteBio). Briefly, purified CD3XCLEC12A WT Fc IgG1, DM-Fc IgG1 or TM-Fc IgG1 was captured to Protein L biosensors (FortéBio, Cat no 18-5085) at a concentration of 50 µg/ml in 0.1 M phosphate buffer/0.002%Tween20 containing 1.0mg/ml BSA pH6.0 (FcRn-Binding buffer) at RT. Subsequently soluble human FcRn (Sino Biological Inc, CT009-H08H) was added at concentration of 1 µg/ml in FcRn-Binding buffer) at RT. Data analysis using the Octet QK analysis software showed upon normalization for IgG binding to the ProtL sensor that the subsequent binding of CD3XCLEC12A bsAb with DM or TM silenced Fc to human FcRn was comparable to CD3XCLEC12A bsAb with wild-type Fc-tail (FIG. 7) and Fc silencing did thus not affect FcRn binding.

[0064] Binding of CD3XCLEC12A bsAb with silenced Fc to CD16, CD32 and CD64 is determined by Bio-Layer Interferometry (BLI, Octet QK, FortéBio). Protocol in short: purified CD3XCLEC12A WT Fc IgG1, DM-Fc IgG1 or TM-Fc IgG1 is captured to Protein L biosensors (FortéBio, Cat no 18-5085) at a concentration of 50 µg/ml in 1x Kinetics Buffer (FortéBio 18-5032) at RT. Subsequently recombinant CD16 (Sino Biological Inc, 10389-H08H1), CD32 (Sino Biological Inc, 10374-H08H) and CD64 (Sino Biological Inc, 10256-H08H) protein is added at concentration of 1.0 µg/ml in Kinetics Buffer (FortéBio 18-5032) at RT. Binding of FcR receptors to bsAb is analyzed using Octet QK analysis software.

[0065] Binding of CD3XCLEC12A bsAb with silenced Fc to human C1q is determined by capture ELISA. To this end purified CD3XCLEC12A WT Fc IgG1, DM-Fc IgG1 or TM-Fc IgG1 is coated in a concentration range of 25-0.012 µg/ml in PBS on Nunc-Immuno maxisorp F96 plate (Nunc, 439454) O/N at 4C. Subsequently human C1q (Quidel, A400) is added at 2.0 µg/ml in ELISA buffer (2%MILK/PBST). The complex is then visualized using sheep-anti-human C1q polyclonal IgG (Meridian, K90020C) and rabbit-anti-sheep HRP conjugated polyclonal IgG (Southern Biotech, 6150-05). Finally, using TMB substrate (BD 51-2606KC/51-2607KC) binding is developed and OD450 is quantified using a Micro plate reader (Multiskan EX, Thermo Electron Corporation).

Example 9: Evaluation of *in vivo* efficacy of CD3xCLEC12A bispecific IgG.

[0066] Animal xenograft studies using luciferase expressing HL60 cells (HL60(-Luc) cells) are performed to confirm and extend the *in vitro* findings using the CD3xCLEC12A bispecific IgG1. More specifically these studies are performed to determine the steady state plasma concentrations at effective doses, which will be taken into account in setting the starting dose for the Phase 1 clinical evaluation. To this purpose NOD/SCID mice (or comparable immune-compromised mice) are injected subcutaneously with an amount of viable HL60(-Luc) cells that results in the establishment of subcutaneous HL60 tumors in the majority of the animals within two weeks upon injection. In parallel with the HL60(Luc) inoculation, or upon initial tumor take, 5x10E6 or 1x10E7 human PBMC are administered. CD3xCLEC12A bispecific IgG or control monospecific or control bispecific IgG are administered intravenously at several dose levels at the first day of PBMC administration, and 3, 6, and 9 days later. Tumor dimensions are scored 1 week after the initial HL60(Luc) inoculation. The arithmetic average of tumor dimensions (either denoted as tumor volumes or as total bioluminescence) from each group is plotted against time.

Example 10: Use of a bispecific full length IgG1 antibody CD3xCLEC12A in a phase Ia/Ib study.

[0067] The final lead CD3xCLEC12A bispecific full length IgG1 candidate is used to manufacture GMP grade material and is clinically evaluated in AML patients. First, a formal non-clinical safety analysis of the product candidate is performed to establish a safe starting dose for first in man studies. Hereafter, an open-label, multi-centre dose escalation Phase Ia/b trial is performed in relapsed and/or refractory AML and in patients unfit for intensive treatment, to explore the safety and tolerability of the CD3xCLEC12A bispecific IgG upon i.v. administration. Secondary endpoints include pharmacokinetic and pharmacodynamic characterization and preliminary efficacy analysis. Overall response rates are assessed by evaluation of the AML blast reduction in the bone marrow. In Phase Ia the maximum tolerated dose (MTD) is assessed upon single/multiple dose escalation. After interim PK analysis, the Phase 1b part of the study entails a dose extension cohort at the MTD or entails further exploration of the dosing frequency.

Example 11: Capacity of CD3xCLEC12A bsAb to induce T cell proliferation.

[0068] In patients with AML T cell numbers are low compared to the amount of AML blasts at diagnosis. It is well known that T cells undergo proliferation upon activation resulting in an increased number of T cells. Moreover, in example 1 we have demonstrated that a CD3xCLEC12A bsAb can activate T cells and has the potency to induce T-cell mediated tumor cell lysis. We hypothesized that AML patients treated with CD3xCLEC12A bsAb benefit from expansion of T cell subsets upon CD3xCLEC12A bispecific molecule mediated T cell activation as T cell proliferation will result in an increased number of effector T cells. To demonstrate that CD3xCLEC12A bsAb induces *in vitro* T cell proliferation, resting T cells were purified, labeled with carboxyfluorescein diacetate succimidyl ester (CFSE) and co-cultured with autologous CLEC12A+ monocytes in the presence of CD3xCLEC12A bsAb or control Abs. To specifically investigate the CD3xCLEC12A induced T cell proliferation without non-specific Fcgamma activation CD3xCLEC12A bsAbs with the DM-Fc tail, as described in Examples 7 and 8, was used. As controls, a CD3xisotype control WT-Fc bsAb, a CD3xisotype control DM-Fc bsAb, a monoclonal CD3 with WT-Fc and an irrelevant isotype control (IgG with WT-FC) were included. Monocytes and T cells from healthy donor peripheral blood were isolated by standard density gradient isolation to enrich for peripheral blood mononuclear cells (PBMC), followed by a CD14 positive selection for monocytes using CD14 microbeads (human CD14 microbeads, Miltenyi Biotec, cat.no. 130-050-201) and a negative selection of untouched T cells using magnetic beads against other leukocytes (pan T-cell isolation kit, Miltenyi Biotec, cat.no. 130-096-535). The pan T-cell isolation kit allows isolation of resting (untouched) T cells (i.e. not stained with antibodies) avoiding the possibility of pre-activation of T cells.

[0069] CFSE-labeled purified resting T cells were subsequently incubated with purified monocytes and bsAbs in medium with 10% normal human serum (HS) at an effector: target cell ratio of 5:1 for seven days. At day 7 decrease of CFSE signal as read-out for T cell proliferation was measured by flow cytometry. Results were expressed CFSE signal per CD3+, CD3+CD4+ or CD3+CD8+ T cells in histograms.

[0070] Positive control CD3 WT-Fc Ab induced T cell proliferation whereas isotype control IgG with WT-Fc did not induce T cell proliferation (Figure 8). As expected the CD3xisotype control WT-Fc bsAb did induce T cell proliferation, but to a lower extend when compared to the bivalent monospecific anti-CD3 IgG control. In contrast, the CD3xisotype control DM-Fc bsAb did not induce T cell proliferation due to its silenced Fc-tail. The CD3xCLEC12A DM-Fc bsAb also induced the desired T cell proliferation mediated by specifically bridging CD3 with the CLEC12A antigen

[0071] This shows that a CD3xCLEC12A bsAb is not only capable of target specific induction of T cell mediated tumor lysis as demonstrated previously, but can also potently induce target specific T cell proliferation resulting in an increased number of T cells. Moreover this further demonstrates that Fc silencing by CH2/lower hinge engineering not only contributes to target-specific killing of aberrant cells but also to target-specific induction of T cell proliferation by the

CD3xCLEC12A DM-Fc bsAb IgG.

Example 12: Evaluation of CD3xCLEC12A induced expansion of T_{EMRA} subset from AML patients.

[0072] As activation of T cell proliferation was demonstrated for CD3xCLEC12 DM-Fc bsAb, we next wished to investigate whether CD3xCLEC12A DM-Fc bsAb is capable of inducing proliferation of the CD8+ cytotoxic T cell compartment in AML patients. $CD8^+$ cytotoxic T cells have been recognized as the main effectors mediating tumor regression (Sluijter et al., 2010). CD8+ T cells can be divided into four subsets: naive (CCR7+CD45RA+), central memory (T_{CM}, CCR7+CD45RA-), effector memory (T_{EM}, CCR7-CD45RA-), and CD45RA+ effector memory (T_{EMRA}, CCR7-CD45RA+) cells. Studies have shown that naive and memory CD8+ T-cell subsets have different capacities to proliferate and differentiate in response to TCR stimulation (Geginat et al., 2003).

[0073] First the CD8+ compartment in peripheral blood of AML patients in clinical remission was analyzed in comparison to healthy donors. To this end PBMC were isolated from frozen peripheral blood samples from AML patients and healthy donors by standard density gradient isolation. Next, PBMCs were stained with CCR7, CD3, CD4, CD8, CD45RA and CD45RO antibodies to analyze for the CD8+ T cell subsets by flow cytometry. Results were expressed as percentage of a subset in the total CD8+ T cell compartment.

[0074] Analogous to what was previously described, it was observed that the naive CD8+ T cell subset was reduced in blood from AML patients compared to the naive CD8+ T cell subset from healthy individuals, whereas the T_{EMRA} compartment (CCR7-CD45RA+) was increased in AML patients compared to healthy donors (Figure 9).

[0075] Next, experiments are performed to study tumor target specific T cell proliferation of the AML patient T cell compartment. More specifically, these experiments are performed to determine if the CD3xCLEC12A DM-Fc bsAb can enhance T cell proliferation and outgrowth of the effector T cell subsets (T_{EM} and T_{EMRA}) of AML patients relative to the naive CD8+ T cells of AML patients.

