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DESCRIPTION

Introduction

[0001] The present invention relates to the modification of natural killer (NK) cells and NK cell lines to produce derivatives thereof with a more cytotoxic phenotype. Furthermore, the present invention relates to methods of producing modified NK cells and NK cell lines, compositions containing the cells and cell lines and uses of said compositions in the treatment of cancer.

Background to the Invention

[0002] Typically, immune cells require a target cell to present antigen via major histocompatibility complex (MHC) before triggering an immune response resulting in the death of the target cell. This allows cancer cells not presenting MHC class I to evade the majority of immune responses.

[0003] NK cells are able, however, to recognize cancer cells in the absence of MHC class I expression. Hence they perform a critical role in the body's defence against cancer.

[0004] On the other hand, in certain circumstances, cancer cells demonstrate an ability to dampen the cytotoxic activity of NK cells, through expression of ligands that bind inhibitory receptors on the NK cell membrane. Resistance to cancer can involve a balance between these and other factors.

[0005] Cytotoxicity, in this context, refers to the ability of immune effector cells, e.g. NK cells, to induce cancer cell death, e.g. by releasing cytolytic compounds or by binding receptors on cancer cell membranes and inducing apoptosis of said cancer cells. Cytotoxicity is affected not only by signals that induce release of cytolytic compounds but also by signals that inhibit their release. An increase in cytotoxicity will therefore lead to more efficient killing of cancer cells, with less chance of the cancer cell dampening the cytotoxic activity of the NK, as mentioned above.

[0006] Genetic modification to remove inhibitory receptor function on NK cells has been suggested as a method for increasing the cytotoxicity of NK cells against cancer cells that lack MHC class I expression but are able to dampen NK cytotoxicity (Bodduluru *et al.* 2012). NKG2A has been established as an inhibitory receptor worth silencing under these circumstances, as certain cancer cells are known to express MICA which binds NKG2A and inhibits NK cell cytotoxicity in the absence of MHC class I expression (Shook *et al.* 2011; WO 2006/023148).

[0007] Another method of downregulating NKG2A expression has been shown in NK-92 cells,

in which transfection with a gene encoding IL-15 was shown to be associated with a reduction in NKG2A expression (Zhang et al. 2004). However, despite an observed increase in the cytotoxicity of the NK cells, the increase was likely a result of a concomitant increase in expression of the activating receptor NKG2D. This is supported by the observation that blocking NKG2A receptors on NK-92 cells was not associated with an increase in cytotoxicity against multiple myeloma cells (Heidenreich et al. 2012). Nevertheless, it is worth noting that the NK-92 cell line is a highly cytotoxic cell line with very low expression of inhibitory receptors. Therefore, any increase in cytotoxicity associated with decreased NKG2A expression might have been too trivial to detect.

[0008] Similar studies have been carried out in mice. For example, mice express a receptor called Ly49 on NK cells, which is analogous to human inhibitory KIR receptors. It has been shown that by blocking the Ly49 receptor with antibody fragments, NK cells are more cytotoxic and capable of killing murine leukemia cells *in vitro* and *in vivo* (Koh et al. 2001).

[0009] It is a consequence of reducing inhibitory receptor function, however, that 'normal' cells in the body also become more susceptible to attack by modified NK cells, as the modified NK cells become less capable of distinguishing between 'normal' cells and cancer cells. This is a significant disadvantage of reducing 'classical' inhibitory receptor function.

[0010] Another way in which NK cells are known to kill cancer cells is by expressing TRAIL on their surface. TRAIL ligand is able to bind TRAIL receptors on cancer cells and induce apoptosis of said cancer cells. One speculative approach describes overexpressing TRAIL on NK cells, in order to take advantage of this anti-cancer mechanism (EP1621550). Furthermore, IL-12 has been reported to upregulate TRAIL expression on NK cells (Smyth et al. 2001). Albeit, NK cells themselves express TRAIL receptors and are thus a potential target for TRAIL-mediated killing (Mirandola et al. 2004). Nevertheless, cancer cells have developed evasive and protective mechanisms for dealing with NK cells expressing TRAIL. Decoy TRAIL receptors are often expressed on cancer cell membranes, and binding of TRAIL to these decoy receptors is unable to induce apoptosis; methods of overcoming such mechanisms have not yet been pursued.

[0011] Acute myeloid leukemia (AML) is a hematopoietic malignancy involving precursor cells committed to myeloid development, and accounts for a significant proportion of acute leukemias in both adults (90%) and children (15-20%) (Hurwitz, Mounce et al. 1995; Lowenberg, Downing et al. 1999). Despite 80% of patients achieving remission with standard chemotherapy (Hurwitz, Mounce et al. 1995; Ribeiro, Razzouk et al. 2005), survival remains unsatisfactory because of high relapse rates from minimal residual disease (MRD). The five-year survival is age-dependent; 60% in children (Rubnitz 2012), 40% in adults under 65 (Lowenberg, Downing et al. 1999) and 10% in adults over 65 (Ferrara and Schiffer 2013). These outcomes can be improved if patients have a suitable hematopoietic cell donor, but many do not, highlighting the need for an alternative approach to treatment.

[0012] Natural killer (NK) cells are cytotoxic lymphocytes, with distinct phenotypes and effector

functions that differ from e.g. natural killer T (NK-T) cells. For example, while NK-T cells express both CD3 and T cell antigen receptors (TCRs), NK cells do not. NK cells are generally found to express the markers CD16 and CD56, wherein CD16 functions as an Fc receptor and mediates antibody dependent cell-mediated cytotoxicity (ADCC) which is discussed below. KHYG-1 is a notable exception in this regard. Despite NK cells being naturally cytotoxic, NK cell lines with increased cytotoxicity have been developed. NK-92 and KHYG-1 represent two NK cell lines that have been researched extensively and show promise in cancer therapeutics (Swift et al. 2011; Swift et al. 2012).

[0013] Adoptive cellular immunotherapy for use in cancer treatment commonly involves administration of natural and modified T cells to a patient. T cells can be modified in various ways, e.g. genetically, so as to express receptors and/or ligands that bind specifically to certain target cancer cells. Transfection of T cells with high-affinity T cell receptors (TCRs) and chimeric antigen receptors (CARs), specific for cancer cell antigens, can give rise to highly reactive cancer-specific T cell responses. A major limitation of this immunotherapeutic approach is that T cells must either be obtained from the patient for autologous *ex vivo* expansion or MHC-matched T cells must be used to avoid immunological eradication immediately following transfer of the cells to the patient or, in some cases, the onset of graft-vs-host disease (GVHD). Additionally, successfully transferred T cells often survive for prolonged periods of time in the circulation, making it difficult to control persistent side-effects resulting from treatment.

[0014] In haplotype transplantation, the graft-versus-leukemia effect is believed to be mediated by NK cells when there is a KIR inhibitory receptor-ligand mismatch, which can lead to improved survival in the treatment of AML (Ruggeri, Capanni et al. 2002; Ruggeri, Mancusi et al. 2005). Furthermore, rapid NK recovery is associated with better outcome and a stronger graft-vs-leukemia (GVL) effect in patients undergoing haplotype T-depleted hematopoietic cell transplantation (HCT) in AML (Savani, Mielke et al. 2007). Other trials have used haploidentical NK cells expanded *ex vivo* to treat AML in adults (Miller, Soignier et al. 2005) and children (Rubnitz, Inaba et al. 2010).

[0015] Several permanent NK cell lines have been established, and the most notable is NK-92, derived from a patient with non-Hodgkin's lymphoma expressing typical NK cell markers, with the exception of CD16 (Fc gamma receptor III). NK-92 has undergone extensive preclinical testing and exhibits superior lysis against a broad range of tumours compared with activated NK cells and lymphokine-activated killer (LAK) cells (Gong, Maki et al. 1994). Cytotoxicity of NK-92 cells against primary AML has been established (Yan, Steinherz et al. 1998).

[0016] Another NK cell line, KHYG-1, has been identified as a potential contender for clinical use (Suck et al. 2005) but has reduced cytotoxicity so has received less attention than NK-92. KHYG-1 cells are known to be pre-activated. Unlike endogenous NK cells, KHYG-1 cells are polarized at all times, increasing their cytotoxicity and making them quicker to respond to external stimuli. NK-92 cells have a higher baseline cytotoxicity than KHYG-1 cells.

[0017] It is therefore clear that current adoptive immunotherapy protocols are affected by donor variability in the quantity and quality of effector cells, variables that could be eliminated if effective cell lines were available to provide more standardized therapy.

[0018] A considerable amount of research into NK cell cytotoxicity has been performed using mouse models. One example is the finding that perforin and granzyme B mRNA are constitutively transcribed in mouse NK cells, but minimal levels of protein are detected until stimulation or activation of the NK cells (Fehniger et al, 2007). Although this work and other work using mouse NK cells is of interest, it cannot be relied upon as conclusive evidence for NK cell cytotoxicity in humans. In contrast to the above example, human NK cells express high levels of perforin and granzyme B protein prior to stimulation (Leong et al, 2011). The result being that when either mouse or human NK cells are freshly isolated in culture, the mouse NK cells have weak cytolytic activity, whereas the human NK cells exhibit strong cytolytic capabilities.

[0019] Mouse and human NK cells also vary greatly in their expression markers, signalling cascades and tissue distribution. For example, CD56 is used as a marker for human NK cells, whereas mouse NK cells do not express this marker at all. Furthermore, a well-established mechanism for regulating NK cell cytotoxicity is via ligand binding NK activation and inhibitory receptors. Two of the most prominent human NK activation receptors are known to be NKp30 and NKp44, neither of which are expressed on mouse NK cells. With regards to NK inhibitory receptors, whilst human NK cells express KIRs that recognise MHC class I and dampen cytotoxic activity, mouse NK cells do not express KIRs at all but, instead, express Ly49s (Trowsdale et al, 2001). All in all, despite mouse NK cells achieving the same function as human NK cells in their natural physiological environment, the mechanisms that fulfil this role vary significantly between species.

[0020] Thus there exists a need for alternative and preferably improved human NK cells and human NK cell lines, e.g. with a more cytotoxic profile.

[0021] An object of the invention is to provide NK cells and NK cell lines with a more cytotoxic phenotype. A further object is to provide methods for producing modified NK cells and NK cell lines, compositions containing the cells or cell lines and uses of said compositions in the treatment of cancers. More particular embodiments aim to provide treatments for identified cancers, e.g. blood cancers, such as leukemias. Specific embodiments aim at combining two or more modifications of NK cells and NK cell lines to further enhance the cytotoxicity of the modified cells.

Summary of the Invention

[0022] There are provided herein modified NK cells and NK cell lines with a more cytotoxic phenotype, and methods of making the cells and cell lines. Also provided are compositions of modified NK cells and NK cell lines, and uses of said compositions for treating cancer.

[0023] The invention provides a human natural killer (NK) cell or NK cell line that has been modified to express a TRAIL variant, wherein the TRAIL variant has at least a 25% increase in affinity for DR4, relative to wildtype TRAIL.

[0024] Furthermore, the invention provides a human natural killer (NK) cell or NK cell line for use in treating cancer, wherein the NK cell or NK cell line has been modified to express a TRAIL variant with at least a 25% increase in affinity for DR4, relative to wildtype TRAIL

[0025] Furthermore, compositions of the invention include NK cells and NK cell lines in which two or more modifications are provided, wherein multiple modifications further enhance the cytotoxic activity of the composition.

[0026] According to the disclosure, there are further provided methods of treating cancer, e.g. blood cancer, using modified NK cell lines, e.g. derivatives of KHYG-1 cells, wherein the modified NK cell lines are engineered to lack expression of checkpoint inhibitory receptors, express TRAIL ligand variants and/or express CARs and/or Fc receptors.

[0027] Diseases particularly treatable according to the invention include cancers, blood cancers, leukemias and specifically acute myeloid leukemia. Tumours and cancers in humans in particular can be treated. References to tumours herein include references to neoplasms.

Details of the Invention

[0028] Accordingly, the present disclosure provides a natural killer (NK) cell or NK cell line that has been genetically modified to increase its cytotoxicity.

[0029] As described in detail below in examples, NK cells and NK cell lines have been genetically modified so as to increase their cytotoxic activity against cancer.

[0030] Together, the NK cells and NK cell lines of the invention will be referred to as the NK cells (unless the context requires otherwise).

[0031] In certain embodiments of the invention NK cells are provided having, in addition, reduced or absent checkpoint inhibitory receptor function. Thus in examples below, NK cells are produced that have one or more checkpoint inhibitory receptor genes knocked out. Preferably, these receptors are specific checkpoint inhibitory receptors. Preferably still, these checkpoint inhibitory receptors are one or more or all of CD96 (TACTILE), CD152 (CTLA4), CD223 (LAG-3), CD279 (PD-1), CD328 (SIGLEC7), SIGLEC9, TIGIT and/or TIM-3.

[0032] In other embodiments, NK cells are provided in which additionally one or more inhibitory receptor signaling pathways are knocked out or exhibit reduced function - the result again being reduced or absent inhibitory receptor function. For example, signaling pathways

mediated by SHP-1, SHP-2 and/or SHIP are knocked out by genetic modification of the cells.

[0033] The resulting NK cells exhibit improved cytotoxicity and are of greater use therefore in cancer therapy, especially blood cancer therapy, in particular treatment of leukemias and multiple myeloma.

[0034] In an embodiment, the genetic modification occurs before the cell has differentiated into an NK cell. For example, pluripotent stem cells (e.g. iPSCs) can be genetically modified to lose the capacity to express one or more checkpoint inhibitory receptors. The modified iPSCs are then differentiated to produce genetically modified NK cells with increased cytotoxicity.

[0035] It is preferred to reduce function of checkpoint inhibitory receptors over other inhibitory receptors, due to the expression of the former following NK cell activation. The normal or 'classical' inhibitory receptors, such as the majority of the KIR family, NKG2A and LIR-2, bind MHC class I and are therefore primarily involved in reducing the problem of self-targeting. Preferably, therefore, checkpoint inhibitory receptors are knocked out. Reduced or absent function of these receptors according to the invention prevents cancer cells from suppressing immune effector function (which might otherwise occur if the receptors were fully functional). Thus a key advantage of these embodiments of the invention lies in NK cells that are less susceptible to suppression of their cytotoxic activities by cancer cells; as a result they are useful in cancer treatment.

[0036] As used herein, references to inhibitory receptors generally refer to a receptor expressed on the plasma membrane of an immune effector cell, e.g. a NK cell, whereupon binding its complementary ligand resulting intracellular signals are responsible for reducing the cytotoxicity of said immune effector cell. These inhibitory receptors are expressed during both 'resting' and 'activated' states of the immune effector cell and are often associated with providing the immune system with a 'self-tolerance' mechanism that inhibits cytotoxic responses against cells and tissues of the body. An example is the inhibitory receptor family 'KIR' which are expressed on NK cells and recognize MHC class I expressed on healthy cells of the body.

[0037] Also as used herein, checkpoint inhibitory receptors are usually regarded as a subset of the inhibitory receptors above. Unlike other inhibitory receptors, however, checkpoint inhibitory receptors are expressed at higher levels during prolonged activation and cytotoxicity of an immune effector cell, e.g. a NK cell. This phenomenon is useful for dampening chronic cytotoxicity at, for example, sites of inflammation. Examples include the checkpoint inhibitory receptors PD-1, CTLA-4 and CD96, all of which are expressed on NK cells.

[0038] The invention hence also provides a NK cell that is additionally modified to be lacking a gene encoding a checkpoint inhibitory receptor selected from CD96 (TACTILE), CD152 (CTLA4), CD223 (LAG-3), CD279 (PD-1), CD328 (SIGLEC7), SIGLEC9, TIGIT and TIM-3.

[0039] A NK cell lacking a gene can refer to either a full or partial deletion, mutation or

otherwise that results in no functional gene product being expressed. In embodiments, the NK cell lacks genes encoding two or more of the inhibitory receptors.

[0040] More specific embodiments comprise a NK cell lacking a gene encoding a checkpoint inhibitory receptor selected from CD96 (TACTILE), CD152 (CTLA4) and CD279 (PD-1). Preferred embodiments comprise a NK cell being a derivative of KHYG-1.

[0041] In examples described below, the inventors have reliably shown the cytotoxic effects of using siRNA to knock down expression of the checkpoint inhibitory receptor CD96 in KHYG-1 cells. CD96 knockdown (KD) KHYG-1 cells demonstrated enhanced cytotoxicity against leukemia cells at a variety of effector:target (E:T) ratios.

[0042] As further described in examples below, cytotoxicity-enhancing modifications of NK cells hence also include increased expression of both TRAIL ligand and/or mutated TRAIL ligand variants.

[0043] The resulting NK cells exhibit increased binding to TRAIL receptors and, as a result, increased cytotoxicity against cancers, especially blood cancers, in particular leukemias.

[0044] The mutants / variants preferably have lower affinity (or in effect no affinity) for 'decoy' receptors, compared with the binding of wild type TRAIL to decoy receptors. Such decoy receptors represent a class of TRAIL receptors that bind TRAIL ligand but do not have the capacity to initiate cell death and, in some cases, act to antagonize the death signaling pathway. Mutant / variant TRAIL ligands may be prepared according to WO 2009/077857.