[0076] To this end resting T cells from AML patients in clinical remission are purified according to example 11. Composition of the CD8+ T cell subsets at day = 0 is analyzed by staining of the PBMC with CCR7, CD3, CD4, CD8, CD45RA and CD45RO antibodies, followed by flow cytometric analysis. In addition, resting T cells are either labeled with CFSE or not labeled (CFSE labeling as described in example 11) and co-cultured with HL60 leukemia cells at an E:T ratio 5:1 with control or test antibodies for 7 days. CFSE labeled T cells are used for quantification of T cell proliferation, whereas unlabeled T cells are used to determine the percentage of proliferated T cell subsets. CFSE-labeled and unlabeled T cells are incubated with PBS, isotype control WT-Fc Ab, CD3xCLEC12A DM-Fc bsAb, CD3xisotype control DM-Fc bsAb and CD3 monoclonal Ab with WT-Fc at 1 µg/ml. After 7 days, CFSE labeled T cell are stained with CD3, CD4 and CD8 antibodies and subjected to FACS analysis to determine the absolute T cells numbers and number of cell divisions, whereas unlabelled CFSE T cells are stained with CCR7, CD3, CD4, CD8, CD45RA and CD45RO antibodies to determine composition of the proliferated CD8+ T cell subsets by flow cytometry. T cell proliferation is expressed as CFSE signal per T cell subset in histograms and the size of the four CD8+ T cells subsets is expressed as percentage within the total CD8+ T cell compartment.

Example 13: Efficacy of CD3xCLEC12A bsAb to induce AML patient T cell mediated tumour cell lysis.

[0077] In example 1 it was demonstrated that a CD3xCLEC12A bsAb can induce killing of CLEC12A-positive HL60 cells by resting T cells from healthy donors. Next we investigated the capacity of the CD3xCLEC12A bsAb to induce target-specific activation of AML patient T cells and its capacity to induce AML patient T cell mediated killing of HL60 cells.

[0078] T cells were isolated from frozen peripheral blood of AML patients (AML FAB classification AML-M1/M2, M4 or M5) in clinical remission using pan T-cell isolation kit as described in example 11. Purified AML patient derived resting T-cells were subsequently incubated with CSFE-labeled HL60 cells in medium supplemented with 10% normal HS at an effector: target cell ratio of 5:1 for two days, in the presence of PBS, isotype control WT-Fc Ab, CD3xCLEC12A DM-Fc, CD3xisotype DM-Fc, and positive control CD3 WT-Fc Ab (all antibodies at concentration of 1 µg/ml). After two days of co-culture, T cell activation was determined by flow cytometric analysis for CD3, CD4, and CD25. These results were expressed as percentage CD25+ cells per CD4+ T cells. Moreover, surviving CFSE-positive HL60 cells were quantified by flow cytometry. Results were expressed as the percentage of specific lysis relative to IgG.

[0079] These data show that the antigen-specific activation of healthy donor and AML patient T cells mediated by CD3xCLEC12A DM-Fc bsAb was comparable (Figure 10A). As expected the CD3xisotype control DM-Fc bsAb did not induce T cell activation of health donor nor AML patient derived T cells. It was demonstrated that the CD3xCLEC12A DM-Fc bsAb mediated lysis of HL60 cells by AML patient derived T-cells (68% HL60 cell lysis) was comparable to that by healthy donor T cells (69% HL60 cell lysis, Figure 10B). As expected, the CD3xisotype control DM-Fc bsAb did not induce killing of HL60 cells, neither by AML patient T cells nor by healthy donor T cells. Thus, the CD3xCLEC12A bispecific molecule is a potent inducer of T cell mediated tumor cell lysis, regardless of whether these T cells are AML patient derived or from healthy donors.

[0080] As it was shown that the CD3xCLEC12A bsAb has the capacity to induce potent lysis of HL60 tumor cells by AML patient T cells, subsequently the capacity of the CD3xCLEC12A bsAb to target specific activation of AML T cells was evaluated. In addition, the capacity of the CD3xCLEC12A bsAb to induce lysis of primary CLEC12A-positive AML blasts by AML patient derived autologous T cells was determined. First, frozen stored bone marrow samples from AML patients at diagnosis samples containing >70% of primary AML blasts as determined by flow cytometric analysis were thawed, cultured overnight (O/N) in IMDM medium supplemented with 10% FCS, 100ng/ml GM-CSF, 100ng/ml G-CSF, 50ng/ml IL-3, 25ng/ml SCF and 20ng/ml Flt3L as previously described (Norde et al., 2009). After O/N culture, primary AML blasts were phenotyped for surface expression of CLEC12A, CD3, CD4, CD8, CD14, CD19, CD33, CD34, CD38, CD45 and CD117 by flow cytometry and labelled with CFSE. Resting autologous patient derived T cells, collected when the patient had achieved clinical remission, were isolated from the peripheral blood using the pan T-cell isolation kit as described in example 11. Subsequently, AML blasts were co-cultured with resting autologous T cells at an E:T ratio of 5:1 in medium with 10% HS for two days. The conditions tested included PBS, isotype control Ab WT-Fc, CD3xCLEC12A DM-Fc, CD3xisotype control DM-Fc and positive control CD3 WT-Fc Ab (all antibodies at 1 µg/ml). After two days of co-culture, T cell activation was determined by flow cytometric analysis for CD3, CD4, CD8, and CD25. These results were expressed as percentage CD25+ cells per CD4+ or CD8+ AML T cells. AML blast lysis was determined by quantification of the surviving CFSE^{+}/CD45^{low} double positive AML blasts by flow cytometry. Results were expressed as the percentage of specific blast lysis relative to IgG.

[0081] These data demonstrate that the CD3xCLEC12A DM-Fc bsAb has the capacity to induce AML blast target specific activation of AML T cells comparable to the monoclonal CD3 WT-Fc positive control Ab (Figure 11A/B). Moreover these data demonstrate that the CD3xCLEC12A bsAb induced potent killing of autologous AML blasts by AML patient-derived T cells is as potent as the killing induced by the monoclonal CD3 WT-Fc positive control Ab (Figure 11C). As expected, no or minor AML blast killing was induced by the CD3xisotype control DM-Fc Ab, which indicates that the observed AML blast killing mediated by the CD3xCLEC12A bsAb is primarily the result of antigen-specific activation of T cells and specific lysis of CLEC12A+ AML tumor cells. Overall, this study demonstrates that CD3xCLEC12A bsAb can efficiently induce killing of CLEC12A positive tumor cells by AML patient T cells.

Example 14: Effect of Fc-silencing on non-specific cytokine release

[0082] In examples 7 and 8 it was demonstrated that CD3xCLEC12A bsAb IgG1 format with Fc silencing by CH2/lower hinge engineering (DM-Fc) resulted in reduced affinity for Fcgamma receptors and abrogated non-specific Fc receptor mediated cytotoxicity of the leukemia-derived HL60 cell line. Next, it was investigated whether the bsAb IgG1 format with DM-Fc silencing abrogated non-specific Fc receptor mediated cytotoxicity in the presence of Fc receptor-positive bystander cells such as NK cells. In this study, autologous healthy donor derived resting T cells were redirected against CLEC12A-positive monocytes in the presence of other Fc receptor positive bystander innate effector cells such as NK cells. To this end PBMC were isolated from heparinized peripheral blood from healthy donors by density gradient centrifugation and were plated at a density of 1*10^6 cells/ml. PBMC were cultured for two days in medium with 10% FBS in the presence of either PBS, isotype control Ab, CD3xCLEC12A WT-Fc bsAb, CD3xCLEC12A DM-Fc bsAb, CD3xisotype control WT-Fc bsAb, CD3xisotype control DM-Fc bsAb or CD3 monoclonal Ab with WT-Fc. After two days culture, surviving monocytes were quantified by flow cytometry based on CD14-expression. Results were expressed as the percentage of specific lysis related to IgG.

[0083] It was demonstrated that, for the CD3xCLEC12A bispecific antibody, Fc silencing through the presence of the DM-Fc region only had a minor effect on monocytes lysis (Figure 12). In contrast, for the CD3xisotype control bsAb, Fc silencing through the presence of the DM-Fc region significantly reduced the non-specific lysis of monocytes. It is thus concluded that Fc silencing in the CD3xCLEC12A bsAb further contributes to target-specific killing: the CD3xCLEC12A DM-Fc bsAb specifically recruits T cells and diminishes non-specific immune activation mediated by normal Fcγ receptor expressing accessory cells.

[0084] Next it was questioned whether the Fc silencing by the DM mutation in the CD3xCLEC12A bsAb abrogates the Fc receptor-mediated release of cytokines, known to be associated with cytokine release syndrome (CRS), a common clinical event with antibody therapies brought about by accessory cells. To this end the cytokine profile in the supernatants of the monocyte killing assay described in Figure 13 was analyzed using the cytokine human 10-plex panel for the Luminex platform (Invitrogen, LHC0001) according to manufacturer instructions. The profile of the following human cytokines was measured in day 2 supernatant: GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α. Results shown are of cytokine concentration measured in pg/ml. The levels of GM-CSF, IL-4 and IL-5 cytokines were below detection limit of this assay (data not shown).

[0085] The data show that CD3xCLEC12A and CD3xisotype control bsAb, both with WT-Fc tail induced release of IL-1β, IL-6, TNF-α, IL-10, IL-2 and IFN-γ (Figue 13). However, no or only very low levels of those cytokines were found in CD3xCLEC12A and CD3xisotype control bsAb when carrying the DM-Fc tail, with an exception for IL-8. As monocytes are the main source of IL-8, the high IL-8 levels are assumed to be released from the lysed monocytes and are not a result from a-specific FcR mediated release. It is concluded that Fc silencing through the DM mutations in the bsAb IgG format significantly eliminates the Fc receptor mediated release of IL-1b, IL-6, TNF-α, IL-2 and IFN-γ cytokines associated with CRS. Overall, these data demonstrate that the Fc silencing by the DM mutation in the CH2/lower hinge region contributes to the enhancement of the efficiency and specific recruitment of effector cells by CD3xCLEC12A DM-bsAb by diminishing the potential non-specific immune activation mediated by normal Fcγ receptor expressing accessory cells and associated release of proinflammatory cytokines.

Example 15

[0086] The binding of candidate 3896 as full length bivalent monoclonal anti-CD3 IgG to membrane bound CD3 was compared with candidate 3056 as full length bivalent monoclonal anti-CD3 IgG by FACS analysis using CD3 expressing HPB-ALL cells. An irrelevant human

IgG1 served as an isotype control IgG. Flow cytometry was performed according to standard procedures known in the art. As shown in Figure 14A, the 3896 CD3 IgG dose-dependently bound to CD3 on HPB-ALL cells, as did the 3056 CD3 IgG.

[0087] Next, the ability of 3896 CD3 IgG to induce T-cell proliferation was tested in direct comparison to murine OKT3 CD3 antibody, 3056 CD3 IgG, and isotype control IgG. Briefly, the antibodies were serially diluted and immobilized onto 96-well plates. Upon removal of unbound IgG, CFSE-labeled T cells were added and incubated at 37°C. At day 5, the level of induced T cell proliferation was analyzed by flow cytometry. Results are expressed as the percentage of viable T cells displaying at least a twofold reduction in CFSE expression level and are shown in figure 14B. It was demonstrated that the 3896 CD3 IgG as a bivalent monospecific antibody was less potent in inducing T cell proliferation as compared to the candidate 3056 CD3 IgG and murine OKT3. These data suggest that the reduced level of T cell proliferation as induced by 3896 when compared to the 3056 CD3 IgG reflect the reduced CD3 binding capacity as analyzed by flow cytometry. This difference in binding allows for choosing an arm with a desired affinity, resulting in a bispecific antibody that displays a favorable balance between the binding affinities for CD3 and CLEC12A, so that T cells and CLEC12A-positive AML tumor cells are efficiently brought together, and T cell mediated lysis of CLEC12A-positive AML tumor cells is optimally induced.

[0088] To test the potency of the new 3896 anti-CD3 arm versus the 3056 anti-CD3 arm in a CD3xCLEC12A bispecific antibody format, the 3896xCLEC12A benchmark bispecific antibody of example 4 (candidate 3896x3116) and the 3056xCLEC12A benchmark bs antibody of example 1 (candidate 3056x3116) were directly compared in the HL60 cytotoxicity assay as previously described. The results are shown in figure 15. It was observed that the 3896xCLEC12A benchmark bsAb has similar potency as the 3056xCLEC12A benchmark bsAb. Hence, as both bispecific antibodies differ only in their CD3 Fab arm whilst the CLEC12A Fab arm is the same, it is concluded that the functionality of the 3896 CD3 Fab arm is similar to that of the 3056 CD3 Fab arm in a CD3xCLEC12A bispecific Ab. It is noted that at lower concentrations the candidate 3896x3116 is even better than the candidate 3056x3116. This is favourable because it provides a larger therapeutic window, as explained herein before.

Example 16

[0089] In example 3, a panel of CLEC12A-specific Fab arms was selected from phage display libraries. All CLEC12A binding molecules contained the huVk1-39 light chain. Three CLEC12A binding molecules were selected: Fabs 3918, 4327 and 4331. These Fabs were expressed as full length human IgG1: 3918 CLEC12A IgG, 4327 CLEC12A IgG and 4331 CLEC12A IgG. The nucleotide and amino acid sequences of the VH of 3918 CLEC12A IgG, the VH of 4327 CLEC12A IgG, the VH of 4331 CLEC12A IgG and the common VL (IGKV1-39; 012) are provided in Figure 20.

[0090] The full length CLEC12A antibodies were tested for binding to CLEC12A expressed by

HL60 cells.

[0091] The binding of 3918 CLEC12A IgG, 4327 CLEC12A IgG and 4331 CLEC12A IgG to membrane bound CLEC12A was compared with the CLEC12A benchmark antibody (3116) by FACS analysis using CLEC12A expressing HL60 cells. An irrelevant human IgG1 served as an isotype control IgG. Flow cytometry was performed according to standard procedures known in the art. As shown in Figure 16, the 4327 CLEC12A IgG bound to CLEC12A in a similar fashion as the CLEC12A benchmark antibody. The other two antibodies, 3918 CLEC12A IgG and 4331 CLEC12A IgG also demonstrated a good dose-dependent binding to CLEC12A on HL60 cells. Their binding to CLEC12A seemed somewhat lower as compared to the CLEC12A benchmark antibody. In conclusion, Fabs 3918, 4327 and 4331 are good CLEC12A binding arms.

Example 17

[0092] It was tested whether bispecific molecules containing the 3896 CD3 Fab arm and the CLEC12A Fab arm 3981, 4327 or 4331 were functional.

[0093] For this, the VH sequence of the 3896 CD3 Fab arm and the VH region of either the CLEC12A benchmark antibody, the 3918 CLEC12A Fab, the 4327 CLEC12A Fab or the 4331 CLEC12A Fab were cloned into expression vectors using methods known in the art for production of bispecific IgG1 (Gunasekaran et al., WO2009/089004) in conjunction with the rearranged huVκ1-39 light chain to result in bispecific antibodies; 3896xCLEC12A benchmark, 3896x3918, 3896x4327 and 3896x4331.

[0094] These bispecifics were tested for functionality in the previously described HL60 cytotoxicity assay. Resting T cells from two healthy donors (HD1 and HD2) were co-cultured with CFSE-labeled HL60 cells in the presence of various concentrations of bispecific antibody at an E:T ratio 5:1 or 48 hours in the presence of 10% HS. Surviving CFSE-positive HL60 cells were quantified by flow cytometry at day 2. Results in Figure 17 are expressed as the percentage specific lysis. For the two individual experiments with T cells from donor 1 (HD1; Figure 17 upper panel) and T cells for donor 2 (HD2; figure 17 lower panel), it was demonstrated that all bispecifics were as potent as the 3896xCLEC12A benchmark bispecific when incubated at high concentration.

[0095] Of note, especially at lower concentrations of bispecific antibodies, it was observed that the 3896x4327 and 3896x4331 bispecific antibodies were more potent than the 3896xCLEC12A benchmark bispecific. Hence, as these bispecific antibodies differ only in their CLEC12A Fab arm whilst the CD3 Fab arm is the same, it can be concluded that the functionality of the 4327 and 4331 CLEC12A Fab arms is more potent as compared to the CLEC12A benchmark Fab arm. Without wishing to be bound to theory, the observed differences between the 3896x4327 and 3896x4331 versus the 3896xCLEC12A benchmark bispecific IgG may reflect a difference in binding affinity of these novel anti-CLEC12A Fab arms or they might be targeting a different CLEC12A epitope that allows a more efficient crosslinking of the tumor cells with CD3 expressing T cells.

Example 18

[0096] In example 2 it was demonstrated that the CLEC12A Fabs 3918 and 4331 competed for binding to an epitope on CLEC12A when tested in ELISA as Fab format against Fab fragments of the CLEC12A benchmark antibody. The 4327 CLEC12A Fab, however, did not compete with CLEC12A benchmark IgG for binding in this assay (Table 2).

[0097] In this experiment, it was tested whether the full length IgG of the 4327 CLEC12A IgG competed for binding to CLEC12A with the CLEC12A benchmark antibody. Briefly, HL60 cells were pre-incubated with primary antibody at 50 µg/ml on ice for 20 minutes. Subsequently, Oregon Green (OG)-labeled (Invitrogen, cat.no. A10476) second antibody was added at 1 µg/ml to the cells plus first antibody (concentration of first antibody after addition of OG-labeled IgG -45 µg/ml). After 20 minutes cells were washed and analyzed by FACS.

[0098] The results are shown in Figure 18: it was concluded that 4327 CLEC12A IgG and CLEC12A benchmark IgG compete for binding to CLEC12A. This suggests that both IgGs bind either a closely related epitope on the CLEC12A antigen or that they bind to different epitopes which do not allow simultaneous binding of both IgGs due to steric hindrance.

Example 19

[0099] In previous examples it was demonstrated that the CLEC12A Fab arms 4327, 4331, 3918 as well as 3116 are good binders to CLEC12A and potent inducers of T cell mediated killing in a CD3xCLEC12A bispecific format. So-far, bispecific antibodies were obtained using known methods for driving immunoglobulin heavy chain heterodimerization (Gunasekaran et al.).

[0100] In our co-pending US and PCT applications (US regular application NO: 13/866,747 and PCT/NL2013/050294; incorporated herein by reference) we have disclosed methods and means for producing bispecific antibodies from a single cell, whereby means are provided that favor the formation of bispecific antibodies over the formation of monospecific antibodies. These methods can also be favorably employed in the present invention. Specifically, preferred mutations to produce essentially only bispecific full length IgG molecules are the amino acid substitutions L351K and T366K (numbering according to Kabat) in the first CH3 domain (the 'KK-variant' heavy chain) and the amino acid substitutions L351D and L368E in the second CH3 domain (the 'DE-variant' heavy chain), or vice versa. It was previously demonstrated in our co-pending US 13/866,747 and PCT/NL2013/050294 applications that the DE-variant and KK-variant preferentially pair to form heterodimers (so-called 'DEKK' bispecific molecules). Homodimerization of DE-variant heavy chains (DEDE homodimers) or KK-variant heavy chains

(KKKK homodimers) hardly occurs due to strong repulsion between the charged residues in the CH3-CH3 interface between identical heavy chains.

[0101] To demonstrate that the effect of CD3xCLEC12A bispecific molecules is not influenced by either the known mutations for heterodimerization (Gunasekaran) or the DEKK mutations, the DE-variant and KK-variant heavy chains were used to drive heterodimerization of the different heavy chains for making CD3xCLEC12A bispecifics. In addition the CH2 / lower hinge double mutations (L235G and G236R; DM) were introduced in these DE- and KK-variant heavy chains. The Fc tail of these resulting bispecific molecules is referred to as 'DM DEKK'.

[0102] Briefly, the VH regions of either the 3116, 4327 or 4331 CLEC12A Fab arms were cloned into expression vectors containing the DE-variant + DM heavy chain whereas the VH region of the 3056 CD3 antibody was cloned into an expression vector containing the KK-variant + DM heavy chain (US regular application NO: 13/866,747 and PCT/NL2013/050294) and these expression vectors, together with a nucleic acid molecule encoding the rearranged human IGKV1-39/IGKJ1 (huVκ1-39) light chain, were provided to a host cell such that the host cell expressed and produced bispecific antibodies. The resulting 3056x3116 DM DEKK, 3056x4327 DM DEKK and 3056x4331 DM DEKK bispecific antibodies were subsequently tested for potency in the HL60 cytotoxicity assay as previously described. The results are shown in figure 19: it was demonstrated that all variants are still capable of efficient tumor cell lysis and it was thus concluded that the DM and DEKK mutations can be introduced into the Fc region of the CD3xCLEC12A bispecific antibody, while maintaining the capacity of inducing tumor cell lysis.