[0045] The mutants / variants may separately have increased affinity for TRAIL receptors, e.g. DR4 and DR5. Wildtype TRAIL is typically known to have a K_D of >2 nM for DR4, >5 nM for DR5 and >20 nM for the decoy receptor DcR1 (WO 2009/077857; measured by surface plasmon resonance), or around 50 to 100 nM for DR4, 1 to 10 nM for DR5 and 175 to 225 nM for DcR1 (Truneh, A. et al. 2000; measured by isothermal titration calorimetry and ELISA). Therefore, an increased affinity for DR4 is suitably defined as a K_D of <2 nM or <50 nM, respectively, whereas an increased affinity for DR5 is suitably defined as a K_D of <5 nM or <1 nM, respectively. A reduced affinity for decoy receptor DcR1 is suitably defined as a K_D of >50 nM or >225 nM, respectively. In any case, an increase or decrease in affinity exhibited by the TRAIL variant/mutant is relative to a baseline affinity exhibited by wildtype TRAIL. The affinity is preferably increased at least 10%, more preferably at least 25%, compared with that exhibited by wildtype TRAIL.

[0046] The TRAIL variant preferably has an increased affinity for DR5 as compared with its affinity for DR4, DcR1 and DcR2. Preferably, the affinity is at least 1.5-fold, 2-fold, 5-fold, 10-fold, 100-fold, or even 1,000-fold or greater for DR5 than for one or more of DR4, DcR1 and DcR2. More preferably, the affinity is at least 1.5-fold, 2-fold, 5-fold, 10-fold, 100-fold, or even 1,000-fold or greater for DR5 than for at least two, and preferably all, of DR4, DcR1 and DcR2.

[0047] A key advantage of these embodiments of the invention lies in NK cells that have greater potency in killing cancer cells.

[0048] Further specific embodiments comprise a NK cell expressing a mutant TRAIL ligand that has reduced or no affinity for TRAIL decoy receptors. Preferably, this NK cell is a derivative of KHYG-1. Further specific embodiments comprise a NK cell expressing a mutant TRAIL ligand that has reduced or no affinity for TRAIL decoy receptors and increased affinity for DR4 and/or DR5.

[0049] In examples of the invention, described in more detail below, NK cells were genetically modified to express a mutant TRAIL. Modified KHYG-1 cells expressed mutant TRAIL, and NK-92 expressed a mutant TRAIL. The modified KHYG-1 cells exhibited improved cytotoxicity against cancer cell lines *in vitro.* KHYG-1 cells express TRAIL receptors (e.g. DR4 and DR5), but at low levels. Other preferred embodiments of the modified NK cells express no or substantially no TRAIL receptors, or do so only at a low level - sufficiently low that viability of the modified NK cells is not adversely affected by expression of the mutant TRAIL.

[0050] In an optional embodiment, treatment of a cancer using modified NK cells expressing TRAIL or a TRAIL variant is enhanced by administering to a patient an agent capable of upregulating expression of TRAIL death receptors on cancer cells. This agent may be administered prior to, in combination with or subsequently to administration of the modified NK cells. It is preferable, however, that the agent is administered prior to administering the modified NK cells.

[0051] In a preferred embodiment the agent upregulates expression of DR5 on cancer cells. The agent may optionally be a chemotherapeutic medication, e.g. Bortezomib, and administered in a low dose capable of upregulating DR5 expression on the cancer.

[0052] The invention is not limited to any particular agents capable of upregulating DR5 expression, but examples of DR5-inducing agents include Bortezomib, Gefitinib, Piperlongumine, Doxorubicin, Alpha-tocopheryl succinate and HDAC inhibitors.

[0053] According to a preferred embodiment of the invention, the mutant / variant TRAIL ligand is linked to one or more NK cell costimulatory domains, e.g. 41BB / CD137, CD3zeta / CD247, DAP12 or DAP10. Binding of the variant to its receptor on a target cell thus promotes apoptotic signals within the target cell, as well as stimulating cytotoxic signals in the NK cell.

[0054] According to further preferred embodiments of the invention, NK cells are provided that both have reduced checkpoint inhibitory receptor function and also express a mutant TRAIL ligand, as described in more detail above in relation to these respective NK cell modifications. In even more preferred embodiments, a NK cell expressing a mutant TRAIL ligand that has reduced or no affinity for TRAIL decoy receptors and may be a derivative of KHYG-1, further lacks a gene encoding a checkpoint inhibitory receptor selected from CD96 (TACTILE), CD152 (CTLA4), CD223 (LAG-3), CD279 (PD-1), CD328 (SIGLEC7), SIGLEC9, TIGIT and TIM-3.

[0055] The present disclosure also provides NK cells and NK cell lines, preferably KHYG-1 cells and derivatives thereof, modified to express one or more CARs.

[0056] Suitably for cancer therapy uses, the CARs specifically bind to one or more ligands on cancer cells, e.g. CS1 (SLAMF7) on myeloma cells. For use in treating specific cancers, e.g. multiple myeloma, the CAR may bind CD38. For example, the CAR may include the binding properties of e.g. variable regions derived from, similar to, or identical with those from the known monoclonal antibody daratumumab. Such NK cells may be used in cancer therapy in combination with an agent that inhibits angiogenesis, e.g. lenalidomide. For use in therapy of cancers, especially leukemias and AML in particular, the CAR may bind to CLL-1.

[0057] The CAR-NKs may be bispecific, wherein their affinity is for two distinct ligands / antigens. Bispecific CAR-NKs can be used either for increasing the number of potential binding sites on cancer cells or, alternatively, for localizing cancer cells to other immune effector cells which express ligands specific to the NK-CAR. For use in cancer therapy, a bispecific CAR may bind to a target tumour cell and to an effector cell, e.g. a T cell, NK cell or macrophage. Thus, for example, in the case of multiple myeloma, a bispecific CAR may bind a T cell antigen (e.g. CD3, etc.) and a tumour cell marker (e.g. CD38, etc.). A bispecific CAR may alternatively bind to two separate tumour cell markers, increasing the overall binding affinity of the NK cell for the target tumour cell. This may reduce the risk of cancer cells developing resistance by downregulating one of the target antigens. An example in this case, in multiple myeloma, would be a CAR binding to both CD38 and CS-1/SLAMF7. Another tumour cell marker suitably targeted by the CAR is a "don't eat me" type marker on tumours, exemplified by CD47.

[0058] Optional features of the invention include providing further modifications to the NK cells and NK cell lines described above, wherein, for example, a Fc receptor (which can be CD16, CD32 or CD64, including subtypes and derivatives) is expressed on the surface of the cell. In use, these cells can show increased recognition of antibody-coated cancer cells and improve activation of the cytotoxic response.

[0059] Further optional features of the invention include adapting the modified NK cells and NK cell lines to better home to specific target regions of the body. NK cells of the invention may be targeted to specific cancer cell locations. In preferred embodiments for treatment of blood cancers, NK effectors of the invention are adapted to home to bone marrow. Specific NK cells are modified by fucosylation and/or sialylation to home to bone marrow. This may be achieved by genetically modifying the NK cells to express the appropriate fucosyltransferase and/or sialyltransferase, respectively. Increased homing of NK effector cells to tumour sites may also be made possible by disruption of the tumour vasculature, e.g. by metronomic chemotherapy, or by using drugs targeting angiogenesis (Melero et al, 2014) to normalize NK cell infiltration via cancer blood vessels.

[0060] Yet another optional feature of the invention is to provide modified NK cells and NK cell lines with an increased intrinsic capacity for rapid growth and proliferation in culture. This can

be achieved, for example, by transfecting the cells to overexpress growth-inducing cytokines IL-2 and IL-15. Moreover, this optional alteration provides a cost-effective alternative to replenishing the growth medium with cytokines on a continuous basis.

[0061] The invention further provides a method of making the modified NK cell or NK cell line, comprising genetically modifying the cell or cell line as described herein so as to increase its cytotoxicity. This genetic modification can be a stable knockout of a gene, e.g. by CRISPR, or a transient knockdown of a gene, e.g. by siRNA.

[0062] In a preferred embodiment, a stable genetic modification technique is used, e.g. CRISPR, in order to provide a new NK cell line with increased cytotoxicity, e.g. a derivative of KHYG-1 cells.

[0063] In embodiments, the method is for making a NK cell or NK cell line that has been modified so as to reduce inhibitory receptor function. Preferably, these inhibitory receptors are checkpoint inhibitory receptors.

[0064] More specific embodiments comprise a method for making a NK cell or NK cell line with reduced inhibitory receptor function, wherein the checkpoint inhibitory receptors are selected from CD96 (TACTILE), CD152 (CTLA4), CD223 (LAG-3), CD279 (PD-1), CD328 (SIGLEC7), SIGLEC9, TIGIT and TIM-3.

[0065] In preferred embodiments, the method comprises modifying the NK cells to reduce function of two or more of the inhibitory receptors.

[0066] The invention still further provides a method of making the modified NK cell or NK cell line comprising genetically modifying the cell or cell line to express TRAIL ligand or mutant TRAIL (variant) ligand.

[0067] In embodiments, the method comprises modifying a NK cell or NK cell line to express mutant TRAIL ligand that has an increased affinity for TRAIL receptors. Preferably, the TRAIL receptors are DR4 and/or DR5. Preferred embodiments provide a method of modifying the NK cells or NK cell lines to express a mutant TRAIL ligand that has a reduced affinity for decoy TRAIL receptors.

[0068] In further preferred embodiments, the method comprises modifying a NK cell or NK cell line to remove function of a checkpoint inhibitory receptor and also to express a mutant TRAIL ligand with reduced or no binding affinity for decoy TRAIL receptors.

[0069] Further typical embodiments provide a method for making a NK cell or NK cell line, in which function of one or more checkpoint inhibitory receptors has been removed and/or a mutant TRAIL ligand is expressed, which has reduced or no binding affinity for decoy TRAIL receptors, and the cell is further modified to express a CAR or bispecific CAR. The properties of the CAR are optionally as described above.

[0070] In embodiments, the method comprises making a NK cell or NK cell line, in which function of one or more checkpoint inhibitory receptors has been removed and/or a mutant TRAIL ligand is expressed, which has reduced or no binding affinity for decoy TRAIL receptors, and the cell is optionally modified to express a CAR or bispecific CAR, and the cell is further modified to express one or more Fc receptors. Suitable Fc receptors are selected from CD16 (FcRIII), CD32 (FcRII) and CD64 (FcRI).

[0071] Preferred embodiments of all the above comprise a method of making NK cells and NK cell lines being a derivative of KHYG-1.

[0072] As per the objects of the invention, the modified NK cell, NK cell line or composition thereof with increased cytotoxicity are for use in treating cancer in a patient, especially blood cancer.

[0073] In preferred embodiments, the modified NK cell, NK cell line or composition is for use in treating blood cancers including acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), Hodgkin's lymphoma, non-Hodgkin's lymphoma, including T-cell lymphomas and B-cell lymphomas, asymptomatic myeloma, smoldering multiple myeloma (SMM), active myeloma or light chain myeloma.

[0074] In even more preferred embodiments, the invention is a NK cell line obtained as a derivative of KYHG-1 by reducing checkpoint inhibitory receptor function in a KHYG-1 cell in addition to expressing a mutant TRAIL ligand in the KHYG-1 cell for use in treating blood cancer.

[0075] Modified NK cells, NK cell lines and compositions thereof described herein, above and below, are suitable for treatment of cancer, in particular cancer in humans, e.g. for treatment of cancers of blood cells or solid cancers. The NK cells and derivatives are preferably human NK cells. For human therapy, human NK cells are preferably used.

[0076] Various routes of administration will be known to the skilled person to deliver active agents and combinations thereof to a patient in need. Embodiments of the invention are for blood cancer treatment. Administration of the modified NK cells and/or NK cell lines can be systemic or localized, such as for example via the intraperitoneal route.

[0077] In other embodiments, active agent is administered more directly. Thus administration can be directly intratumoural, suitable especially for solid tumours.

[0078] NK cells in general are believed suitable for the methods, uses and compositions of the invention. As per cells used in certain examples herein, the NK cell can be a NK cell obtained from a cancer cell line. Advantageously, a NK cell, preferably treated to reduce its tumourigenicity, for example by rendering it mortal and/or incapable of dividing, can be

obtained from a blood cancer cell line and used in methods of the invention to treat blood cancer.

[0079] To render a cancer-derived cell more acceptable for therapeutic use, it is generally treated or pre-treated in some way to reduce or remove its propensity to form tumours in the patient. Specific modified NK cell lines used in examples are safe because they have been rendered incapable of division; they are irradiated and retain their killing ability but die within about 3-4 days. Specific cells and cell lines are hence incapable of proliferation, e.g. as a result of irradiation. Treatments of potential NK cells for use in the methods herein include irradiation to prevent them from dividing and forming a tumour *in vivo* and genetic modification to reduce tumourigenicity, e.g. to insert a sequence encoding a suicide gene that can be activated to prevent the cells from dividing and forming a tumour *in vivo.* Suicide genes can be turned on by exogenous, e.g. circulating, agents that then cause cell death in those cells expressing the gene. A further alternative is the use of monoclonal antibodies targeting specific NK cells of the therapy. CD52, for example, is expressed on KHYG-1 cells and binding of monoclonal antibodies to this marker can result in antibody-dependent cell-mediated cytotoxicity (ADCC) and KHYG-1 cell death.

[0080] As discussed in an article published by Suck et al, 2006, cancer-derived NK cells and cell lines are easily irradiated using irradiators such as the Gammacell 3000 Elan. A source of Cesium-137 is used to control the dosing of radiation and a dose-response curve between, for example, 1 Gy and 50 Gy can be used to determine the optimal dose for eliminating the proliferative capacity of the cells, whilst maintaining the benefits of increased cytotoxicity. This is achieved by assaying the cells for cytotoxicity after each dose of radiation has been administered.

[0081] There are significant benefits of using an irradiated NK cell line for adoptive cellular immunotherapy over the well-established autologous or MHC-matched T cell approach. Firstly, the use of a NK cell line with a highly proliferative nature means expansion of modified NK cell lines can be achieved more easily and on a commercial level. Irradiation of the modified NK cell line can then be carried out prior to administration of the cells to the patient. These irradiated cells, which retain their useful cytotoxicity, have a limited life span and, unlike modified T cells, will not circulate for long periods of time causing persistent side-effects.

[0082] Additionally, the use of allogeneic modified NK cells and NK cell lines means that MHC class I expressing cells in the patient are unable to inhibit NK cytotoxic responses in the same way as they can to autologous NK cytotoxic responses. The use of allogeneic NK cells and cell lines for cancer cell killing benefits from the previously mentioned GVL effect and, unlike for T cells, allogeneic NK cells and cell lines do not stimulate the onset of GVHD, making them a much preferred option for the treatment of cancer via adoptive cellular immunotherapy.

Examples

[0083] The present invention is now described in more and specific details in relation to the production of NK cell line KHYG-1 derivatives, modified to exhibit more cytotoxic activity and hence ability to cause leukemia cell death in humans.

[0084] The invention is now illustrated in specific embodiments with reference to the accompanying drawings in which:

Fig. 1 shows the DNA sequence of the LIR2 gene target region and marks the gRNA flanking regions;

Fig. 2 shows the DNA sequence of the CTLA4 gene target region and marks the gRNA flanking regions;

Fig. 3 shows the gRNA construct (expression vector) used for transfection;

Fig. 4 shows gel electrophoresis bands for parental and mutated LIR2 DNA, before and after transfection;

Fig. 5 shows gel electrophoresis bands for parental and mutated CTLA4 DNA, before and after transfection;

Fig. 6A is a FACS plot showing successful CD96 knockdown using electroporation;

Fig. 6B is a FACS plot showing successful CD96 knockdown using electroporation;

Fig. 7 is a bar chart showing increased cytotoxicity of CD96 knockdown KHYG-1 cells against K562 cells at various E:T ratios;

Fig. 8 shows knockdown of CD328 (Siglec-7) in NK-92 cells;

Fig. 9 shows enhanced cytotoxicity of NK Cells with the CD328 (Siglec-7) knockdown;

Fig. 10 shows a FACS plot of the baseline expression of TRAIL on KHYG-1 cells;

Fig. 11 shows a FACS plot of the expression of TRAIL and TRAIL variant after transfection of KHYG-1 cells;

Fig. 12 shows a FACS plot of the expression of CD107a after transfection of KHYG-1 cells;

Fig. 13 shows the effects of transfecting KHYG-1 cells with TRAIL and TRAIL variant on cell viability;

Fig. 14 shows a FACS plot of the baseline expression of DR4, DR5, DcR1 and DcR2 on both KHYG-1 cells and NK-92 cells;

Fig.s 15, 16 and 17 show the effects of expressing TRAIL or TRAIL variant in KHYG-1 cells on apoptosis of three target cell populations: K562, RPMI8226 and MM1.S, respectively;

Fig. 18 shows two FACS plots of DR5 expression on RPMI8226 cells and MM1.S cells, respectively, wherein the effects of Bortezomib treatment on DR5 expression are shown;

Fig. 19 shows FACS plots of apoptosis in Bortezomib-pretreated/untreated MM1.S cells co-cultured with KHYG-1 cells with/without the TRAIL variant;

Fig. 20 shows a FACS plot of perforin expression levels in KHYG-1 cells treated with 100 nM CMA for 2 hours;

Fig. 21 shows FACS plots of KHYG-1 cell viability after treatment with 100 nM CMA or vehicle;

Fig. 22 shows FACS plots of apoptosis in MM1.S cells co-cultured with KHYG-1 cells with/without the TRAIL variant and pretreated with/without CMA;

Fig. 23 shows FACS plots of apoptosis in K562 cells co-cultured with KHYG-1 cells with CD96-siRNA and/or TRAIL variant expression; and

Fig. 24 shows FACS plots of apoptosis in MM1.S cells co-cultured with KHYG-1 cells with CD96-siRNA and/or TRAIL variant expression.

[0085] DNA, RNA and amino acid sequences are referred to below, in which:

SEQ ID NO: 1 is the full LIR2 DNA sequence;

SEQ ID NO: 2 is the LIR2 amino acid sequence;

SEQ ID NO: 3 is the LIR2 g9 gRNA sequence;

SEQ ID NO: 4 is the LIR2 g18 gRNA sequence;

SEQ ID NO: 5 is the LIR2 forward primer sequence;

SEQ ID NO: 6 is the LIR2 reverse primer sequence;

SEQ ID NO: 7 is the full CTLA4 DNA sequence;

SEQ ID NO: 8 is the CTLA4 amino acid sequence;

SEQ ID NO: 9 is the CTLA4 g7 gRNA sequence;

SEQ ID NO: 10 is the CTLA4 g15 gRNA sequence;

SEQ ID NO: 11 is the CTLA4 forward primer sequence; and

SEQ ID NO: 12 is the CTLA4 reverse primer sequence.

Example 1 - Knockout of Inhibitory Receptor Function

CRISPR/Cas9

[0086] Cells were prepared as follows, having inhibitory receptor function removed. gRNA constructs were designed and prepared to target genes encoding the 'classical' inhibitory receptor LIR2 and the 'checkpoint' inhibitory receptor CTLA4 in the human genome of NK cells. CRISPR/Cas9 genome editing was then used to knock out the LIR2 and CTLA4 target genes.

[0087] Two gRNA candidates were selected for each target gene and their cleavage efficacies in K562 cells determined. The sequences of the gRNA candidates are shown in Table 1 and the Protospacer Adjacent Motif (PAM) relates to the last 3 bases of the sequence. The flanking regions of the gRNA sequences on the LIR2 gene (SEQ ID NO: 1) and the CTLA4 gene (SEQ ID NO: 7) are shown in Figures 1 and 2, respectively.

Table 1. gRNA candidates and sequences

Gene	Plasmid Name	Sequence
hLIR2	SM682.LIR2.g9	GAGTCACAGGTGGCATTTGG**CGG** (SEQ ID NO: 3)
	SM682.LIR2.g18	CGAATCGCAGGTGGTCGCAC**AGG** (SEQ ID NO: 4)
hCTLA4	SM683.CTLA4.g7	CACTCACCTTTGCAGAAGAC**AGG** (SEQ ID NO: 9)
	SM683.CTLA4.g15	CCTTGTGCCGCTGAAATCCA**AGG** (SEQ ID NO: 10)

[0088] K562 cells were transfected with the prepared gRNA constructs (Figure 3) and subsequently harvested for PCR amplification. The presence of GFP expression was used to report successful incorporation of the gRNA construct into the K562 cells. This confirmed expression of the Cas9 gene and therefore the ability to knock out expression of the LIR2 and CTLA4 genes.

[0089] The cleavage activity of the gRNA constructs was determined using an *in vitro* mismatch detection assay. T7E1 endonuclease I recognises and cleaves non-perfectly matched DNA, allowing the parental LIR2 and CTLA4 genes to be compared to the mutated genes following CRISPR/Cas9 transfection and non-homologous end joining (NHEJ).

[0090] Figure 4 shows the resulting bands following agarose gel electrophoresis after knockout of the LIR2 gene with the g9 and g18 gRNA sequences. The three bands corresponding to each mutation relate to the parental gene and the two resulting strands following detection of a mismatch in the DNA sequence after transfection. The g9 gRNA sequence resulted in an 11% success rate of transfection, whereas the g18 gRNA resulted in 10%.

[0091] Figure 5 shows the resulting bands following agarose gel electrophoresis after knockout

of the CTLA4 gene with the g7 and g15 gRNA sequences. The g7 gRNA sequence resulted in a 32% success rate of transfection, whereas the g15 gRNA resulted in 26%.

[0092] Following the successful knockout of LIR2 and CTLA4 in K562 cells, KHYG-1 cells were transfected with gRNA constructs.

[0093] KHYG-1 derivative clones having homozygous deletions were selected. A Cas9 / puromycin acetyltransferase (PAC) expression vector was used for this purpose. Successfully transfected cells were selected, based on their resistance to the antibiotic puromycin.

Cas9 RNP

[0094] Another protocol used for knockout of checkpoint inhibitory receptors in NK cells was that of Cas9 RNP transfection. An advantage of using this protocol was that similar transfection efficiencies were achievable but with significantly lower toxicity compared to using the DNA plasmids of the CRISPR/Cas9 protocol.

[0095] $1x10^6$ KHYG1 cells were harvested for each transfection experiment. The cells were washed with PBS and spun down in a centrifuge. The supernatant was then discarded. The CRISPR RNP (RNA binding protein) materials were then prepared as follows:

1. (1) a 20μM solution of the required synthesized crRNA and tRNA (purchased from Dharmacon) was prepared.
2. (2) 4μl of crRNA (20μM) and 4μl of tRNA (20μM) were mixed together.
3. (3) The mixture was then added to 2μl Cas9 protein (5μg/μl).
4. (4) All of the components were mixed and incubated at room temperature for 10 minutes.

[0096] Following the Neon® Transfection System, the cells were mixed with Cas9 RNP and electroporation was performed using the following parameters:

Voltage: 1450v

Pulse width: 30ms

Pulse number: 1

[0097] The cells were then transferred to one well of a 12-well plate containing growth medium (inc. IL-2 and IL-15).

[0098] The cells were harvested after 48-72 hours to confirm gene editing efficiency by T7 endonuclease assay and/or Sanger sequencing. The presence of indels were confirmed, indicating successful knockout of CTLA4, PD1 and CD96 in KHYG1 cells.

Site-specific nucleases

[0099] Another protocol used for knockout of checkpoint inhibitory receptors in NK cells was that of XTN TALEN transfection. An advantage of using this protocol was that a particularly high level of specificity was achievable compared to wildtype CRISPR.

Step 1: Preparation of Reagents

[0100] KHYG-1 cells were assayed for certain attributes including transfection efficiency, single cell cloning efficiency and karyotype/copy number. The cells were then cultured in accordance with the supplier's recommendations.

[0101] Depending on the checkpoint inhibitory receptor being knockout out, nucleases were prepared by custom-design of at least 2 pairs of XTN TALENs. The step of custom-design includes evaluation of gene locus, copy number and functional assessment (i.e. homologs, off-target evaluation).

Step 2: Cell Line Engineering

[0102] The cells were transfected with the nucleases of Step 1; this step was repeated up to 3 times in order to obtain high levels of cutting and cultures were split and intermediate cultures maintained prior to each transfection.

[0103] Initial screening occurred several days after each transfection; the pools of cells were tested for cutting efficiency via the Cel-1 assay. Following the level of cutting reaching acceptable levels or plateaus after repeated transfections, the cells were deemed ready for single cell cloning.

[0104] The pooled cells were sorted to one cell per well in a 96-well plate; the number of plates for each pool was dependent on the single cell cloning efficiency determined in Step 1. Plates were left to incubate for 3-4 weeks.

Step 3 - Screening and Expansion

[0105] Once the cells were confluent in the 96-well plates, cultures were consolidated and split

into triplicate 96-well plates; one plate was frozen as a backup, one plate was re-plated to continue the expansion of the clones and the final plate was used for genotype confirmation.

[0106] Each clone in the genotype plate was analyzed for loss of qPCR signal, indicating all alleles had been modified. Negative clones were PCR amplified and cloned to determine the nature of the indels and lack of any wildtype or in-frame indels. Clones with the confirmed knockout were consolidated into no more than one 24-well plate and further expanded; typically 5-10 frozen cryovials containing $1x10^6$ cells per vial for up to 5 individual clones were produced per knockout.

Step 4 - Validation

[0107] Cells were banked under aseptic conditions.

[0108] Basic release criteria for all banked cells included viable cell number (pre-freeze and post-thaw), confirmation of identity via STR, basic sterility assurance and mycoplasma testing; other release criteria were applied when necessary (karyotype, surface marker expression, high level sterility, knockout evaluation of transcript or protein, etc).

Example 2 - Knockdown of Checkpoint Inhibitory Receptor CD96 Function via RNAi

[0109] siRNA knockdown of CD96 in KHYG-1 cells was performed by electroporation. The Nucleofection Kit T was used, in conjunction with the Amaxa Nucleofector II, from Lonza, as it is appropriate for use with cell lines and can successfully transfect both dividing and non-dividing cells and achieves transfection efficiencies of up to 90%..

[0110] Control siRNA (catalog number: sc-37007) and CD96 siRNA (catalog number: sc-45460) were obtained from Santa Cruz Biotechnology. Antibiotic-free RPMI-1640 containing 10% FBS, 2mM L-glutamine was used for post-Nucleofection culture. Mouse anti-human CD96-APC (catalog number: 338409) was obtained from Biolegend for staining.

[0111] A 20µM of siRNA stock solution was prepared. The lyophilized siRNA duplex was resuspended in 33µl of the RNAse-free water (siRNA dilution buffer: sc-29527) to FITC-control/control-siRNA, in 165µl of the RNAse-free water for the target gene siRNA (siRNA CD96). The tube was heated to 90°C for 1 minute and then incubated at 37°C for 60 minutes. The siRNA stock was then stored at -20°C until needed.

[0112] The KHYG-1 cells were passaged one to two days before Nucleofection, as the cells must be in logarithmic growth phase.

[0113] The Nucleofector solution was warmed to room temperature (100ul per sample). An

aliquot of culture medium containing serum and supplements was also pre-warmed at 37°C in a 50ml tube. 6-well plates were prepared by adding 1.5ml of culture medium containing serum and supplements. The plates were pre-incubated in a humidified 37°C / 5% CO_2 incubator.

[0114] $2x10^6$ cells in 100µl Nucleofection solution was mixed gently with 4µl 20µM siRNA solution (1.5µg siRNA). Air bubbles were avoided during mixing. The mixture was transferred into Amaxa certified cuvettes and placed into the Nucleofector cuvette holder and program U-001 selected.

[0115] The program was allowed to finish, and the samples in the cuvettes were removed immediately. 500µl pre-equilibrated culture medium was then added to each cuvette. The sample in each cuvette was then gently transferred to a corresponding well of the prepared 6-well plate, in order to establish a final volume of 2ml per well.

[0116] The cells were then incubated in a humidified 37°C / 5% CO_2 incubator until transfection analysis was performed. Flow cytometry analysis was performed 16-24 hours after electroporation, in order to measure CD96 expression levels. This electroporation protocol was carried out multiple times and found to reliably result in CD96 knockdown in KHYG-1 cells (see e.g. Figures 6A and 6B).

Example 3 - Enhanced Cytotoxicity of NK Cells with a CD96 Knockdown

[0117] KHYG-1 cells with and without the CD96 knockdown were co-cultured with K562 cells at different effector:target (E:T) ratios.

[0118] Cytotoxicity was measured 4 hours after co-culture, using the DELFIA EuTDA Cytotoxicity Kit from PerkinElmer (Catalog number: AD0116).

[0119] Target cells K562 were cultivated in RPMI-1640 medium containing 10% FBS, 2mM L-glutamine and antibiotics. 96-well V-bottom plates (catalog number: 83.3926) were bought from SARSTEDT. An Eppendorf centrifuge 5810R (with plate rotor) was used to spin down the plate. A VARIOSKAN FLASH (with ScanIt software 2.4.3) was used to measure the fluorescence signal produced by lysed K562 cells.

[0120] K562 cells were washed with culture medium and the number of cells adjusted to $1x10^6$ cells/mL with culture medium. 2-4mL of cells was added to 5µl of BATDA reagent and incubated for 10 minutes at 37°C. Within the cell, the ester bonds are hydrolysed to form a hydrophilic ligand, which no longer passes through the membrane. The cells were centrifuged at 1500RPM for 5 mins to wash the loaded K562 cells. This was repeated 3-5 times with medium containing 1mM Probenecid (Sigma P8761). After the final wash the cell pellet was resuspended in culture medium and adjusted to about $5x10^4$ cells/mL.

[0121] Wells were set up for detection of background, spontaneous release and maximum release. 100µL of loaded target cells (5,000 cells) were transferred to wells in a V-bottom plate and 100µL of effector cells (KHYG-1 cells) were added at varying cell concentrations, in order to produce effector to target ratios ranging from 1:1 to 20:1. The plate was centrifuged at 100xg for 1 minute and incubated for 4 hours in a humidified 5% CO_2 atmosphere at 37°C. For maximum release wells 10µL of lysis buffer was added to each well 15 minutes before harvesting the medium. The plate was centrifuged at 500xg for 5 minutes.

[0122] 20µL of supernatant was transferred to a flat-bottom 96 well plate 200µL of pre-warmed Europium solution added. This was incubated at room temperature for 15 mins using a plate shaker. As K562 cells are lysed by the KHYG-1 cells, they release ligand into the medium. This ligand then reacts with the Europium solution to form a fluorescent chelate that directly correlates with the amount of lysed cells.

[0123] The fluorescence was then measured in a time-resolved fluorometer by using VARIOSKAN FLASH. The specific release was calculated using the following formula:

% specific release = Experiment release – Spontaneous release / Maximum release – Spontaneous release

[0124] Statistical analysis was performed using Graphpad Prism 6.04 software. A paired t test was used to compare the difference between siRNA CD96 knockdown KHYG-1 cells and control groups (n=3).

[0125] The specific release was found to be significantly increased in co-cultures containing the CD96 knockdown KHYG-1 cells. This was the case at all E:T ratios (see Figure 7).

[0126] As fluorescence directly correlates with cell lysis, it was confirmed that knocking down CD96 expression in KHYG-1 cells resulted in an increase in their ability to kill K562 cancer target cells.

Example 4 - Enhanced Cytotoxicity of NK Cells with a CD328 (Siglec-7) Knockdown

<u>SiRNA-mediated knock-down of CD328 in NK-92 cells</u>

<u>Materials, reagents and instruments</u>

[0127] Control siRNA (catalog number: sc-37007) and CD328 siRNA (catalog number: sc-106757) were bought from Santa Cruz Biotechnology. To achieve transfection efficiencies of up to 90% with high cell viability (>75%) in NK-92 cells with the Nucleofector™ Device

(Nucleofector II, Lonza), a Nucleofector™ Kit T from Lonza was used. RPMI-1640 containing 10% FBS, 2mM L-glutamine, antibiotics free, was used for post-Nucleofection culture. Mouse anti-human CD328-APC (catalog number: 339206) was bought from Biolegend.

Protocol

[0128] To make 10µM of siRNA stock solution

- Resuspend lyophilized siRNA duplex in 66µl of the RNAse-free water (siRNA dilution buffer: sc-29527) to FITC-control/control-siRNA, in 330µl of the RNAse-free water for the target gene siRNA (siRNA CD328).
- Heat the tube to 90°C for 1 minute.
- Incubate at 37 °C for 60 minutes.
- Store siRNA stock at -20 °C if not used directly.
- One Nucleofection sample contains (for 100µl standard cuvette)
- Cell number: $2x10^6$ cells
- siRNA: 4µl of 10µM stock
- Nucleofector solution: 100µl

Nucleofection

[0129]

- Cultivate the required number of cells. (Passage one or two day before Nucleofection, cells must be in logarithmic growth phase).
- Prepare siRNA for each sample.
- Pre-warm the Nucleofector solution to room temperature (100µl per sample).
- Pre-warm an aliquot of culture medium containing serum and supplements at 37 °C in a 50ml tube. Prepare 6-well plates by filling with 1.5ml of cullture medium containing serum and supplements and pre-incubate plates in a humidified 37 °C /5% CO2 incubator.
- Take an aliquot of cell culture and count the cells to determine the cell density.
- Centrifuge the required number of cells at 1500rpm for 5 min. Discard supernatant completely so that no residual medium covers the cell pellet.
- Resuspend the cell pellet in room temperature Nucleofector Solution to a final concentration of $2x10^6$ cells/100µl. Avoid storing the cell suspension longer than 15-20 min in Nucleofector Solution, as this reduces cell viability and gene transfer efficiency.
- Mix 100µl of cell suspension with siRNA.
- Transfer the sample into an amaxa certified cuvette. Make sure that the sample covers the bottom of the cuvette, avoid air bubbles while pipetting. Close the cuvette with the

blue cap.