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Patentkrav

1. Bispecifikt humant IgG-fuldlængdeantistof, hvor det bispecifikke IgG-antistof omfatter én arm, der specifikt genkender CLEC12A, og en anden arm, der specifikt genkender CD3, og hvor den arm, der specifikt genkender CLEC12A, omfatter en variabel tungkædesekvens bestående af en sekvens, der er identisk med QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGSTSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCAKGTTGDWFDYWGQGTLVTVSS; eller en variabel tungkædesekvens, der består af en sekvens, der er identisk med EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGSTSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGNYGDEFDYWGQGTLVTVSS, og hvor begge arme omfatter en fælles letkæde, der omfatter en variabel letkædesekvens, der består af en sekvens, der er identisk med DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPPTFGQGTKVEIK.

2. Bispecifikt IgG-antistof ifølge krav 1, hvor antistoffet specifikt genkender CD3ε.

3. Bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-2, hvor det bispecifikke antistof er et humant IgGl.

4. Bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-3, hvor den anden arm, der specifikt genkender CD3, omfatter en tungkæde-CDR1-sekvens, der består af sekvensen SYGMH, og en tungkæde-CDR2-sekvens, der består af sekvensen IIWYSGSKKNYADSVKG, og en tungkæde-CDR3-sekvens, der består af sekvensen GTGYNWFDP.

5. Bispecifikt IgG-antistof ifølge krav 4, hvor den anden arm, der specifikt genkender CD3, omfatter en variabel tungkædesekvens, der består af sekvensen QVQLVESGGGVVQPGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAIIWYSGSKKNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWGQGTLVTVSS.

6. Bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-5, hvor det bispecifikke IgG-antistof har muterede CH2- og/eller lavere hængselsdomæner, således at interaktion af det bispecifikke IgG-antistof med Fcy-receptorer reduceres signifikant.

7. Bispecifikt IgG-antistof ifølge krav 6, hvor de muterede CH2- og/eller lavere hængselsdomæner omfatter én substitution ved aminosyrepositionen 235 og/eller 236

(nummerering ifølge Kabat).

8. Bispecifikt IgG-antistof ifølge krav 6 eller 7, hvor de muterede CH2- og/eller lavere hængselsdomæner omfatter substitution L235G og/eller G236R, fortrinsvis L235G og G236R.

9. Fremgangsmåde til fremstilling af et bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-8 ud fra en enkelt celle, hvor det bispecifikke IgG-antistof omfatter to CH3-domæner, der er i stand til at danne en grænseflade, hvor fremgangsmåden omfatter tilvejebringelse af:

- en celle med a) en første nukleinsyresekvens, der koder for den IgG-tungkæde, der specifikt genkender CLEC12A, og der indeholder et første CH3-domæne, og b) en anden nukleinsyresekvens, der koder for den IgG-tungkæde, der specifikt genkender CD3, og der indeholder et andet CH3-domæne, hvor cellen har en tredje nukleinsyresekvens, der koder for den fælles letkæde, og hvor nukleinsyresekvenserne er forsynet med mutationer for præferentiel parring af det første og det andet CH3-domæne, hvilken fremgangsmåde endvidere omfatter trinnet med dyrkning af cellen og at tillade ekspression af nukleinsyresekvenserne og høst af det bispecifikke IgG-antistof fra kulturen.

10. Fremgangsmåde ifølge krav 9 til fremstilling af et bispecifikt IgG1-antistof, hvor det første CH3-domæne omfatter aminosyresubstitutionerne L351K og T366K (nummerering ifølge Rabat), og hvor det andet CH3-domæne omfatter aminosyresubstitutionerne L351D og L368E, hvor fremgangsmåden endvidere omfatter trinnet med dyrkning af cellen og at tillade ekspression af nukleinsyresekvenserne og høst af det bispecifikke antistof fra kulturen.

11. Farmaceutisk sammensætning, der omfatter et bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-8 og en farmaceutisk acceptabel bærer.

12. Bispecifikt IgG-antistof ifølge et hvilket som helst af kravene 1-8 til anvendelse som et farmaceutisk middel i behandlingen af myelodysplastisk syndrom (MDS), kronisk myelogen leukæmi (CML) eller fortrinsvis akut myeloid leukæmi (AML).

DRAWINGS

CLL-1: predicted aa sequence Bakker et al.2004
KLRL1: translated aa sequence Han et al.2004 (= Zhang/Cao group; deposited under no. AF247788)
MICL: predicted aa sequence Marshall et al.2004 (deposited under no. AY498550)
DCAL-2: predicted aa sequence Chen et al.2006 (= E.A. Clark group deposited under no. AY426759)
CLEC12B: Predicted aa sequence H.F. Clark et al. 2003 (deposited under no. AY358810)

```
KLRL1     ----------MSEEVTYADLQFQNSSEMEKIPEIGKFGEKAPPAPSHVWRPAALFLTLLCLLLLIGLGVLASMFHVTLK---IEMKKMNKLQNI
MICL      ----------MSEEVTYADLQFQNSSEMEKIPEIGKFGEKAPPAPSHVWRPAALFLTLLCLLLLIGLGVLASMFHVTLK---IEMKKMNKLQNI
DCAL-2    ----------MSEEVTYADLQFQNSSEMEKIPEIGKFGEKAPPAPSHVWRPAALFLTLLCLLLLIGLGVLASMFHVTLK---IEMKKMNKLQNI
CLL-1     MWIDFFTYSSMSEEVTYADLQFQNSSEMEKIPEIGKFGEKAPPAPSHVWRPAALFLTLLCLLLLIGLGVLASMFHVTLK---IEMKKMNKLQNI
CLEC12B   ----------MSEEVTYATLTFQDSAGARNNRDGNNLRKRGHPAPSPIWRHAALGLVTLCLMLLIGLVTLGMMFLQISNDINSDSEKLSQLQKT

KLRL1     SEELQRNISLQLMSNMNI-------SNKIRNLSTTLQTIATKLCRELYSKEQEHKCKPCPRRWIWHKDSCYFLS-DDVQTWQESKMACAAQNAS
MICL      SEELQRNISLQLMSNMNI-------SNKIRNLSTTLQTIATKLCRELYSKEQEHKCKPCPRRWIWHKDSCYFLS-DDVQTWQESKMACAAQNAS
DCAL-2    SEELQRNISLQLMSNMNI-------SNKIRNLSTTLQTIATKLCRELYSKEQEHKCKPCPRRWIWHKDSCYFLS-DDVQTWQESKMACAAQNAS
CLL-1     SEELQRNISLQLMSNMNI-------SNKIRNLSTTLQTIATKLCRELYSKEQEHKCKPCPRRWIWHKDSCYFLS-DDVQTWQESKMACAAQNAS
CLEC12B   IQQQQDNLSQQLGNSNNLSMEEEFLKSQISSVLKRQEQMAIKLCQELIIHTSDHRCNPCPKMWQWYQNSCYYFTTNEEKTWANSRKDCIDKNST

KLRL1     LLKINNKNALEFIKSQSRS--YDYWLGLSPEEDSTRGMRVDNIINSSAW-VIRNAPDL-NNMYCGYINRLYVQYYHCTYKQRMICEKMANPVQL
MICL      LLKINNKNALEFIKSQSRS--YDYWLGLSPEEDSTRGMRVDNIINSSAW-VIRNAPDL-NNMYCGYINRLYVQYYHCTYKQRMICEKMANPVQL
DCAL-2    LLKINNKNALEFIKSQSRS--YDYWLGLSPEEDSTRGMRVDNIINSSAW-VIRNAPDL-NNMYCGYINRLYVQYYHCTYKQRMICEKMANPVQL
CLL-1     LLKINNKNALEFIKSQSRS--YDYWLGLSPEEDSTRGMRVDNIINSSAW-VIRNAPDL-NNMYCGYINRLYVQYYHCTYKKRMICEKMANPVQL
CLEC12B   LVKIDSLEEKDFLMSQPLLMFSFFWLGLSWDSSGRSWFWEDGSVPSPSLFSTKELDQINGSKGCAYFQKGNIYISRCSAEIFWICEKTAAPVKT

KLRL1     GSTYFREA
MICL      GSTYFREA
DCAL-2    GSTYFREA
CLL-1     GSTYFREA
CLEC12B   EDLD----
```

FIG. 1

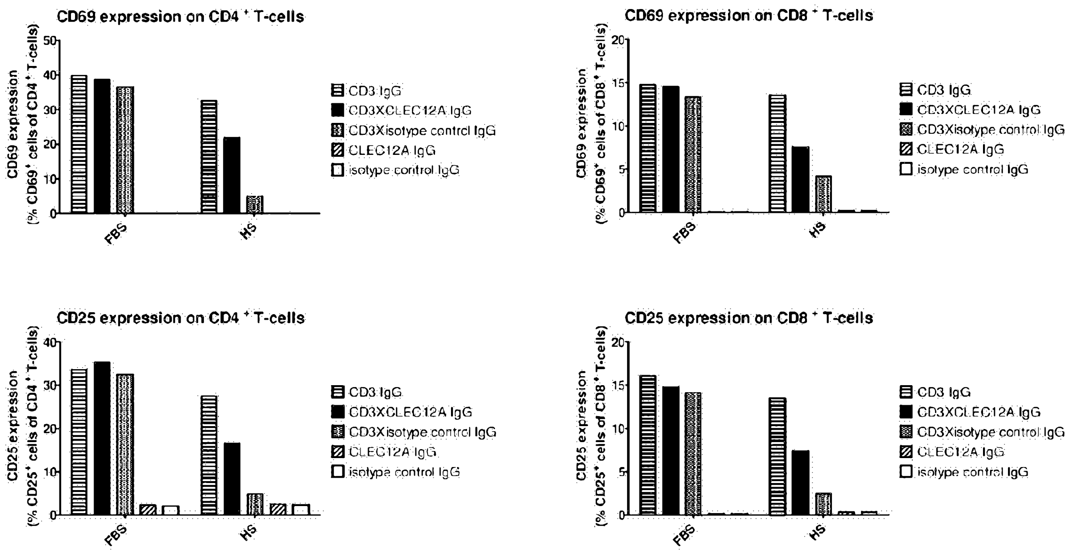

CD69 expression on CD4 + T-cells
CD69 expression
(% CD69+ cells of CD4 + T-cells)
0
10
20
30
40
50
FBS
HS
CD69 expression on CD8 + T-cells
CD69 expression
(% CD69+ cells of CD8+ T-cells)
0
5
10
15
20
CD25 expression on CD4 + T-cells
CD25 expression
(% CD25+ cells of CD4 + T-cells)
0
10
20
30
40
CD25 expression on CD8 + T-cells
CD25 expression
(% CD25+ cells of CD8+ T-cells)
▤ CD3 IgG
■ CD3XCLEC12A IgG
▩ CD3Xisotype control IgG
▨ CLEC12A IgG
☐ isotype control IgG

FIG. 2

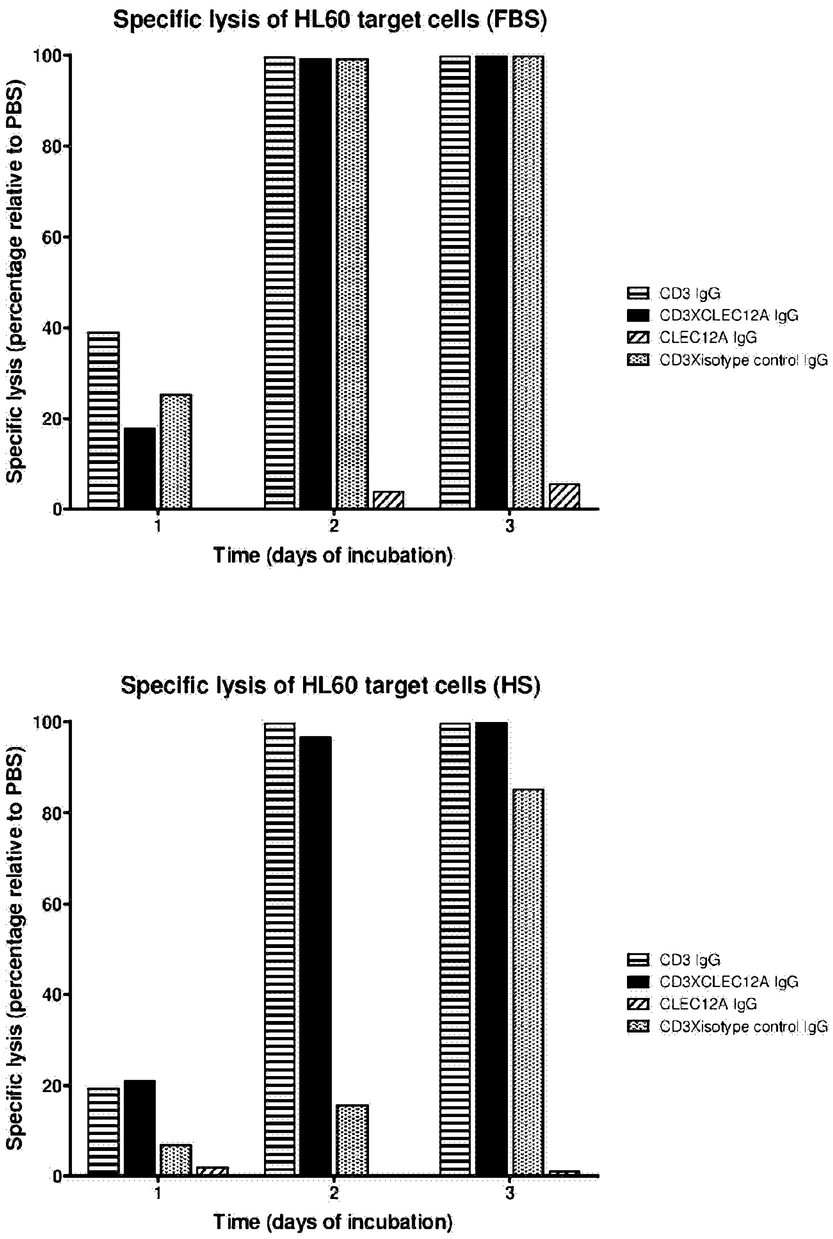

Specific lysis of HL60 target cells (FBS)
Specific lysis (percentage relative to PBS)
100
80
60
40
20
0
1
2
3
Time (days of incubation)
CD3 IgG
CD3XCLEC12A IgG
CLEC12A IgG
CD3Xisotype control IgG
Specific lysis of HL60 target cells (HS)
Specific lysis (percentage relative to PBS)
100
80
60
40
20
0
1
2
3
Time (days of incubation)
CD3 IgG
CD3XCLEC12A IgG
CLEC12A IgG
CD3Xisotype control IgG

FIG. 3

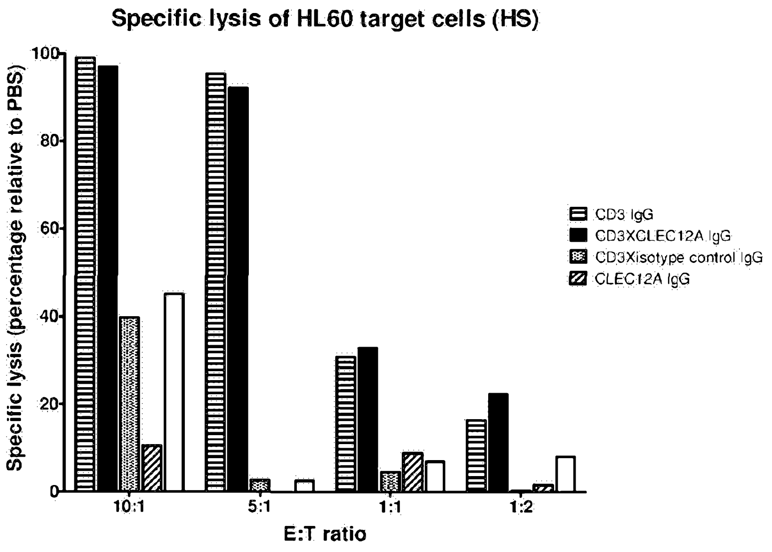

Specific lysis of HL60 target cells (HS)
Specific lysis (percentage relative to PBS)
100
80
60
40
20
0
10:1
5:1
1:1
1:2
E:T ratio
CD3 IgG
CD3XCLEC12A IgG
CD3Xisotype control IgG
CLEC12A IgG

FIG. 4

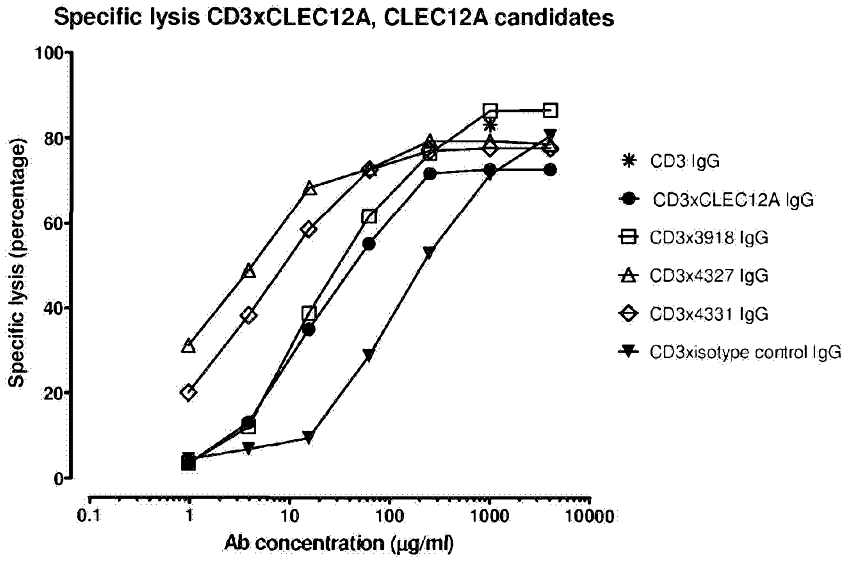

Specific lysis CD3xCLEC12A, CLEC12A candidates
Specific lysis (percentage)
100
80
60
40
20
0
0.1
1
10
100
1000
10000
Ab concentration (µg/ml)
CD3 IgG
CD3xCLEC12A IgG
CD3x3918 IgG
CD3x4327 IgG
CD3x4331 IgG
CD3xisotype control IgG

FIG. 5

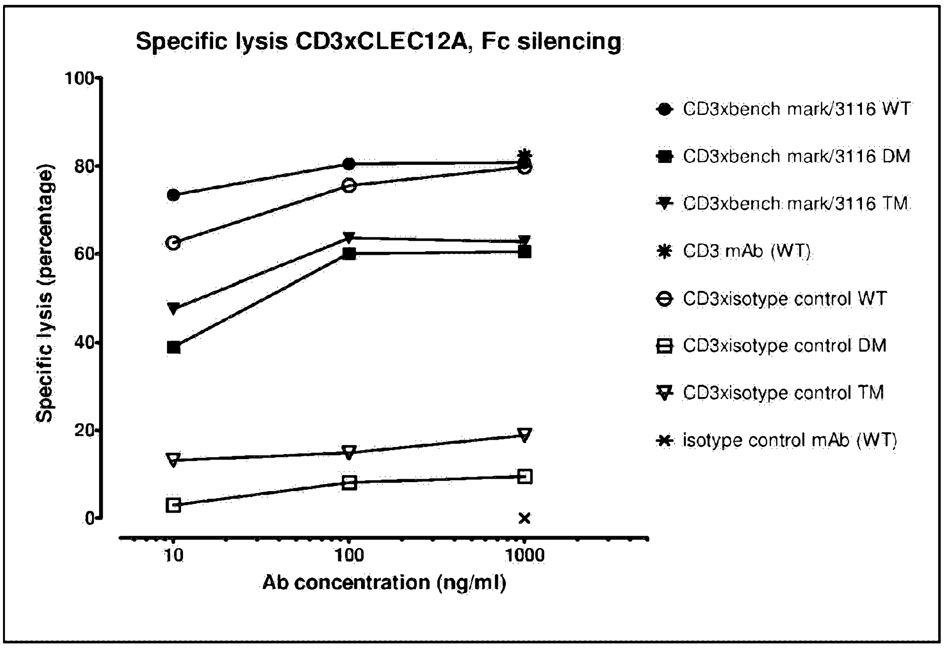

Specific lysis CD3xCLEC12A, Fc silencing
Specific lysis (percentage)
100
80
60
40
20
0
10
100
1000
Ab concentration (ng/ml)
● CD3xbench mark/3116 WT
■ CD3xbench mark/3116 DM
▼ CD3xbench mark/3116 TM
✱ CD3 mAb (WT)
⊖ CD3xisotype control WT
⊟ CD3xisotype control DM
▽ CD3xisotype control TM
✕ isotype control mAb (WT)