- Select the appropriate Nucleofector program (A-024 for NK-92 cells). Insert the cuvette into the cuvette holder (Nucleofector II: rotate the carousel clockwise to the final position) and press the "x" button to start the program.
- To avoid damage to the cells, remove the samples from the cuvette immediately after the program has finished (display showing "OK"). Add 500µl of the pre-warmed culture medium into the cuvette and transfer the sample into the prepared 6-well plate.
- Incubate cells in a humidified 37°C/5% CO_2 incubator. Perform flow cytometric analysis and cytotoxicity assay after 16-24 hours.

[0130] Results: we followed the above protocol and performed flow cytometry analysis of CD328 expression level in NK-92 cells. The results of one representative experiment is shown in Fig. 8, confirming successful knockdown.

Knocking down CD328 enhances cytotoxicity

Materials, reagents and instruments

[0131] DELFIA EuTDA cytotoxicity kit based on fluorescence enhancing ligand (Catalog nmber: AD0116) was bought from PerkinElmer. Target cells K562 were cultivated in RPMI-1640 medium containing 10% FBS, 2mM L-glutamine and antibiotics. 96-well V-bottom plates (catalog number: 83.3926) were bought from SARSTEDT. Eppendrof centrifuge 5810R (with plate rotor) was used to spin down the plate. VARIOSKAN FLASH (with ScanIt software 2.4.3) was used to measure the fluorescence signal produced by lysed K562 cells.

Protocol

[0132]

- Load target K562 cells with the fluorescence enhancing ligand DELFIA BATDA reagent
- Wash K562 cells with medium, adjust the number of cells to $1x10^6$ cells/mL with culture medium. Add 2-4 mL of cells to 5 µl of BATDA reagent, incubate for 10 minutes at 37°C.
- Spin down at 1500RPM for 5minutes to wash the loaded K562 cells for 3-5 times with medium containing 1mM Probenecid (Sigma P8761).
- After the final wash resuspend the cell pellet in culture medium and adjust to about $5x10^4$ cells/mL.

Cytotoxicity assay

[0133]

- Set up wells for detection of background, spontaneously release and maximum release.
- Pipette 100µL of loaded target cells (5,000 cells) to a V-bottom plate.
- Add 100µL of effector cells (NK-92) of varying cell concentrations. Effector to target ratio ranges from 1:1 to 20:1.
- Spin down the plate at 100xg of RCF for 1 minute.
- Incubate for 2 hours in a humidified 5% CO2 atmosphere at 37 °C. For maximum release wells, add 10 µL of lysis buffer to each well 15 minutes before harvesting the medium.
- Spin down the plate at 500xg for 5 minutes.
- Transfer 20 µL of supernatant to a flat-bottom 96 well plate, add 200 µL of pre-warmed Europium solution, incubate at room temperature for 15 minutes using plateshaker.
- Measure the fluorescence in a time-resolved fluorometer by using VARIOSKAN FLASH. The specific release was calculated using the following formula:
- % specific release = Experiment release - Spontaneous release / Maximum release - Spontaneous release

[0134] Results: we followed the above to determine the effect on cytotoxicity of the CD328 knockdown. The results of one representative experiment are shown in figure 9. As seen, cytotoxicity against target cells was increased in cells with the CD328 knockdown.

Example 5 - Protocol for Blood Cancer Therapy by Knockdown / Knockout of Checkpoint Inhibitory Receptors

[0135] As demonstrated in the above Examples, checkpoint inhibitory receptor function can be knocked down or knocked out in a variety of ways. The following protocol was developed for use in treating patients with blood cancer:
Following diagnosis of a patient with a cancer suitably treated with the invention, an aliquot of modified NK cells can be thawed and cultured prior to administration to the patient.

[0136] Alternatively, a transient mutation can be prepared using e.g. siRNA within a day or two, as described above. The MaxCyte Flow Electroporation platform offers a suitable solution for achieving fast large-scale transfections in the clinic.

[0137] The removal of certain checkpoint inhibitory receptors may be more beneficial than others. This is likely to depend on the patient and the cancer. For this reason, the cancer is optionally biopsied and the cancer cells are grown in culture *ex vivo.* A range of NK cells with

different checkpoint inhibitory receptor modifications can thus be tested for cytotoxicity against the specific cancer. This step can be used to select the most appropriate NK cell or derivative thereof for therapy.

[0138] Following successful modification, the cells are resuspended in a suitable carrier (e.g. saline) for intravenous and/or intratumoural injection into the patient.

Example 6 - KHYG-1 Knock-in of TRAIL / TRAIL variant

[0139] KHYG-1 cells were transfected with both TRAIL and TRAIL variant, in order to assess their viability and ability to kill cancer cells following transfection.

[0140] The TRAIL variant used is that described in WO 2009/077857. It is encoded by the wildtype TRAIL gene containing the D269H/E195R mutation. This mutation significantly increases the affinity of the TRAIL variant for DR5, whilst reducing the affinity for both decoy receptors (DcR1 and DcR2).

Baseline TRAIL Expression

[0141] Baseline TRAIL (CD253) expression in KHYG-1 cells was assayed using flow cytometry.

[0142] Mouse anti-human CD253-APC (Biolegend catalog number: 308210) and isotype control (Biolegend catalog number: 400122) were used to stain cell samples and were analyzed on a BD FACS Canto II flow cytometer.

[0143] KHYG-1 cells were cultured in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine, penicillin (100 U/mL)/streptomycin (100 mg/mL) and IL-2 (10ng/mL). 0.5-1.0 × 10^6 cells/test were collected by centrifugation (1500rpm x 5 minutes) and the supernatant was aspirated. The cells (single cell suspension) were washed with 4 mL ice cold FACS Buffer (PBS, 0.5-1% BSA, 0.1% NaN3 sodium azide). The cells were re-suspended in 100 µL ice cold FACS Buffer, add 5uL antibody was added to each tube and incubated for 30 minutes on ice. The cells were washed 3 times by centrifugation at 1500 rpm for 5 minutes. The cells were then re-suspended in 500 µL ice cold FACS Buffer and temporarily kept in the dark on ice.

[0144] The cells were subsequently analyzed on the flow cytometer (BD FACS Canto II) and the generated data were processed using FlowJo 7.6.2 software.

[0145] As can be seen in Fig. 10, FACS analysis showed weak baseline expression of TRAIL on the KHYG-1 cell surface.

TRAIL / TRAIL variant Knock-in by Electroporation

[0146] Wildtype TRAIL mRNA and TRAIL variant (D269H/195R) mRNA was synthesized by TriLink BioTechnologies, aliquoted and stored as -80°C. Mouse anti-human CD253-APC (Biolegend catalog number: 308210) and isotype control (Biolegend catalog number: 400122), and Mouse anti-human CD107a-PE (eBioscience catalog number: 12-1079-42) and isotype control (eBioscience catalog number: 12-4714) antibodies were used to stain cell samples and were analyzed on a BD FACS Canto II flow cytometer. DNA dye SYTOX-Green (Life Technologies catalog number: S7020; 5 mM Solution in DMSO) was used. To achieve transfection efficiencies of up to 90% with high cell viability in KHYG-1 cells with the Nucleofector™ Device (Nucleofector II, Lonza), a Nucleofector™ Kit T from Lonza was used. Antibiotics-free RPMI 1640 containing 10% FBS, L-glutamine (2mM) and IL-2 (10ng/mL) was used for post-Nucleofection culture.

[0147] KHYG-1 and NK-92 cells were passaged one or two days before Nucleofection, as the cells must be in the logarithmic growth phase. The Nucleofector solution was pre-warmed to room temperature (100 µl per sample), along with an aliquot of culture medium containing serum and supplements at 37°C in a 50 mL tube. 6-well plates were prepared by filling with 1.5 mL culture medium containing serum and supplements and pre-incubated in a humidified 37°C / 5% CO_2 incubator. An aliquot of cell culture was prepared and the cells counted to determine the cell density. The required number of cells was centrifuged at 1500rpm for 5 min, before discarding the supernatant completely. The cell pellet was re-suspended in room temperature Nucleofector Solution to a final concentration of $2x10^6$ cells/100µl) (maximum time in suspension = 20 minutes). 100 µl cell suspension was mixed with 10 µg mRNA (volume of RNA < 10 µL). The sample was transferred into an Amaxa-certified cuvette (making sure the sample covered the bottom of the cuvette and avoiding air bubbles). The appropriate Nucleofector program was selected (i.e. U-001 for KHYG-1 cells). The cuvettes were then inserted into the cuvette holder. 500 µl pre-warmed culture medium was added to the cuvette and the sample transferred into a prepared 6-well plate immediately after the program had finished, in order to avoid damage to the cells. The cells were incubated in a humidified 37°C / 5% CO_2 incubator. Flow cytometric analysis and cytotoxicity assays were performed 12-16 hours after electroporation. Flow cytometry staining was carried out as above.

[0148] As can be seen in Fig.s 11 and 12, expression of TRAIL / TRAIL variant and CD107a (NK activation marker) increased post-transfection, confirming the successful knock-in of the TRAIL genes into KHYG-1 cells.

[0149] Fig. 13 provides evidence of KHYG-1 cell viability before and after transfection via electroporation. It can be seen that no statistically significant differences in cell viability are observed following transfection of the cells with TRAIL / TRAIL variant, confirming that the expression of wildtype or variant TRAIL is not toxic to the cells. This observation contradicts corresponding findings in NK-92 cells, which suggest the TRAIL variant gene knock-in is toxic to the cells (data not shown). Nevertheless, this is likely explained by the relatively high expression levels of TRAIL receptors DR4 and DR5 on the NK-92 cell surface (see Fig. 14).

Effects of TRAIL / TRAIL variant on KHYG-1 Cell Cytotoxicity

[0150] Mouse anti-human CD2-APC antibody (BD Pharmingen catalog number: 560642) was used. Annexin V-FITC antibody (ImmunoTools catalog number: 31490013) was used. DNA dye SYTOX-Green (Life Technologies catalog number: S7020) was used. A 24-well cell culture plate (SARSTEDT AG catalog number: 83.3922) was used. Myelogenous leukemia cell line K562, multiple myeloma cell line RPMI8226 and MM1.S were used as target cells. K562, RPMI8226, MM1.S were cultured in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine and penicillin (100 U/mL)/streptomycin (100 mg/mL).

[0151] As explained above, KHYG-1 cells were transfected with TRAIL / TRAIL variant.

[0152] The target cells were washed and pelleted via centrifugation at 1500rpm for 5 minutes. Transfected KHYG-1 cells were diluted to $0.5x10^6$/mL. The target cell density was then adjusted in pre-warmed RPMI 1640 medium, in order to produce effector:target (E:T) ratios of 1:1.

[0153] 0.5 mL KHYG-1 cells and 0.5 mL target cells were then mixed in a 24-well culture plate and placed in a humidified 37°C / 5% CO_2 incubator for 12 hours. Flow cytometric analysis was then used to assay KHYG-1 cell cytotoxicity; co-cultured cells (at different time points) were washed and then stained with CD2-APC antibody (5 µL/test), Annexin V-FITC (5 µL/test) and SYTOX-Green (5 µL/test) using Annexin V binding buffer.

[0154] Data were further analyzed using FlowJo 7.6.2 software. CD2-positive and CD2-negative gates were set, which represent KHYG-1 cell and target cell populations, respectively. The Annexin V-FITC and SYTOX-Green positive cells in the CD2-negative population were then analyzed for TRAIL-induced apoptosis.

[0155] Fig.s 15, 16 and 17 show the effects of both KHYG-1 cells expressing TRAIL or TRAIL variant on apoptosis for the three target cell lines: K562, RPMI8226 and MM1.S, respectively. It is apparent for all target cell populations that TRAIL expression on KHYG-1 cells increased the level of apoptosis, when compared to normal KHYG-1 cells (not transfected with TRAIL). Moreover, TRAIL variant expression on KHYG-1 cells further increased apoptosis in all target cell lines, when compared to KHYG-1 cells transfected with wildtype TRAIL.

[0156] Cells of the invention, expressing the TRAIL variant, offer a significant advantage in cancer therapy, due to exhibiting higher affinities for the death receptor DR5. When challenged by these cells of the invention, cancer cells are prevented from developing defensive strategies to circumvent death via a certain pathway. Thus cancers cannot effectively circumvent TRAIL-induced cell death by upregulating TRAIL decoy receptors, as cells of the invention are modified so that they remain cytotoxic in those circumstances.

Example 7 - Protocol for Blood Cancer Therapy using NK Cells with TRAIL Variants Knocked-in

[0157] KHYG-1 cells were transfected with TRAIL variant, as described above in Example 6. The following protocol was developed for use in treating patients with blood cancer:
Following diagnosis of a patient with a cancer suitably treated with the invention, a DR5-inducing agent, e.g. Bortezomib, is administered, prior to administration of the modified NK cells, and hence is used at low doses to upregulate expression of DR5 on the cancer, making modified NK cell therapy more effective.

[0158] An aliquot of modified NK cells is then thawed, cultured and administered to the patient.

[0159] Since the TRAIL variant expressed by the NK cells used in therapy has a lower affinity for decoy receptors than wildtype TRAIL, there is increased binding of death receptors on the cancer cell surface, and hence more cancer cell apoptosis as a result.

[0160] Another option, prior to implementation of the above protocol, is to biopsy the cancer and culture cancer cells *ex vivo.* This step can be used to identify those cancers expressing particularly high levels of decoy receptors, and/or low levels of death receptors, in order to help determine whether a DR5-inducing agent is appropriate for a given patient. This step may also be carried out during therapy with the above protocol, as a given cancer might be capable of adapting to e.g. reduce its expression of DR5, and hence it may become suitable to treat with a DR5-inducing agent part-way through therapy.

Example 8 - Low Dose Bortezomib Sensitizes Cancer Cells to NK Cells Expressing TRAIL Variant

[0161] Bortezomib (Bt) is a proteasome inhibitor (chemotherapy-like drug) useful in the treatment of Multiple Myeloma (MM). Bortezomib is known to upregulate DR5 expression on several different types of cancer cells, including MM cells.

[0162] KHYG-1 cells were transfected with TRAIL variant, as described above in Example 6, before being used to target MM cells with or without exposure to Bortezomib.

<u>Bortezomib-induced DR5 expression</u>

[0163] Bortezomib was bought from Millennium Pharmaceuticals. Mouse anti-human DR5-AF647 (catalog number: 565498) was bought from BD Pharmingen. The stained cell samples were analyzed on BD FACS Canto II.

1. (1) MM cell lines RPMI8226 and MM1.S were grown in RPMI1640 medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM sodium bicarbonate, 0.01% of antibiotics and 10% fetal bovine serum (Sigma, St Louis, MO, USA), in 5% CO2 atmosphere at 37°C.
2. (2) MM cells were seeded in 6-well plates at $1x10^6$/mL, 2mL/well.
3. (3) MM cells were then treated with different doses of Bortezomib for 24 hours.
4. (4) DR5 expression in Bortezomib treated/untreated MM cells was then analyzed by flow cytometry (Fig. 18).

[0164] Low dose Bortezomib treatment was found to increase DR5 expression in both MM cell lines (Fig. 18). DR5 upregulation was associated with a minor induction of apoptosis (data not shown). It was found, however, that DR5 expression could not be upregulated by high doses of Bortezomib, due to high toxicity resulting in most of the MM cells dying.

Bortezomib-induced sensitization of cancer cells

[0165] KHYG-1 cells were transfected with the TRAIL variant (TRAIL D269H/E195R), as described above in Example 6.

1. (1) Bortezomib treated/untreated MM1.S cells were used as target cells. MM1.S cells were treated with 2.5nM of Bortzeomib or vehicle (control) for 24 hours.
2. (2) 6 hours after electroporation of TRAIL variant mRNA, KHYG-1 cells were then cultured with MM cells in 12- well plate. After washing, cell concentrations were adjusted to $1x10^6$/mL, before mixing KHYG-1 and MM1.S cells at 1:1 ratio to culture for 12 hours.
3. (3) Flow cytometric analysis of the cytotoxicity of KHYG-1 cells was carried out. The co-cultured cells were collected, washed and then stained with CD2-APC antibody (5 uL/test), AnnexinV-FITC (5 uL/test) and SYTOX-Green (5 uL/test) using AnnexinV binding buffer.
4. (4) Data were further analyzed using FlowJo 7.6.2 software. CD2-negative population represents MM1.S cells. KHYG-1 cells are strongly positive for CD2. Finally, the AnnexinV-FITC and SYTOX-Green positive cells in the CD2-negative population were analyzed.

[0166] Flow cytometric analysis of apoptosis was performed in Bortezomib-pretreated/untreated MM1.S cells co-cultured with KHYG-1 cells electroporated with/without TRAIL variant (Fig. 19).

[0167] It was found that Bortezomib induced sensitivity of MM cells to KHYG-1 cells expressing the TRAIL variant. The data therefore indicated that an agent that induced DR5 expression

was effective in the model in increasing cytotoxicity against cancer cells, and hence may be useful in enhancing the cancer therapy of the present invention.

Example 9 - Confirmation of Induced Apoptosis by the TRAIL Variant

[0168] Despite the conclusive evidence of increased NK cell cytotoxicity resulting from TRAIL variant expression in the previous Examples, we wished to confirm whether the increased cytotoxicity resulted from inducing cancer cell apoptosis (most likely) or by inadvertently activating the NK cells to exhibit a more cytotoxic phenotype and hence kill cancer cells via perforin secretion.

[0169] Concanamycin A (CMA) has been demonstrated to inhibit perforin-mediated cytotoxic activity of NK cells, mostly due to accelerated degradation of perforin by an increase in the pH of lytic granules. We investigated whether the cytotoxicity of KHYG-1 cells expressing the TRAIL variant could be highlighted when perforin-mediated cytotoxicity was partially abolished with CMA.

CMA-induced reduction of perforin expression

[0170] Mouse anti-human perforin-AF647 (catalog number: 563576) was bought from BD pharmingen. Concanamycin A (catalog number: SC-202111) was bought from Santa Cruz Biotechnology. The stained cell samples were analyzed using a BD FACS Canto II.

1. (1) KHYG-1 cells were cultured in RPMI1640 medium containing 10%FBS (fetal bovine serum), 2mM L-glutamine, penicillin (100 U/mL)/streptomycin (100 mg/mL), and IL-2 (10ng/mL).
2. (2) KHYG-1 cells (6 hours after electroporation, cultured in penicillin/streptomycin free RPMI1640 medium) were further treated with 100nM CMA or equal volume of vehicle (DMSO) for 2 hours.
3. (3) The cells were collected ($1x10^6$ cells/test) by centrifugation (1500rpm x 5 minutes) and the supernatant was aspirated.
4. (4) The cells were fixed in 4% paraformaldehyde in PBS solution at room temperature for 15 minutes.
5. (5) The cells were washed with 4 mL of FACS Buffer (PBS, 0.5-1% BSA, 0.1% sodium azide) twice.
6. (6) The cells were permeabilized with 1mL of PBS/0.1% saponin buffer for 30 minutes at room temperature.
7. (7) The cells were washed with 4 mL of PBS/0.1% saponin buffer.
8. (8) The cells were re-suspended in 100 uL of PBS/0.1% saponin buffer, before adding 5uL of the antibody to each tube and incubating for 30 minutes on ice.
9. (9) The cells were washed with PBS/0.1% saponin buffer 3 times by centrifugation at

1500 rpm for 5 minutes.

10. (10) The cells were re-suspended in 500 uL of ice cold FACS Buffer and kept in the dark on ice or at 4°C in a fridge briefly until analysis.
11. (11) The cells were analyzed on the flow cytometer (BD FACS Canto II). The data were processed using FlowJo 7.6.2 software.

[0171] CMA treatment significantly decreased the perforin expression level in KHYG-1 cells (Fig. 20) and had no negative effects on the viability of KHYG-1 cells (Fig. 21).

Cytotoxicity of NK cell TRAIL variants in the presence of CMA

[0172] KHYG-1 cells were transfected with the TRAIL variant (TRAIL D269H/E195R), as described above in Example 6.

1. (1) MM1.S cells were used as target cells.
2. (2) 6 hours after electroporation of TRAIL mRNA, KHYG-1 cells were treated with 100mM CMA or an equal volume of vehicle for 2 hours.
3. (3) The KHYG-1 cells were washed with RPMI1640 medium by centrifugation, and re-suspended in RPMI1640 medium containing IL-2, adjusting cell concentrations to $1x10^6$/mL.
4. (4) The MM1.S cells were re-suspended in RPMI1640 medium containing IL-2 adjusting cell concentrations to $1x10^6$/mL.
5. (5) The KHYG-1 and MM1.S cells were mixed at 1:1 ratio and co-cultured for 12 hours.
6. (6) Flow cytometric analysis of the cytotoxicity of KHYG-1 cells was carried out. The co-cultured cells were washed and stained with CD2-APC antibody (5 uL/test).
7. (7) After washing, further staining was performed with AnnexinV-FITC (5 uL/test) and SYTOX-Green (5 uL/test) using AnnexinV binding buffer.
8. (8) Data were further analyzed using FlowJo 7.6.2 software. CD2-negative population represents MM1.S cells. KHYG-1 cells are strongly positive for CD2. The AnnexinV-FITC and SYTOX-Green positive cells in CD2-negative population were then analyzed.

[0173] It was again shown that NK cells expressing the TRAIL variant show higher cytotoxicity than control cells lacking expression of the TRAIL variant (Fig. 22). In this Example, however, it was further shown that CMA was unable to significantly diminish the cytotoxic activity of NK cells expressing TRAIL variant, in contrast to the finding for control NK cells treated with CMA.

[0174] NK cells without the TRAIL variant (control or mock NK cells) were shown to induce 48% cancer cell death in the absence CMA and 35.9% cancer cell death in the presence of CMA (Fig. 22). NK cells expressing the TRAIL variant were able to induce more cancer cell

death than control NK cells both in the presence and absence of CMA. In fact, even with CMA present, NK cells expressing TRAIL variant induced more cancer cell death than control NK cells in the absence of CMA.

[0175] This data thus shows the importance of the TRAIL variant in increasing NK cell cytotoxicity against cancer cells via a mechanism less susceptible to perforin-related downregulation. Since perforin is used commonly by NK cells to kill target cells, and many cancer cells have developed mechanisms for reducing NK cell perforin expression, in order to evade cytotoxic attack, the NK cells of the invention represent a powerful alternative less susceptible to attenuation by cancer cells.

Example 10 - Combined Expression of Mutant TRAIL Variant and Knockdown of Checkpoint Inhibitory Receptor CD96 in KHYG-1 Cells

[0176] Increases in NK cell cytotoxicity were observed when knocking down checkpoint inhibitory receptor CD96 expression and also when expressing TRAIL variant. We also tested combining the two genetic modifications to provoke a synergistic effect on NK cell cytotoxicity.

[0177] CD96 expression was knocked down in KHYG-1 cells, as described in Example 2.

[0178] KHYG-1 cells were transfected with the TRAIL variant (TRAIL D269H/E195R), as described above in Example 6.

1. (1) 12 hours after electroporation KHYG-1 cells were co-cultured with target cells (K562 or MM1.S) at a concentration of $1x10^6$/mL in 12-well plates (2mL/well) for 12 hours. The E:T ratio was 1:1.
2. (2) 12 hours after co-culture, the cells were collected, washed, stained with CD2-APC, washed again and further stained with AnnexinV-FITC (5 uL/test) and SYTOX-Green (5 uL/test) using AnnexinV binding buffer.
3. (3) Cell samples were analyzed using a BD FACS canto II flow cytometer. Data were further analyzed using FlowJo 7.6.2 software. CD2-negative population represents MM1.S cells. KHYG-1 cells are strongly positive for CD2. The AnnexinV-FITC and SYTOX-Green positive cells in the CD2-negative population were then analyzed.

[0179] Simultaneously knocking down CD96 expression and expressing TRAIL variant in KHYG-1 cells was found to synergistically enhance the cells' cytotoxicity against both K562 target cells (Fig. 23) and MM1.S target cells (Fig. 24). This was indicated by the fact that in both target cell groups, more cell death resulted from the simultaneous genetic modification than resulted from the individual modifications in isolation.

[0180] At the same time, further evidence showing knockdown of CD96 increases NK cell

cytotoxicity was obtained (Fig. 23 & 24), in addition to further evidence showing expression of the TRAIL mutant/variant increases NK cell cytotoxicity (Fig. 23 & 24).

[0181] The invention thus provides NK cells and cell lines, and production thereof, for use in blood cancer therapy.

SEQUENCE LISTING

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<120> MODIFIED NATURAL KILLER CELLS AND NATURAL KILLER CELL LINES HAVING INCREASED CYTOTOXICITY

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO2006023148A [0006]
- EP1621550A [0010]
- WO2009077857A [0044] [0045] [0140]

PATENTKRAV

1. Human, naturlig dræber- (NK) celle eller NK-cellelinje, der er modificeret til at udtrykke en TRAIL-variant, hvor TRAIL-varianten har mindst en 25 % stigning i affinitet for DR4, i forhold til vildtype-TRAIL.

2. Human NK-celle eller NK-cellelinje ifølge krav 1, hvor TRAIL-varianten har en reduceret affinitet, i forhold til vildtype-TRAIL, for afflednings-TRAIL-receptorer.

3. Human NK-celle eller NK-cellelinje ifølge et hvilket som helst forudgående krav, der er blevet yderligere modificeret til at reducere ekspression, i forhold til en vildtype-NK-celle eller NK-cellelinje, af én eller flere checkpoint-hæmmerreceptorer.

4. Human NK-celle eller NK-cellelinje ifølge krav 3, hvor checkpoint-hæmmerreceptorerne er udvalgt fra CD96 (TACTILE), CD152 (CTLA4), CD223 (LAG-3), CD279 (PD-1), CD328 (SIGLEC7), SIGLEC9, TIGIT og TIM-3.

5. Human NK-celle eller NK-cellelinje ifølge et hvilket som helst forudgående krav, hvor NK-cellelinjen er KHYG-1 eller et derivat deraf.

6. Human, naturlig dræber- (NK) celle eller NK-cellelinje til anvendelse i behandling af cancer, hvor NK-cellen eller NK-cellelinjen er blevet modificeret til at udtrykke en TRAIL-variant med mindst en 25 % stigning i affinitet for DR4, i forhold til vildtype-TRAIL.

7. Human NK-celle eller NK-cellelinje til anvendelse ifølge krav 6, hvor TRAIL-varianten har en reduceret affinitet, i forhold til vildtype-TRAIL, for afflednings-TRAIL-receptorer.

8. Human NK-celle eller NK-cellelinje til anvendelse ifølge krav 6 eller 7, hvor canceren er en blodcancer.

9. Human NK-celle eller NK-cellelinje til anvendelse ifølge krav 8, hvor blodcanceren er akut lymfocytisk leukæmi (ALL), akut myeloid leukæmi (AML), kronisk lymfocytisk

leukæmi (CLL), kronisk myeloid leukæmi (CML), Hodgkins lymfom, ikke-Hodgkins lymfom, indbefattende T-cellelymfomer og B-cellelymfomer, asymptomatisk myelom, ulmende multipelt myelom (SMM), aktivt myelom eller letkæde-myelom.

10. Human NK-celle eller NK-cellelinje til anvendelse ifølge krav 8, hvor blodcanceren er is leukæmi.

11. Human NK-celle eller NK-cellelinje til anvendelse ifølge krav 10, hvor leukæmien er AML.

12. Human, naturlig dræber- (NK) celle eller NK-cellelinje til anvendelse i behandling af akut myeloid leukæmi (AML), hvor NK-cellen eller NK-cellelinjen er blevet modificeret til at udtrykke en TRAIL-variant med mindst en 25 % stigning i affinitet for DR4, i forhold til vildtype-TRAIL.

13. Human NK-celle eller NK-cellelinje til anvendelse ifølge et hvilket som helst af kravene 6-12, hvor NK-cellelinjen er KHYG-1 eller et derivat deraf.

14. Human NK-celle eller NK-cellelinje ifølge et hvilket som helst af kravene 1-5 eller human NK-celle eller NK-cellelinje til anvendelse ifølge et hvilket som helst af kravene 6-13, hvor NK-cellen eller NK-cellelinjen er blevet modificeret til at udtrykke en TRAIL-variant med mindst en 25 % stigning i affinitet for DR5, i forhold til vildtype-TRAIL.