FIG. 6

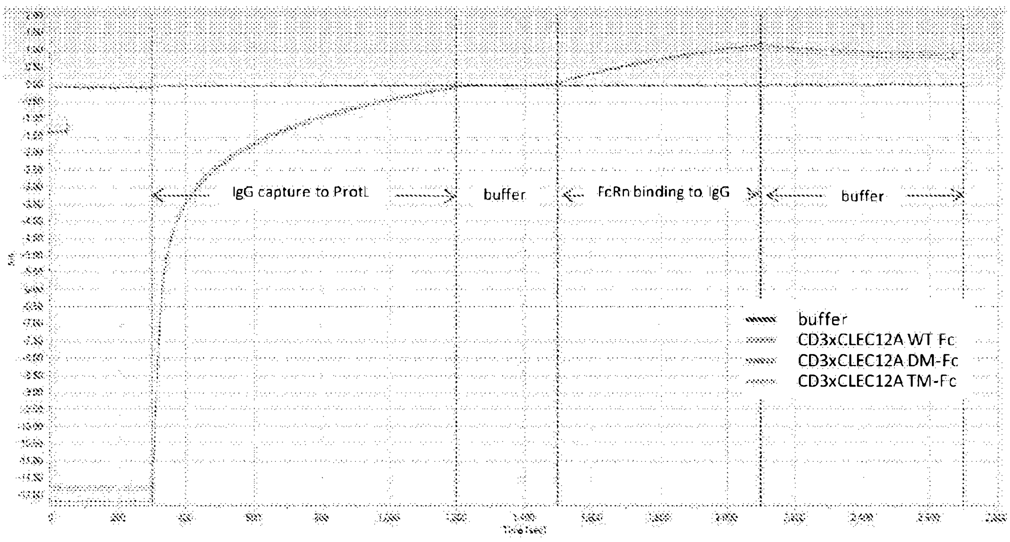

IgG capture to ProtL
buffer
FcRn binding to IgG
buffer
buffer
CD3xCLEC12A WT Fc
CD3xCLEC12A DM-Fc
CD3xCLEC12A TM-Fc

FIG. 7

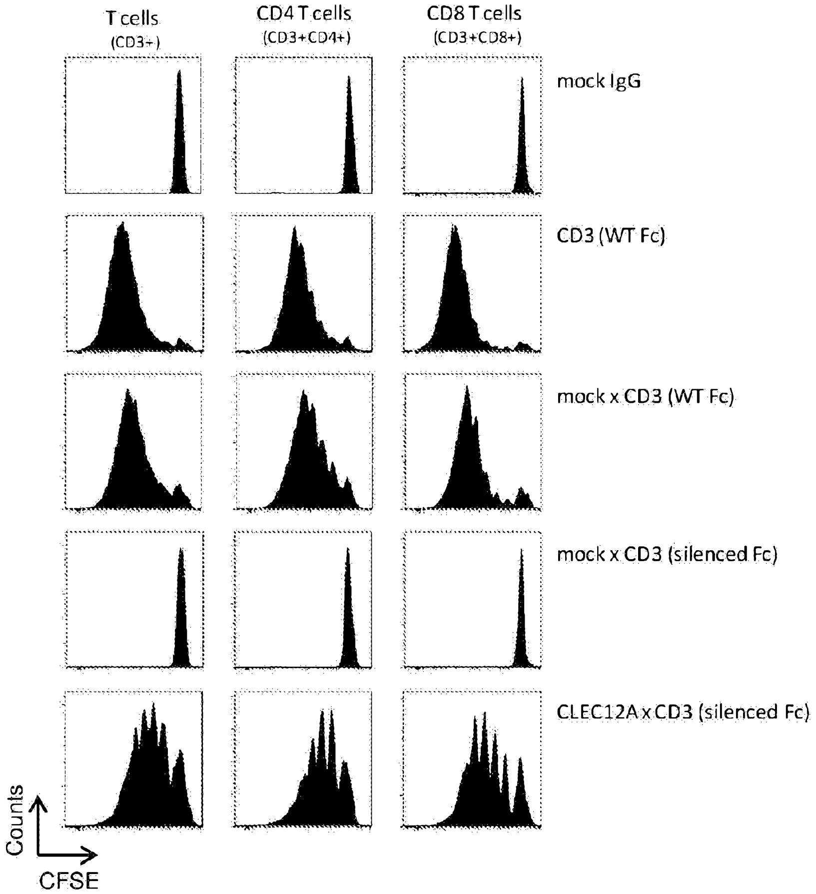

T cells
(CD3+)
CD4 T cells
(CD3+CD4+)
CD8 T cells
(CD3+CD8+)
mock IgG
CD3 (WT Fc)
mock x CD3 (WT Fc)
mock x CD3 (silenced Fc)
CLEC12A x CD3 (silenced Fc)
Counts
CFSE

FIG. 8

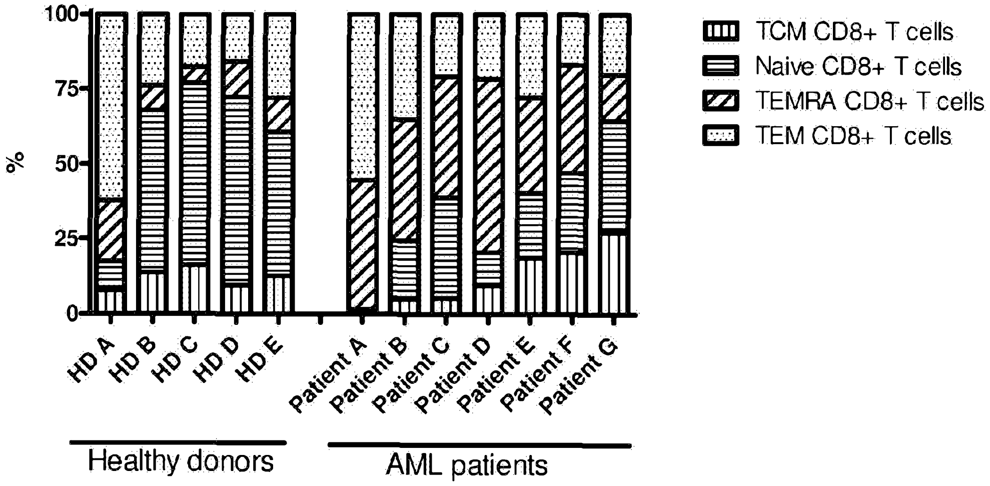

100
75
50
25
0
%
HD A
HD B
HD C
HD D
HD E
Patient A
Patient B
Patient C
Patient D
Patient E
Patient F
Patient G
Healthy donors
AML patients
TCM CD8+ T cells
Naive CD8+ T cells
TEMRA CD8+ T cells
TEM CD8+ T cells

FIG. 9

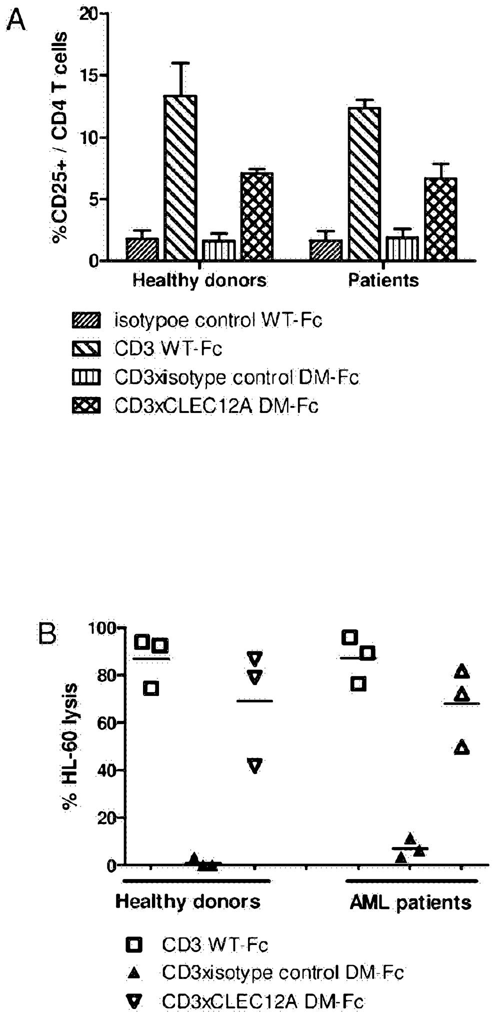

A
%CD25+ / CD4 T cells
20
15
10
5
0
Healthy donors
Patients
▨ isotypoe control WT-Fc
▧ CD3 WT-Fc
▥ CD3xisotype control DM-Fc
▩ CD3xCLEC12A DM-Fc
B
% HL-60 lysis
100
80
60
40
20
0
Healthy donors
AML patients
□ CD3 WT-Fc
▲ CD3xisotype control DM-Fc
▽ CD3xCLEC12A DM-Fc

FIG. 10

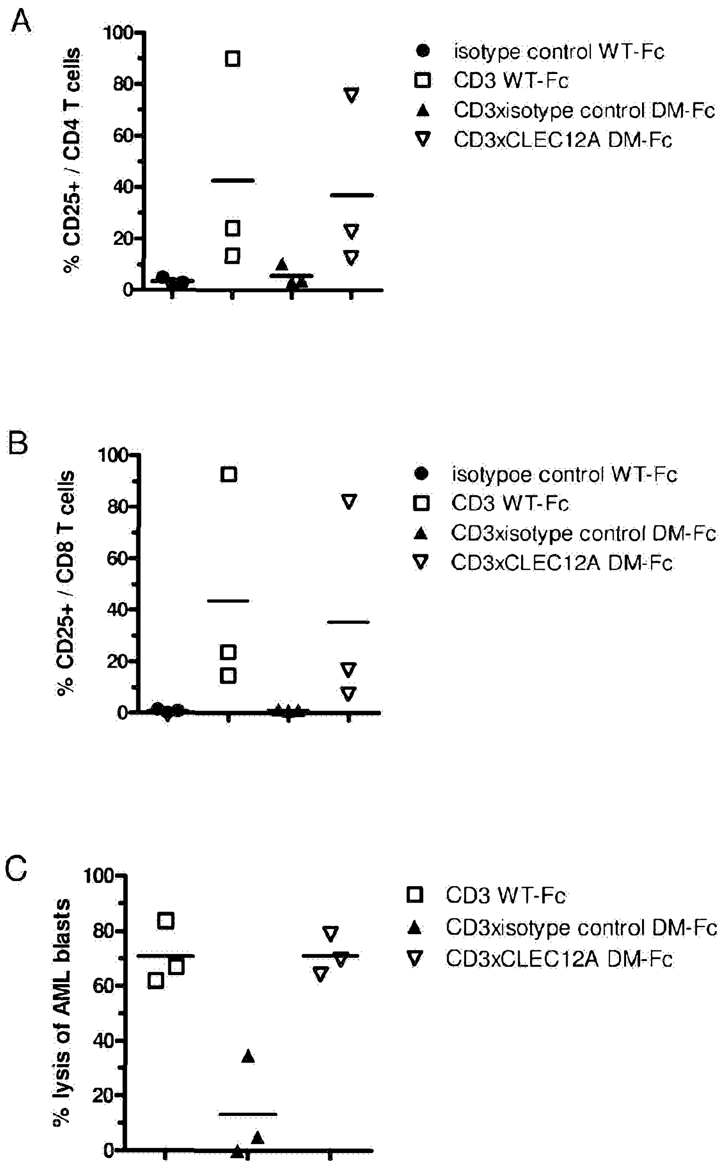

A
% CD25+ / CD4 T cells
100
80
60
40
20
0
● isotype control WT-Fc
□ CD3 WT-Fc
▲ CD3xisotype control DM-Fc
▽ CD3xCLEC12A DM-Fc
B
% CD25+ / CD8 T cells
100
80
60
40
20
0
● isotypoe control WT-Fc
□ CD3 WT-Fc
▲ CD3xisotype control DM-Fc
▽ CD3xCLEC12A DM-Fc
C
% lysis of AML blasts
100
80
60
40
20
0
□ CD3 WT-Fc
▲ CD3xisotype control DM-Fc
▽ CD3xCLEC12A DM-Fc

FIG. 11

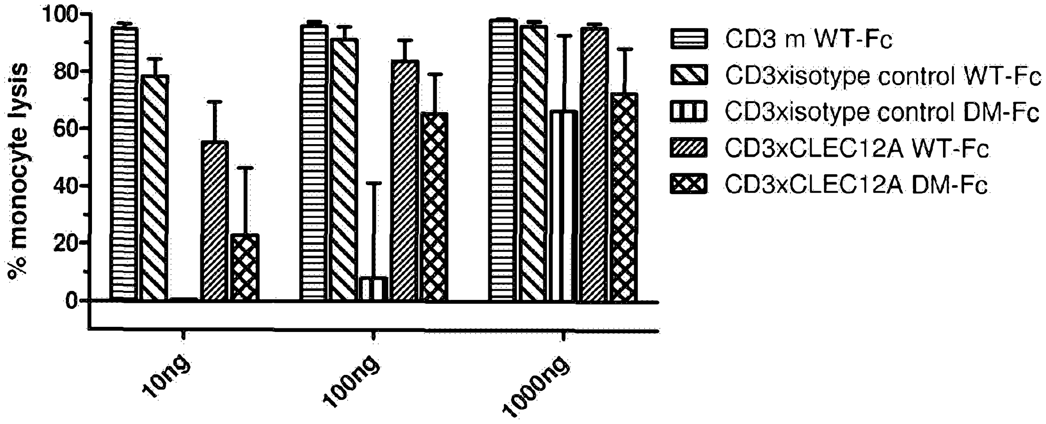
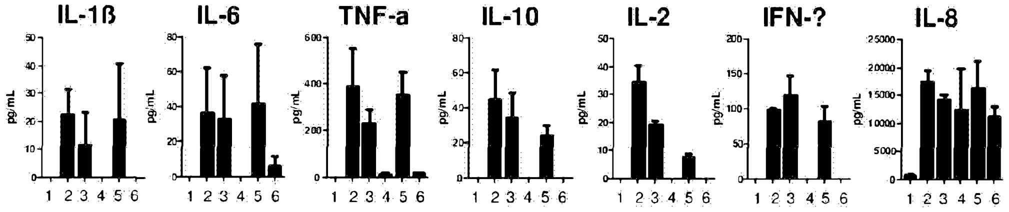


FIG. 12



1: isotype control
2: CD3 mAb (WT)
3: CD3xisotype control bsAb (WT)
4: CD3xisotype control bsAb (DM)
5: CD3xCLEC12A bsAb (WT)
6: CD3xCLEC12A bsAb (DM)