DRAWINGS

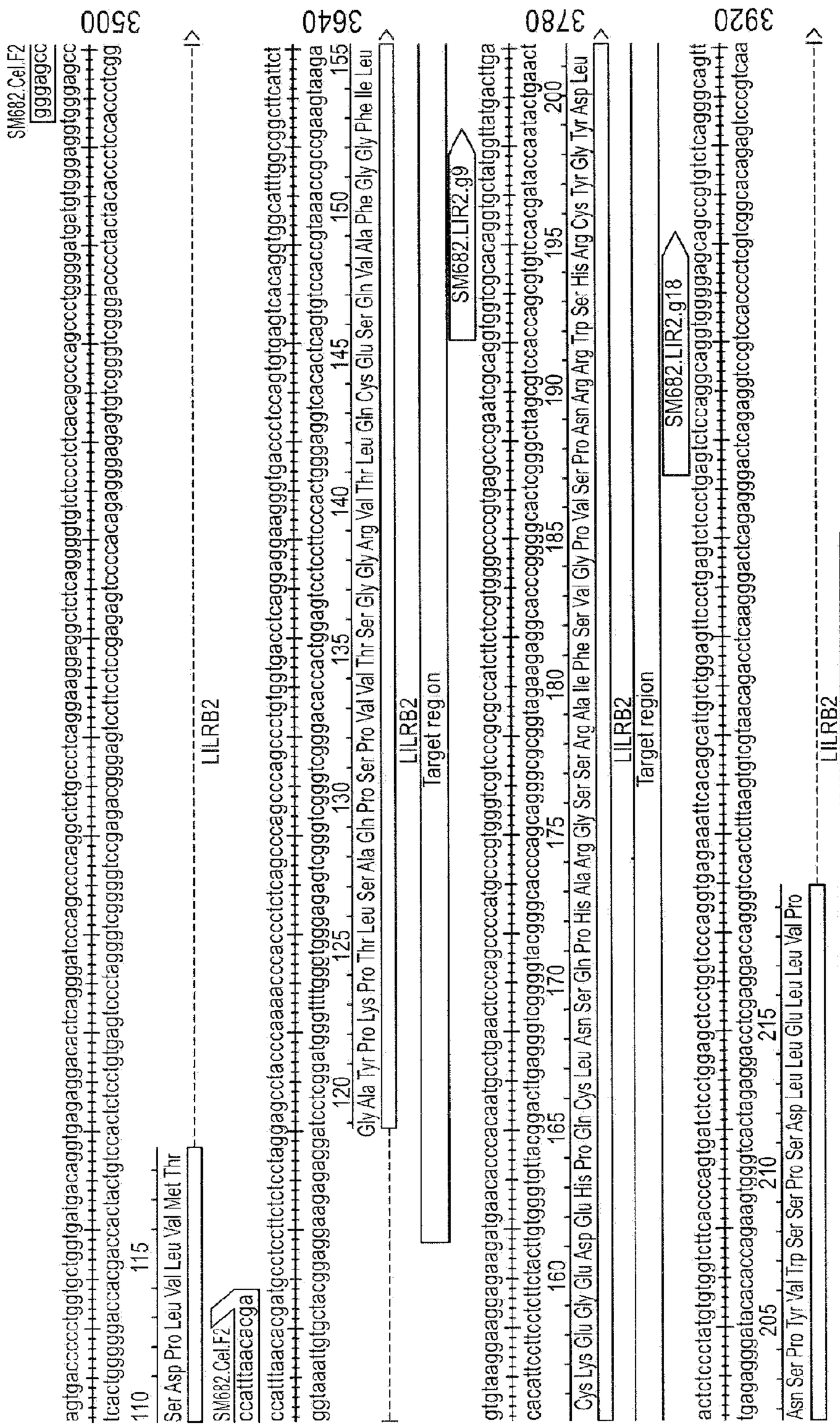


FIG. 1

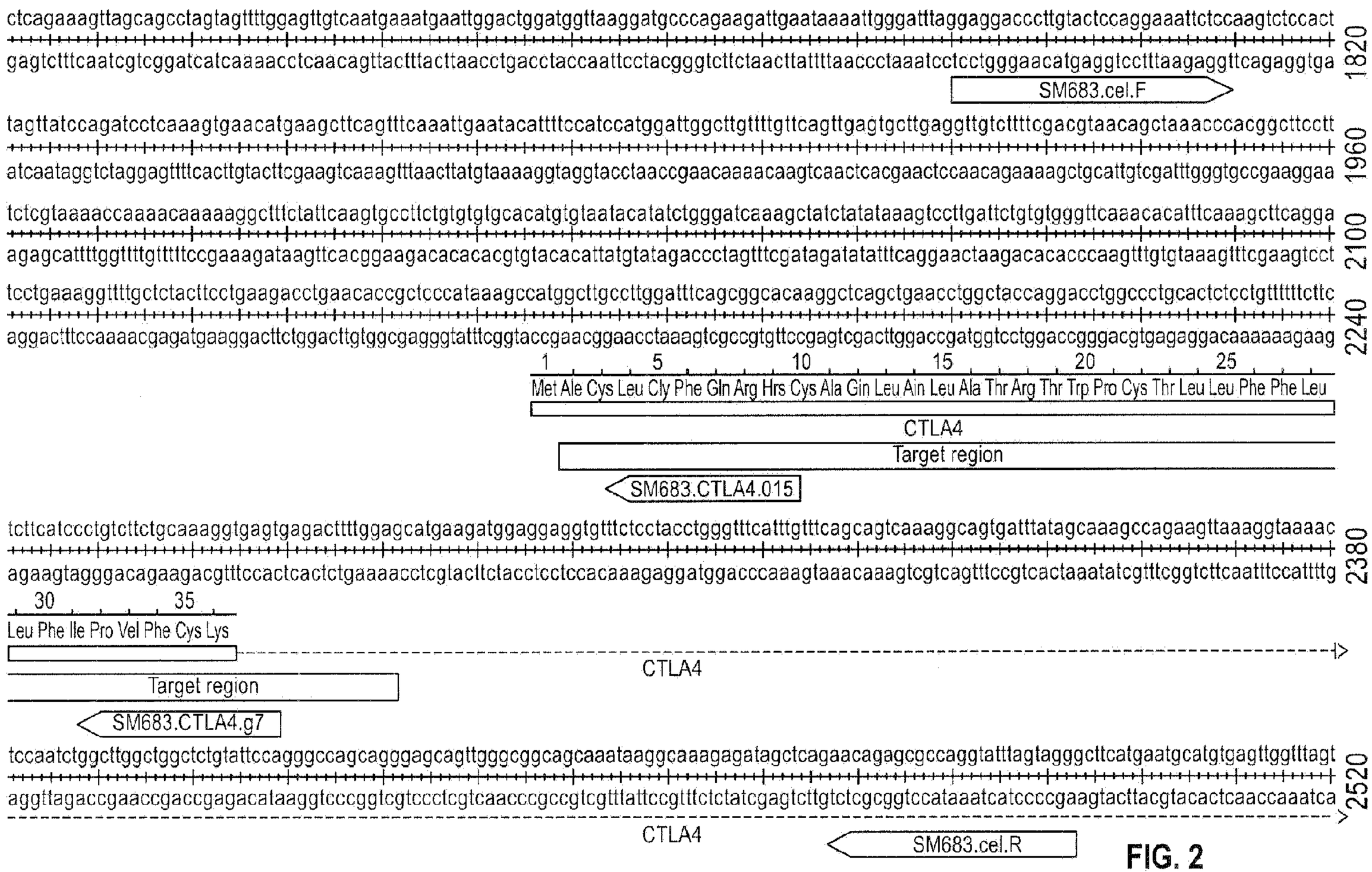

ctcagaaagttagcagcctagtagttttggagttgtcaatgaaatgaattggactggatggttaaggatgcccagaagattgaataaaattgggatttaggaggacccttgtactccaggaaattctccaagtctccact
gagtctttcaatcgtcggatcatcaaaacctcaacagttactttacttaacctgacctaccaattcctacgggtcttctaacttatttaaccctaaatcctcctgggaacatgaggtcctttaagaggttcagaggtga
1820
SM683.cel.F
tagttatccagatcctcaaagtgaacatgaagcttcagtttcaaattgaatacattttccatccatggattggcttgttttgttcagttgagtgcttgaggttgtcttttcgacgtaacagctaaacccacggcttcctt
atcaatagqtctaggagttttcacttgtacttcgaagtcaaagtttaacttatgtaaaaggtaggtacctaaccgaacaaaacaagtcaactcacgaactccaacagaaaagctgcattgtcgatttgggtgccgaaggaa
1960
tctcgtaaaaccaaaacaaaaaggctttctattcaagtgccttctgtgtgtgcacatgtgtaatacatatctgggatcaaagctatctatataaagtccttgattctgtgtgggttcaaacacatttcaaagcttcagga
agagcattttggttttgttttccgaaagataagttcacggaagacacacacgtgtacacattatgtatagaccctagtttcgatagatatatttcaggaactaagacacacccaagtttgtgtaaagtttcgaagtcct
2100
tcctgaaaggttttgctctacttcctgaagacctgaacaccgctcccataaagccatggcttgccttggatttcagcggcacaaggctcagctgaacctggctaccaggacctggccctgcactctcctgttttttcttc
aggactttccaaaacgagatgaaggacttctggacttgtggcgagggtatttcggtaccgaacggaacctaaagtcgccgtgttccgagtcgacttggaccgatggtcctggaccgggacgtgagaggacaaaaaagaag
2240
1 5 10 15 20 25
Met Ale Cys Leu Cly Phe Gln Arg Hrs Cys Ala Gin Leu Ain Leu Ala Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu
CTLA4
Target region
SM683.CTLA4.015
tcttcatccctgtcttctgcaaaggtgagtgagacttttggagcatgaagatggaggaggtgtttctcctacctgggtttcatttgtttcagcagtcaaaggcagtgatttatagcaaagccagaagttaaaggtaaaac
agaagtagggacagaagacgtttccactcactctgaaaacctcgtacttctacctcctccacaaagaggatggacccaaagtaaacaaagtcgtcagtttccgtcactaaatatcgtttcggtcttcaatttccatttg
2380
30 35
Leu Phe Ile Pro Vel Phe Cys Lys
CTLA4
Target region
SM683.CTLA4.g7
tccaatctggcttggctggctctgtattccagggccagcagggagcagttgggcggcagcaaataaggcaaagagatagctcagaacagagcgccaggtatttagtagggcttcatgaatgcatgtgagttggtttagt
aggttagaccgaaccgaccgagacataaggtcccggtcgtccctcgtcaacccgccgtcgtttattccgtttctctatcgagtcttgtctcgcggtccataaatcatccccgaagtacttacgtacactcaaccaaatca
2520
CTLA4
SM683.cel.R

FIG. 2

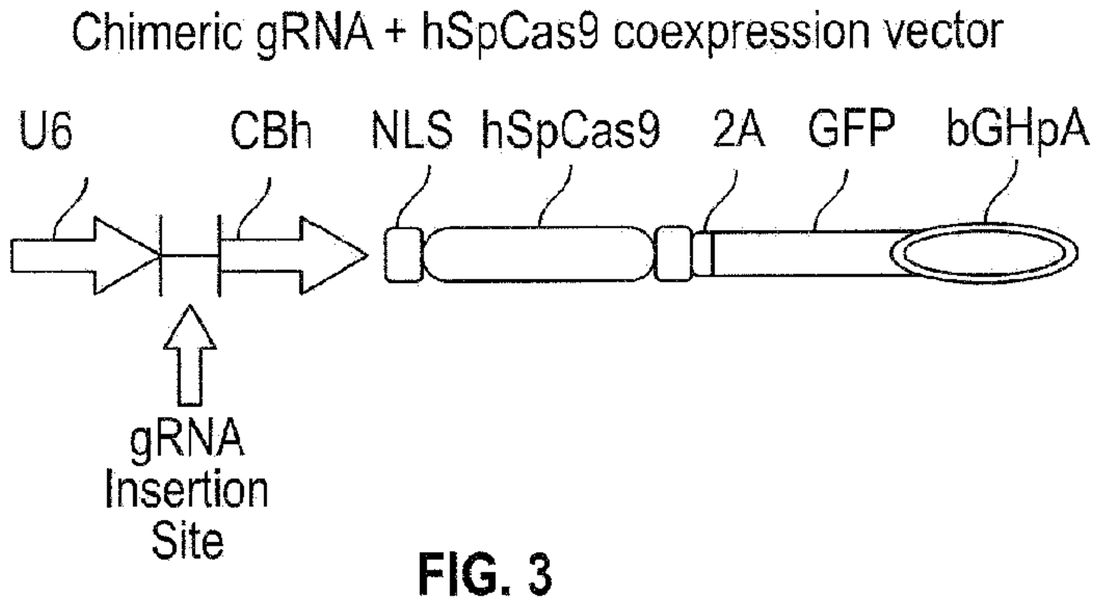
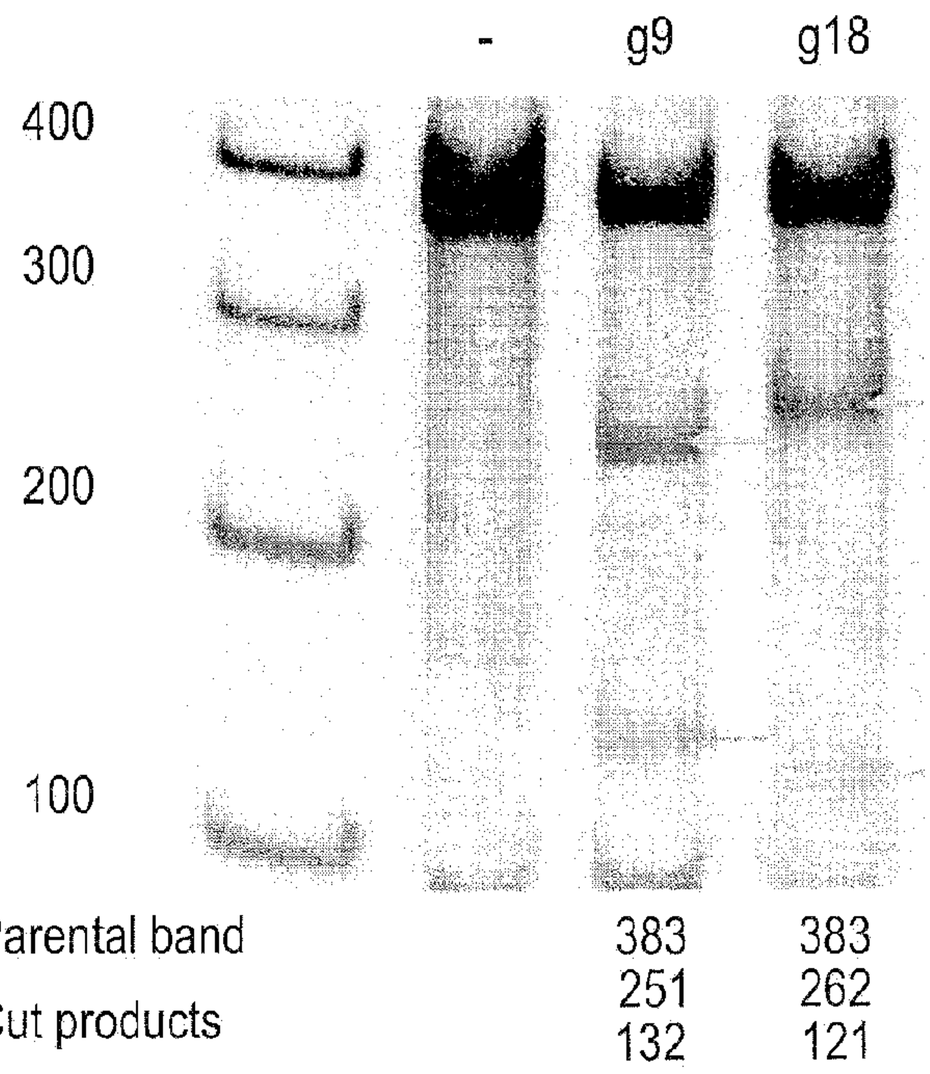


FIG. 3



Parental band		383	383
Cut products		251	262
		132	121

FIG. 4

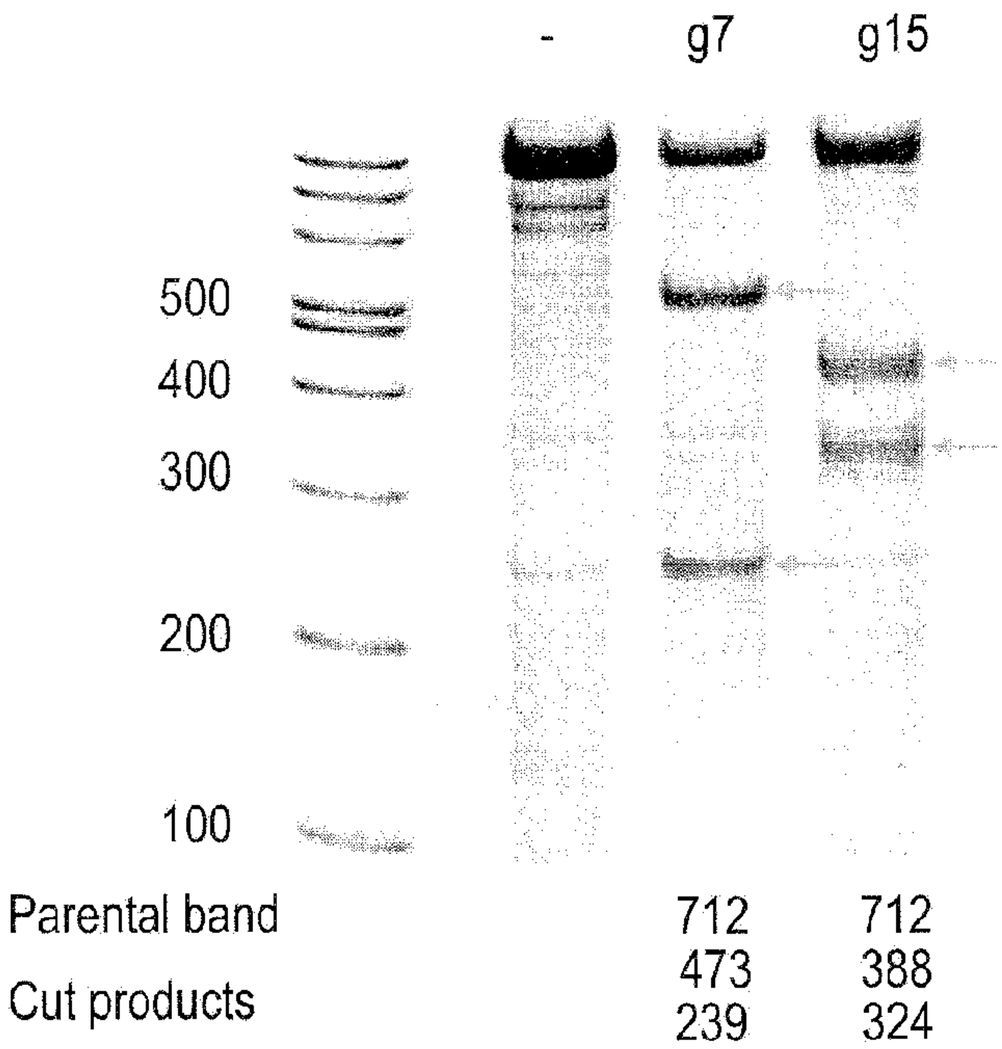

-
g7
g15
500
400
300
200
100
Parental band
712
712
Cut products
473
388
239
324

FIG. 5

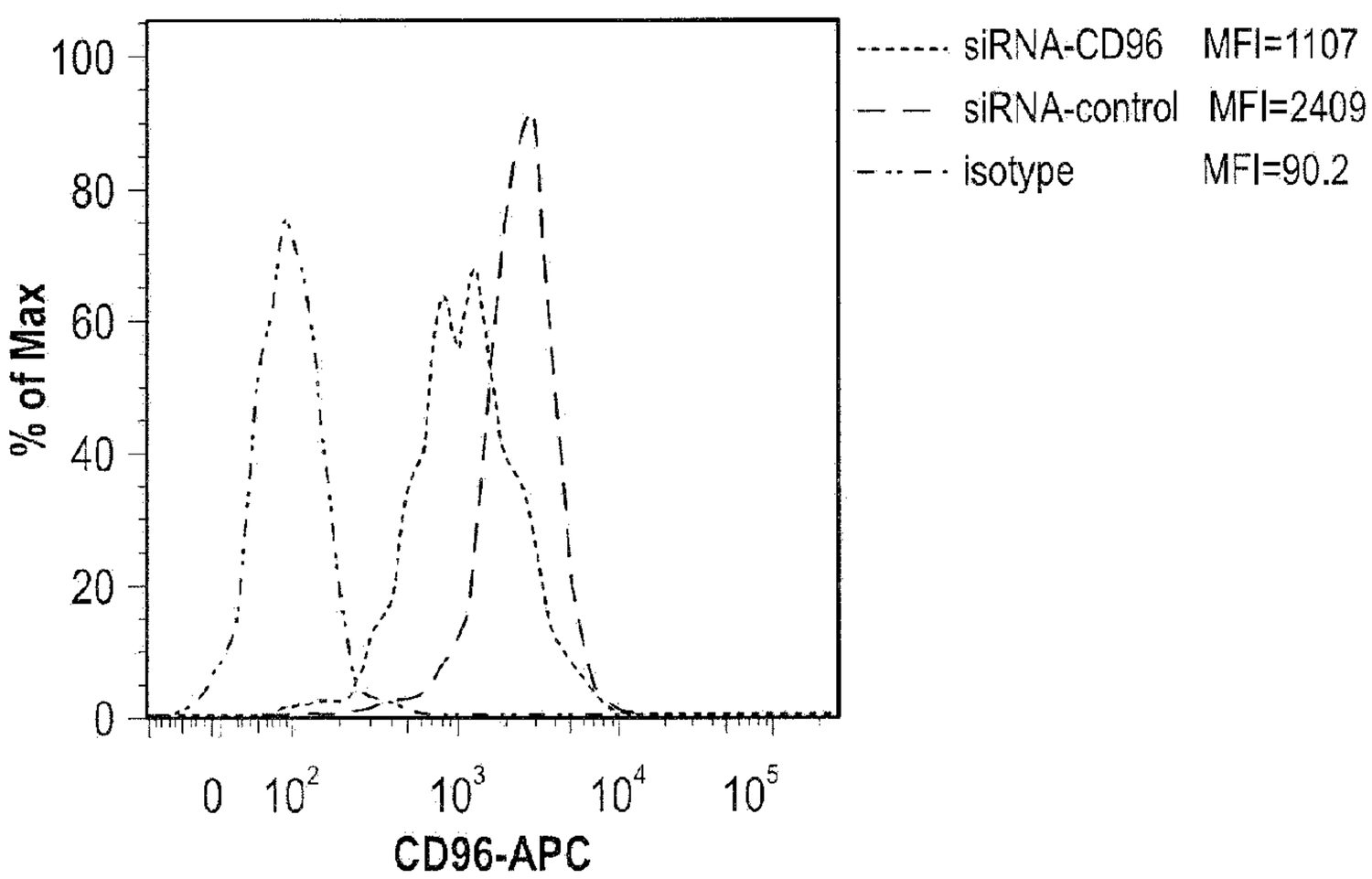

100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
CD96-APC
siRNA-CD96 MFI=1107
siRNA-control MFI=2409
isotype MFI=90.2