FIG. 13

Figure 14A

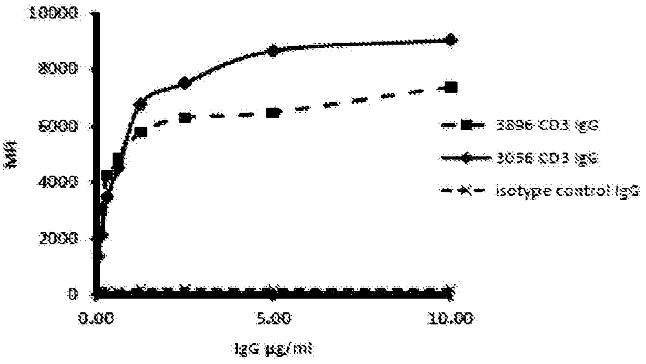


Figure 14B

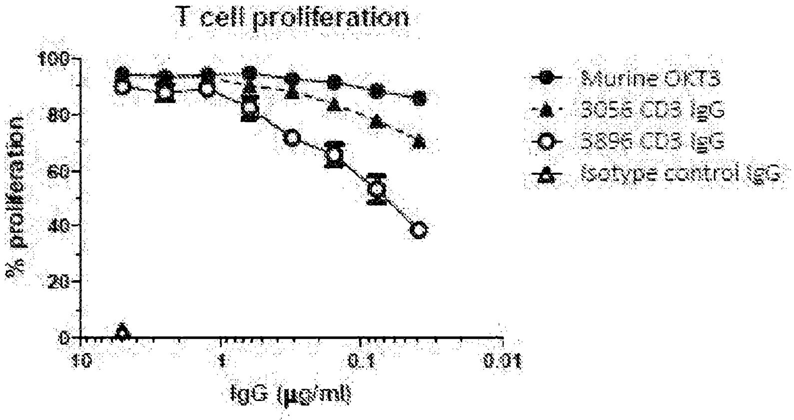


Figure 15

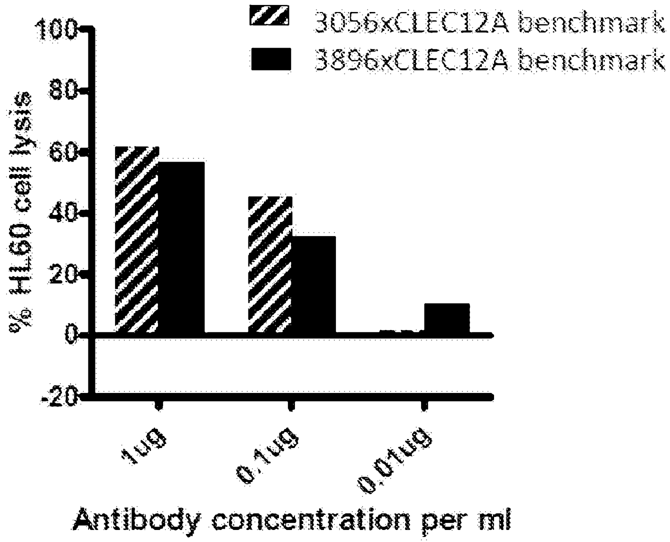


Figure 16

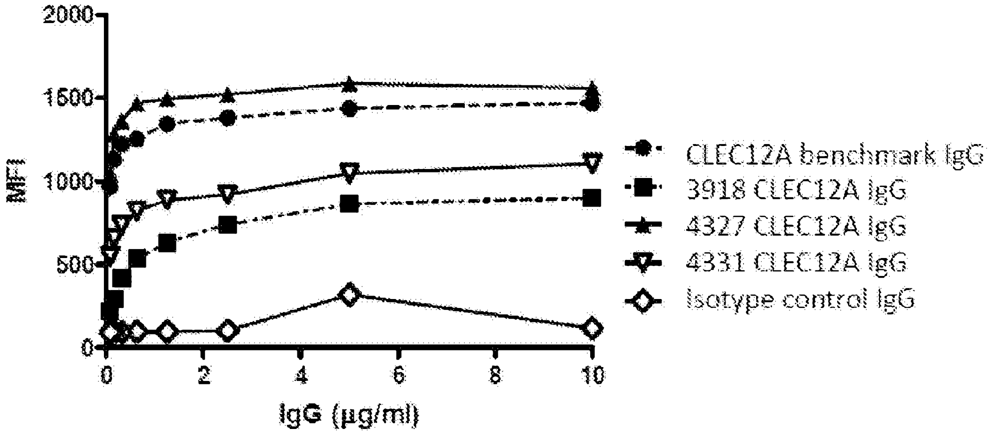


Figure 17

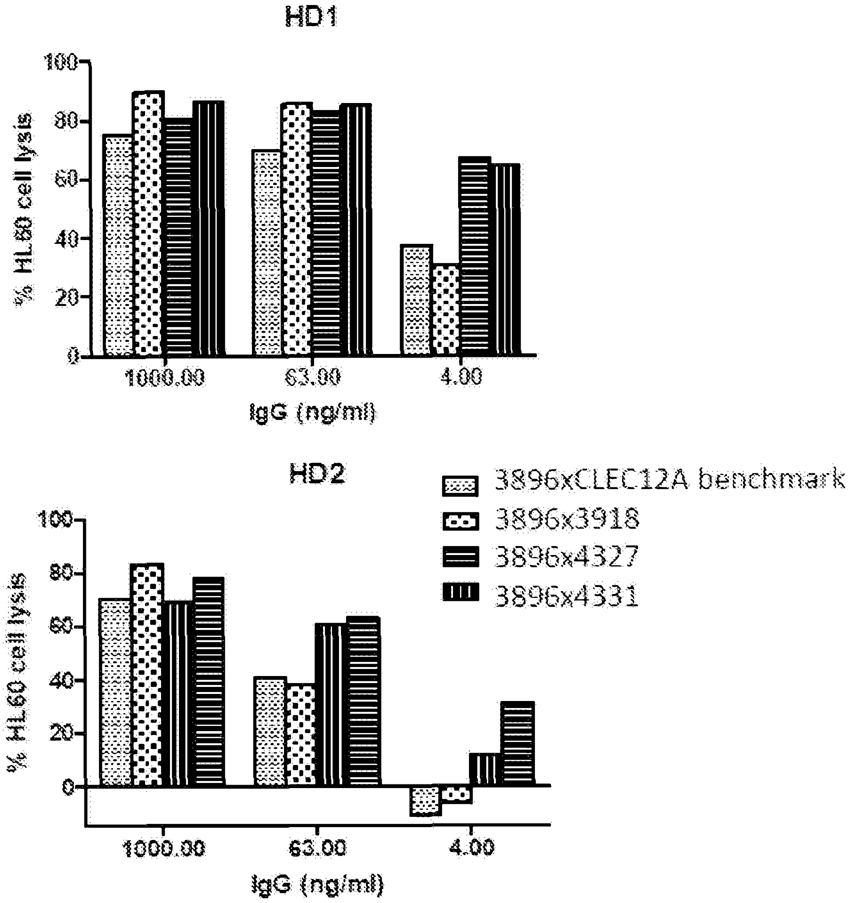

HD1
100
80
60
40
20
0
% HL60 cell lysis
1000.00
63.00
4.00
IgG (ng/ml)
HD2
100
80
60
40
20
0
% HL60 cell lysis
1000.00
63.00
4.00
IgG (ng/ml)
3896xCLEC12A benchmark
3896x3918
3896x4327
3896x4331

Figure 18

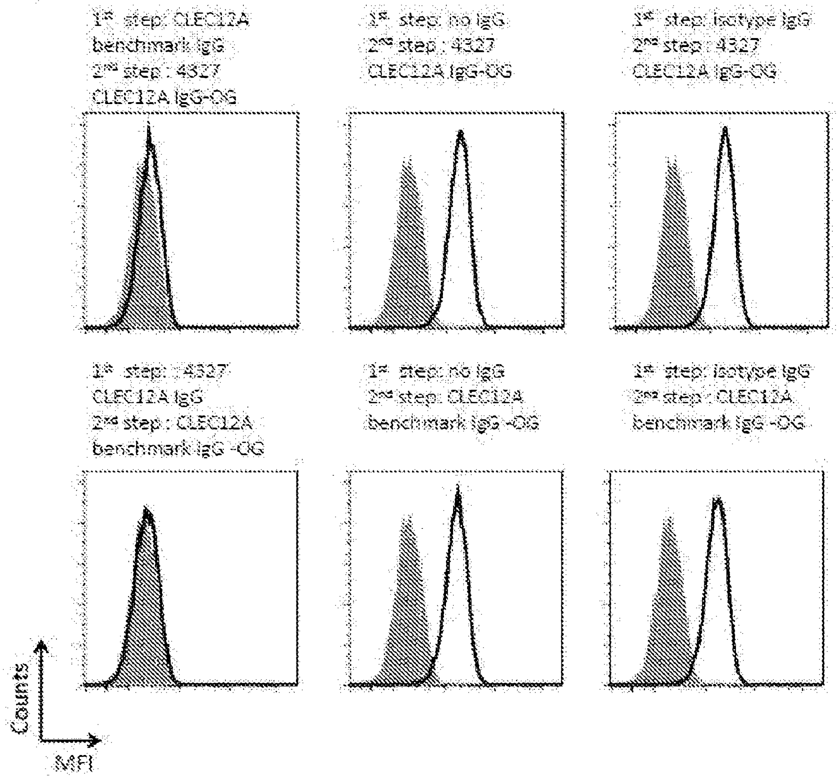

1st step: CLEC12A benchmark IgG
2nd step : 4327 CLEC12A IgG-OG
1st step: no IgG
2nd step : 4327 CLEC12A IgG-OG
1st step: isotype IgG
2nd step : 4327 CLEC12A IgG-OG
1st step : 4327 CLEC12A IgG
2nd step : CLEC12A benchmark IgG -OG
1st step: no IgG
2nd step: CLEC12A benchmark IgG -OG
1st step: isotype IgG
2nd step : CLEC12A benchmark IgG -OG
Counts
MFI

Figure 19

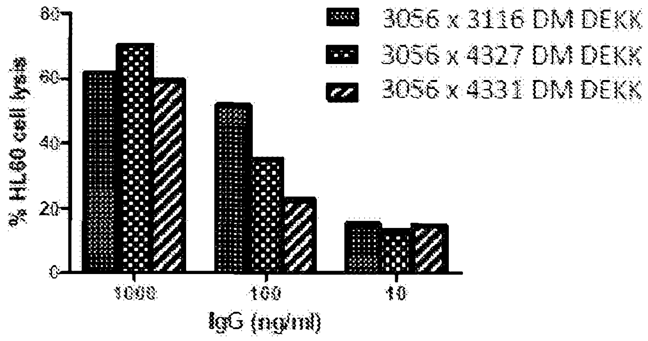

80
60
40
20
0
% HL60 cell lysis
1000
100
10
IgG (ng/ml)
3056 x 3116 DM DEKK
3056 x 4327 DM DEKK
3056 x 4331 DM DEKK

Figure 20

3056
VH (VDJ):

caggtgcagctggtgcagtctggcggcggagtggtgcagcccggcagaagcctgagactgagctgcgtgg
ccagcggcttcaccttcagcagctacggcatgcactgggtccgccaggcccctggcaagggactggaatg
ggtggccgccatctggtacaacggccggaagcaggactacgccgacagcgtgaagggccggttcaccatc
agccgggacaacagcaagaacaccctgtacctgcagatgaacagcctccgggccgaggacaccgccgtgt
actactgtacccggggcaccggctacaattggttcgaccccttggggccagggcaccctggtcaccgtctc
cagt

QVQLVQSGGGVVQPGRSLRLSCVASGFTFSSYGMHWVRQAPGKGLEWVAAIWYNGRKQDYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCTRGTGYNWFDPWGQGTLVTVSS

VL/O12 (VJ):

Gacatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatcacttgccgggca
agtcagagcattagcagctacttaaattggtatcagcagaaaccagggaaagcccctaagctcctgatctatgct
gcatccagtttgcaaagtggggtcccatcaaggttcagtggcagtggatctgggacagatttcactctcaccatc
agcagtctgcaacctgaagattttgcaacttactactgtcaacagagttacagtacccctccaacgttcggccaa
gggaccaaggtggagatcaaac

DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTD
FTLTISSLQPEDFATYYCQQSYSTPPTFGQGTKVEIK

3896
VH:

caggtgcagctggtggagtctggcggcggagtggtgcagcccggcagaagcctgagactgagctgcgccg
ccagcggcttcaccttcagaagctacggcatgcactgggtccgccaggcccctggcaagggactggaatg
ggtggccatcatctggtacagcggcagcaagaagaactacgccgacagcgtgaagggccggttcaccatc
agccgggacaacagcaagaacaccctgtacctgcagatgaacagcctccgggccgaggacaccgccgtgt
actactgtgcccggggcaccggctacaattggttcgaccccttggggccagggcaccctggtcaccgtctc
cagt

QVQLVESGGGVVQPGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAIIWYSGSKKNYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCARGTGYNWFDPWGQGTLVTVSS

VL:
O12

CLEC12A BENCHMARK (3116)

VH:

caggtgcagctgcaggaatctggacccggactggtcaagcccagcgagacactgagcctgacctgtgtgg
tgtccggcggcagcatcagcagcagcaattggtggtcttgggtccgacagccccctggcaagggcctgga
atggatcggcgagatctaccacagcggcagccccgactacaaccccagcctgaagtccagagtgaccatc
agcgtggacaagagccggaaccagttcagcctgaagctgagcagcgtgacagccgccgataccgccgtgt
actactgcgccaaagtgtccaccggcggctttttcgactactggggccagggcacactggtcaccgtctc
cagt

QVQLQESGPGLVKPSETLSLTCVVSGGSISSSNWWSWVRQPPGKGLEWIGEIYHSGSPDYNPSLKSRVTI
SVDKSRNQFSLKLSSVTAADTAVYYCAKVSTGGFFDYWGQGTLVTSS

VL:

O12

3918

VH:

caggtgcagctggtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaggtctcctgcaagg
cttctggatacaccttcaccggctactatatgcactgggtgcgacaggcccctggacaagggcttgagtg
gatgggatggatcaaccctaacagtggtggcacaaactatgcacagaagtttcagggcagggtcacgatg
accagggacacgtccatcagcacagcctacatggagctgagcaggctgagatctgacgacacggccgtgt
attactgtgcaagagatggttacttcgctgacgcctttgattattggggccaaggtaccctggtcaccgt
ctccagt

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPNSGGTNYAQKFQGRVTM
TRDTSISTAYMELSRLRSDDTAVYYCARDGYFADAFDYWGQGTLVTVSS

VL:

O12

4327

VH:

caggtgcagctggtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaggtttcctgcaagg
catctggatacaccttcaccagctactatatgcactgggtgcgacaggcccctggacaagggcttgagtg
gatgggaataatcaaccctagtggtggtagcacaagctacgcacagaagttccagggcagagtcaccatg
accagggacacgtccacgagcacagtctacatggagctgagcagcctgagatctgaggacacggccgtgt
attactgtgcaaagggcactactggtgattggtttgactactggggccaaggtaccctggtcaccgtctc
cagt

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGSTSYAQKFQGRVTM
TRDTSTSTVYMELSSLRSEDTAVYYCAKGTTGDWFDYWGQGTLVTVSS

VL:
O12

4331
VH:

```
gaggtccagctggtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaggtttcctgcaagg
catctggatacaccttcaccagctactatatgcactgggtgcgacaggcccctggacaagggcttgagtg
gatgggaataatcaaccctagtggtggtagcacaagctacgcacagaagttccagggcagagtcaccatg
accagggacacgtccacgagcacagtctacatggagctgagcagcctgagatctgaggacacggccgtgt
attactgtgcaaggggcaattatggtgatgagtttgactactggggccaaggtaccctggtcaccgtctc
cagt
```

```
EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGSTSYAQKFQGRVTM
TRDTSTSTVYMELSSLRSEDTAVYYCARGNYGDEFDYWGQGTLVTVSS
```

VL:
O12