FIG. 6A

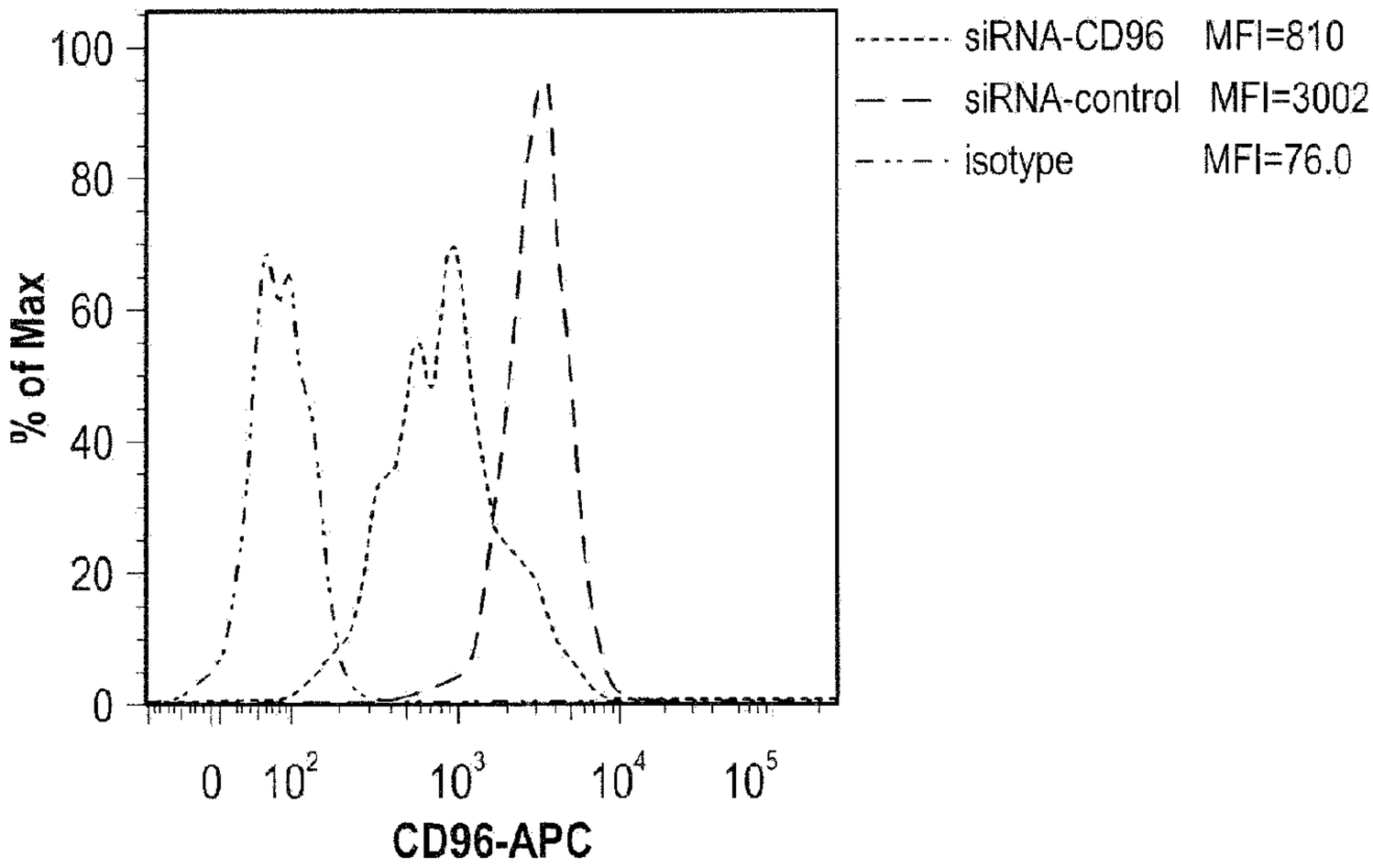

100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
CD96-APC
siRNA-CD96 MFI=810
siRNA-control MFI=3002
isotype MFI=76.0

FIG. 6B

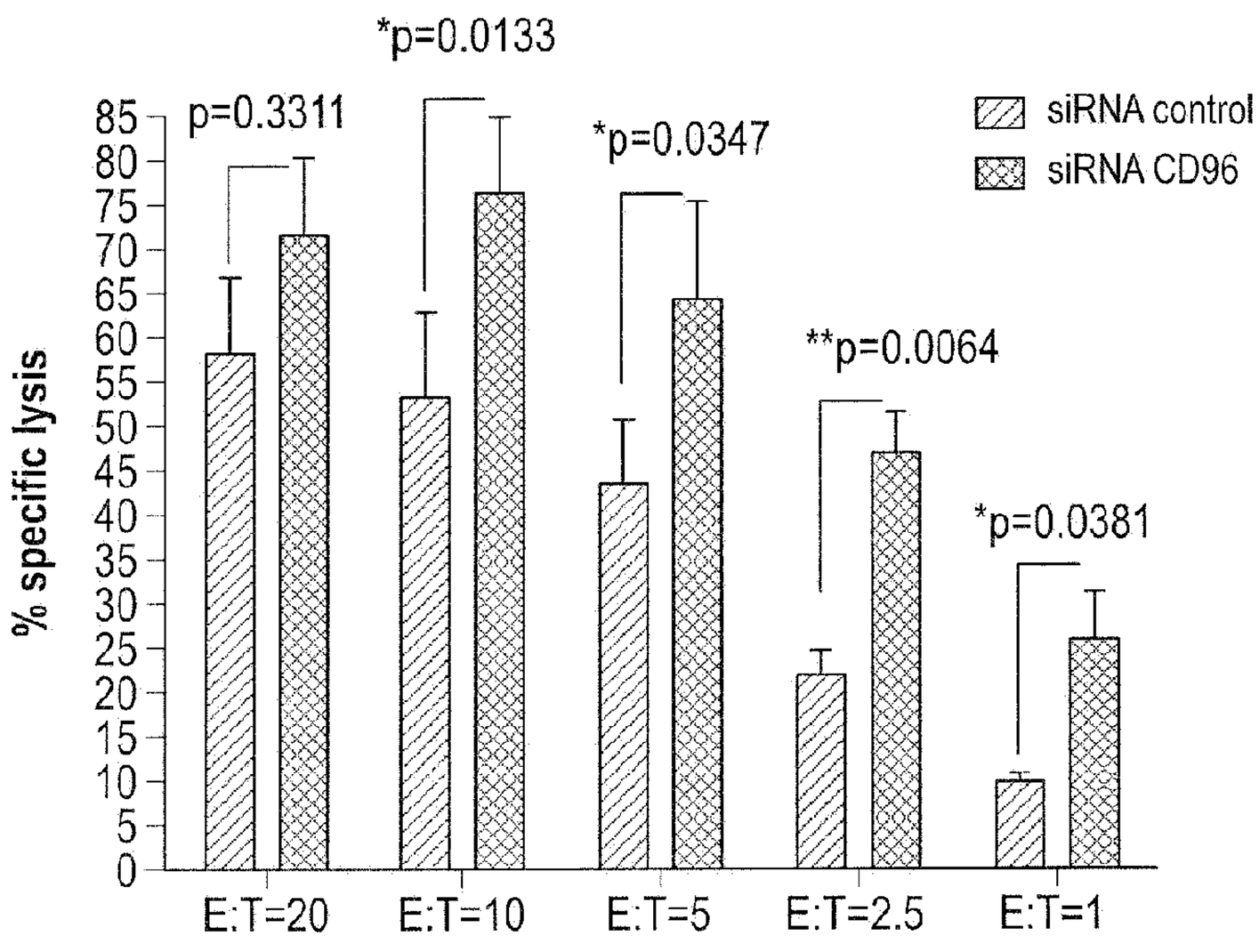

*p=0.0133
p=0.3311
*p=0.0347
**p=0.0064
*p=0.0381
% specific lysis
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
E:T=20
E:T=10
E:T=5
E:T=2.5
E:T=1
siRNA control
siRNA CD96

FIG. 7

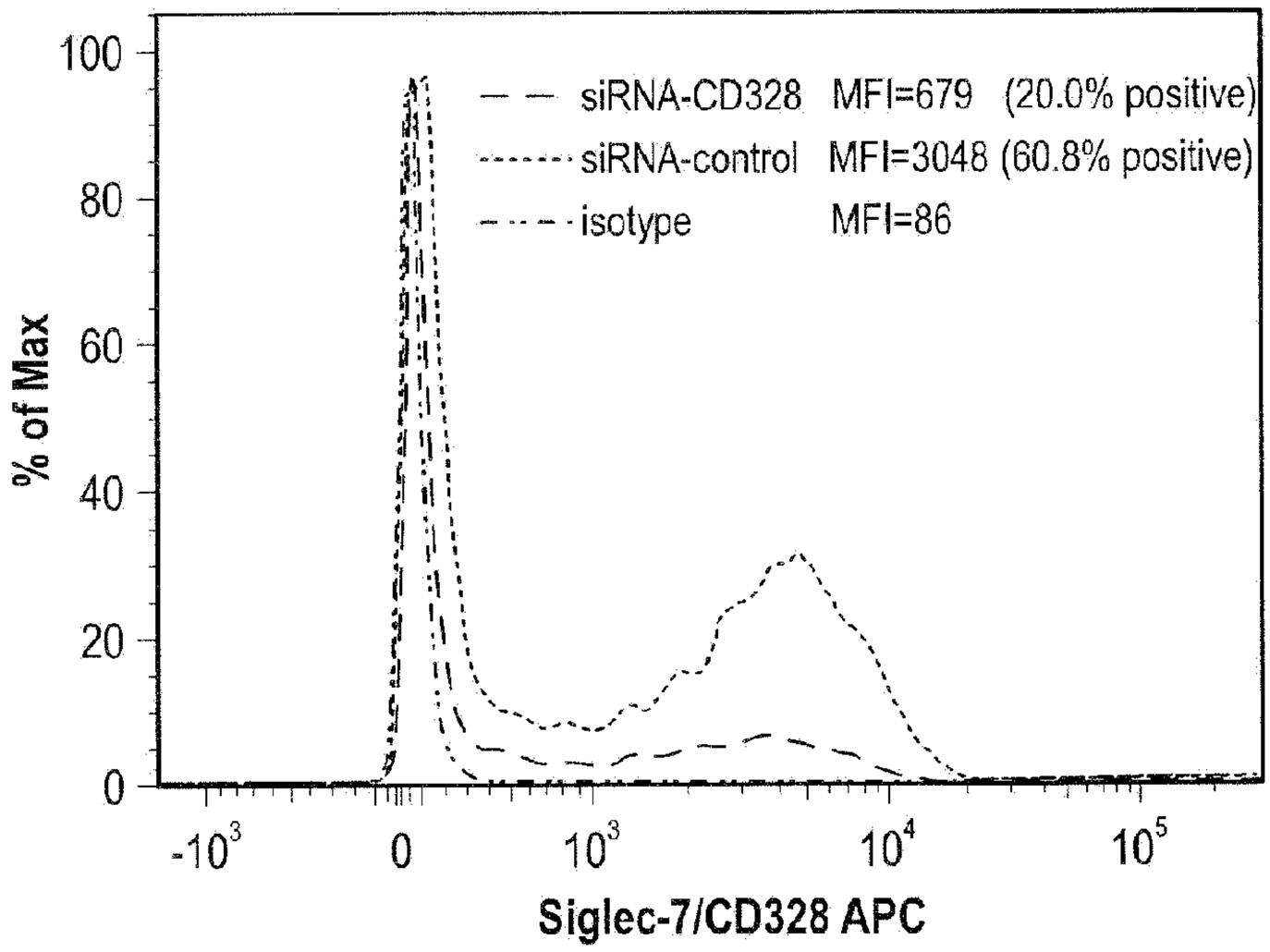

100
80
60
40
20
0
% of Max
siRNA-CD328 MFI=679 (20.0% positive)
siRNA-control MFI=3048 (60.8% positive)
isotype MFI=86
-10^3
0
10^3
10^4
10^5
Siglec-7/CD328 APC

FIG. 8

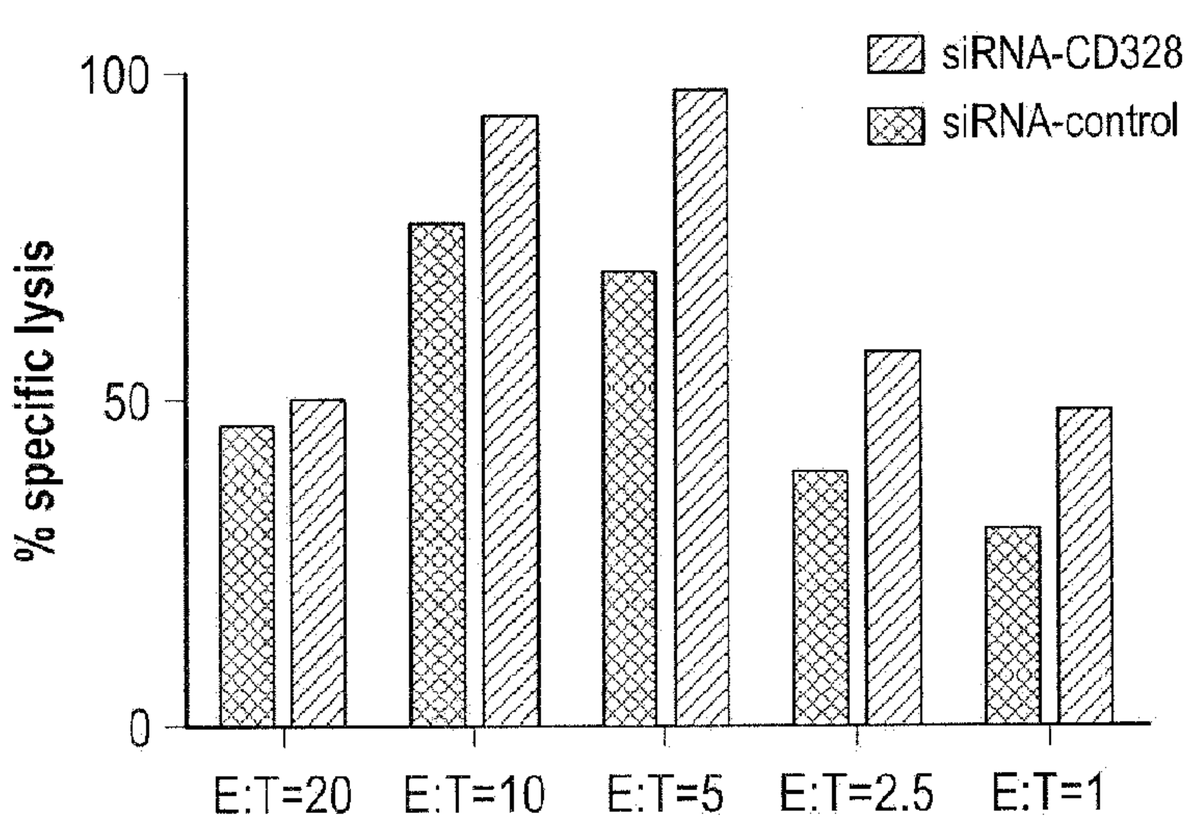

100
50
0
% specific lysis
siRNA-CD328
siRNA-control
E:T=20
E:T=10
E:T=5
E:T=2.5
E:T=1

FIG. 9

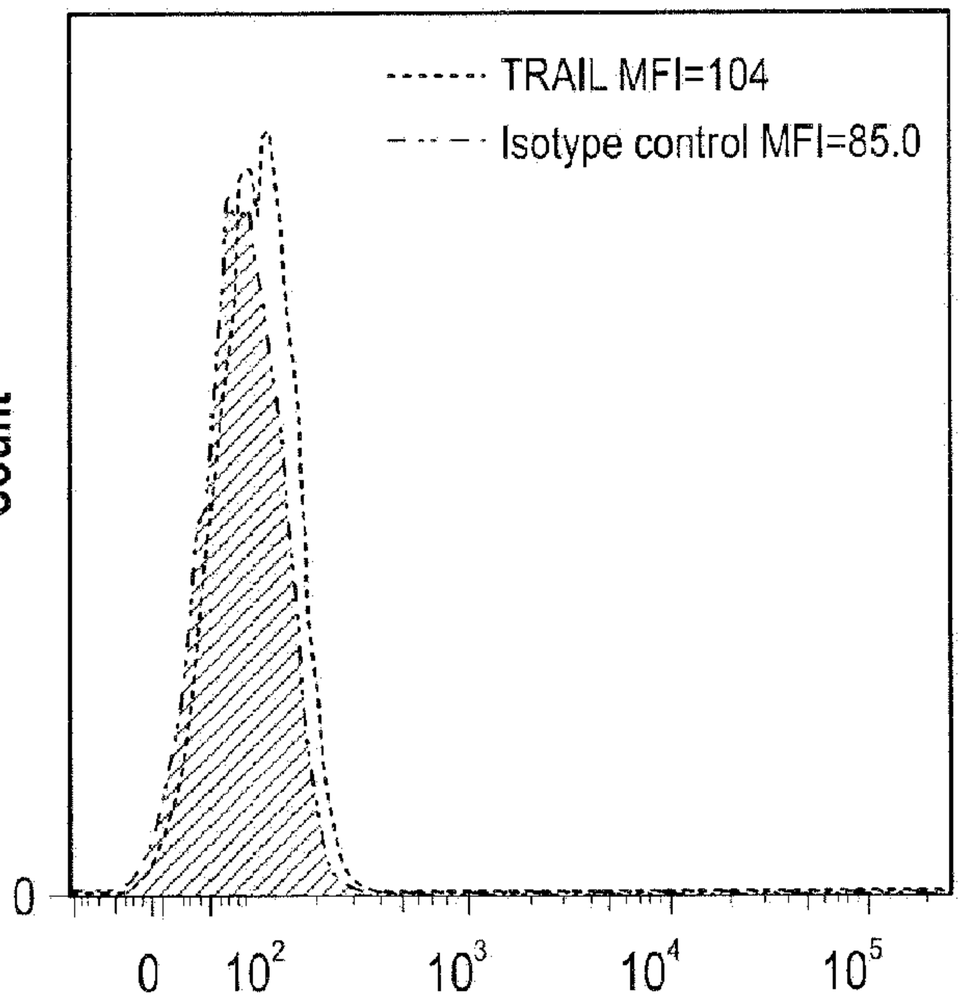

TRAIL MFI=104
Isotype control MFI=85.0
Count
0
0
10^2
10^3
10^4
10^5
TRAIL(CD253)-APC

FIG. 10

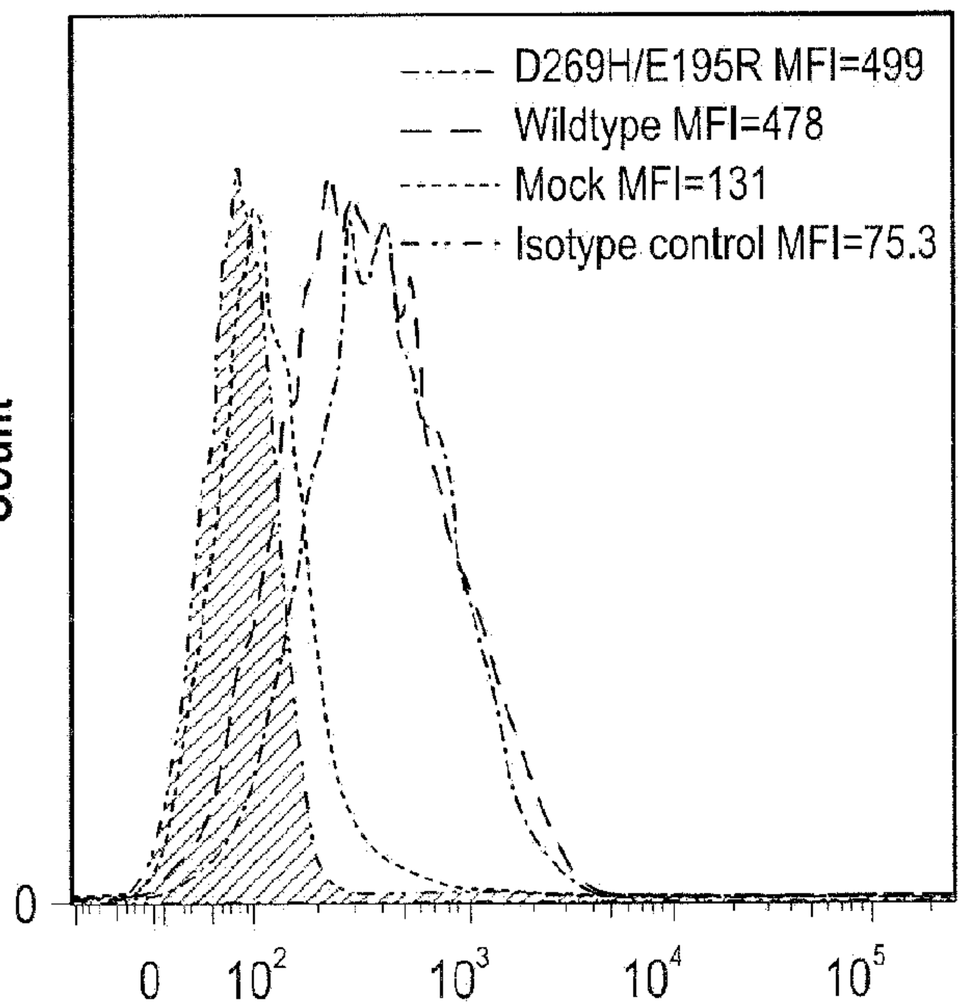

D269H/E195R MFI=499
Wildtype MFI=478
Mock MFI=131
Isotype control MFI=75.3
Count
0
0
10^2
10^3
10^4
10^5
TRAIL-APC

FIG. 11

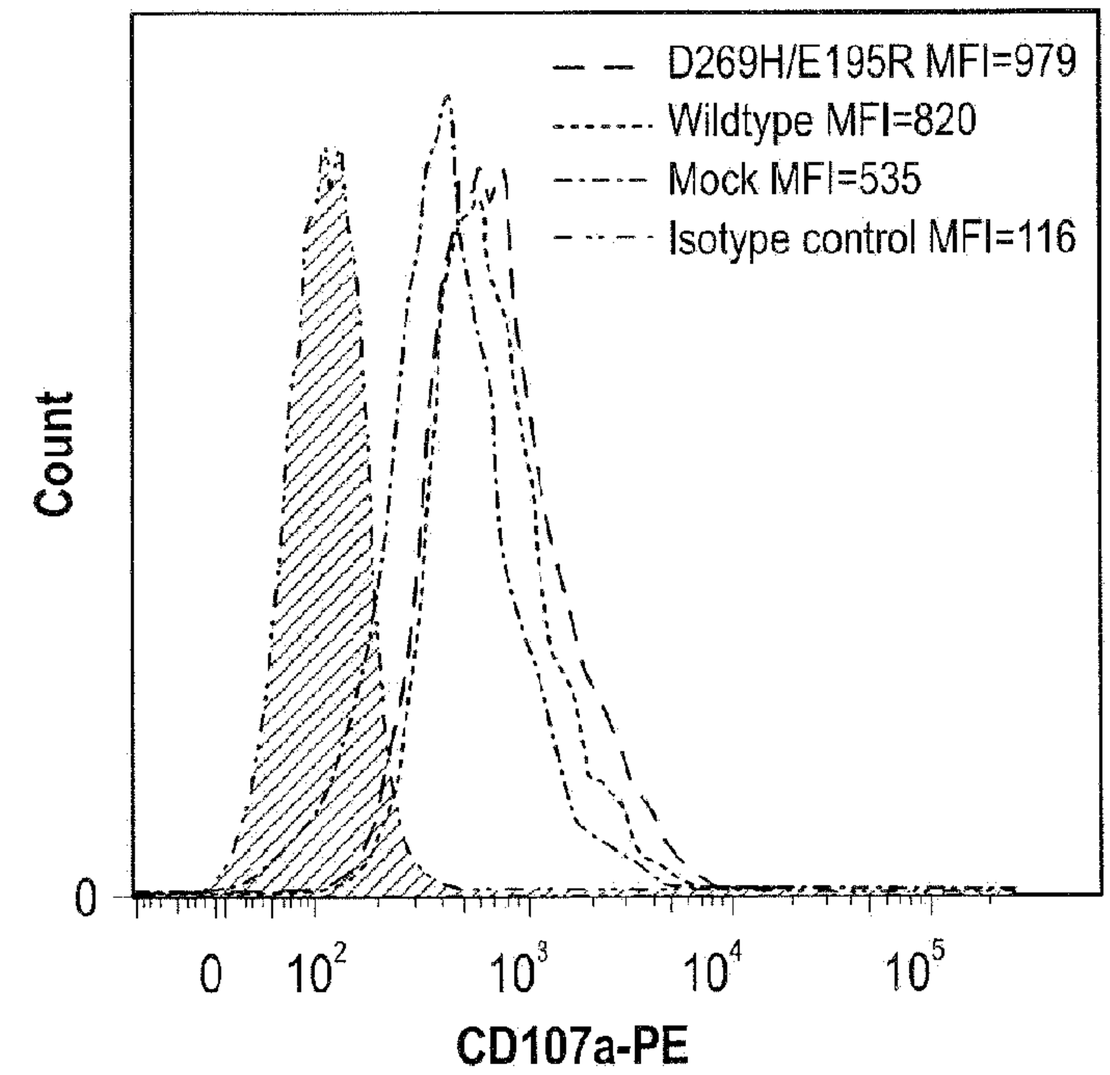

D269H/E195R MFI=979
Wildtype MFI=820
Mock MFI=535
Isotype control MFI=116
Count
0
0
10^2
10^3
10^4
10^5
CD107a-PE

FIG. 12

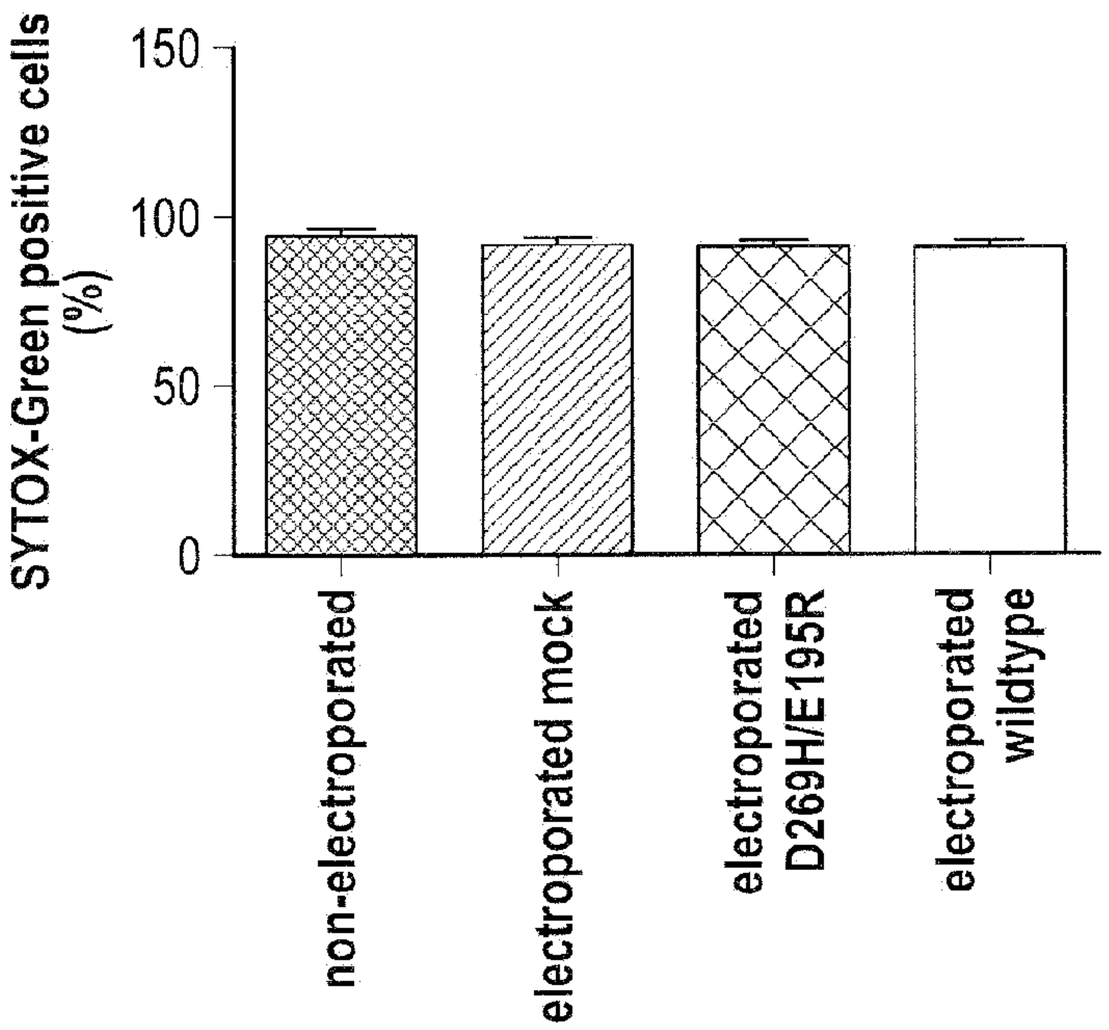

SYTOX-Green positive cells (%)
150
100
50
0
non-electroporated
electroporated mock
electroporated D269H/E195R
electroporated wildtype

FIG. 13

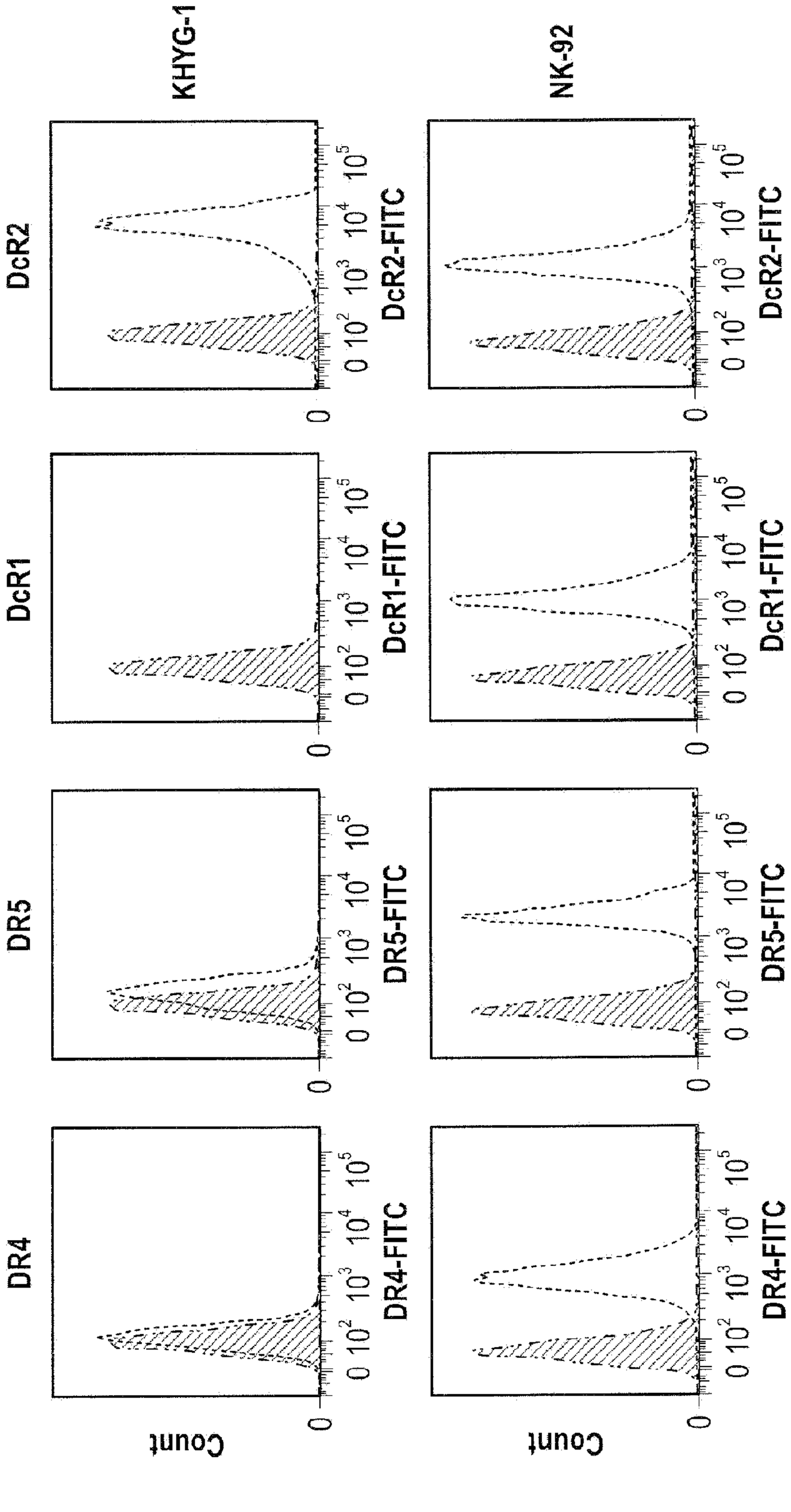

KHYG-1
NK-92
DcR2
DcR1
DR5
DR4
Count
0 10^2 10^3 10^4 10^5
DcR2-FITC
DcR1-FITC
DR5-FITC
DR4-FITC

FIG. 14

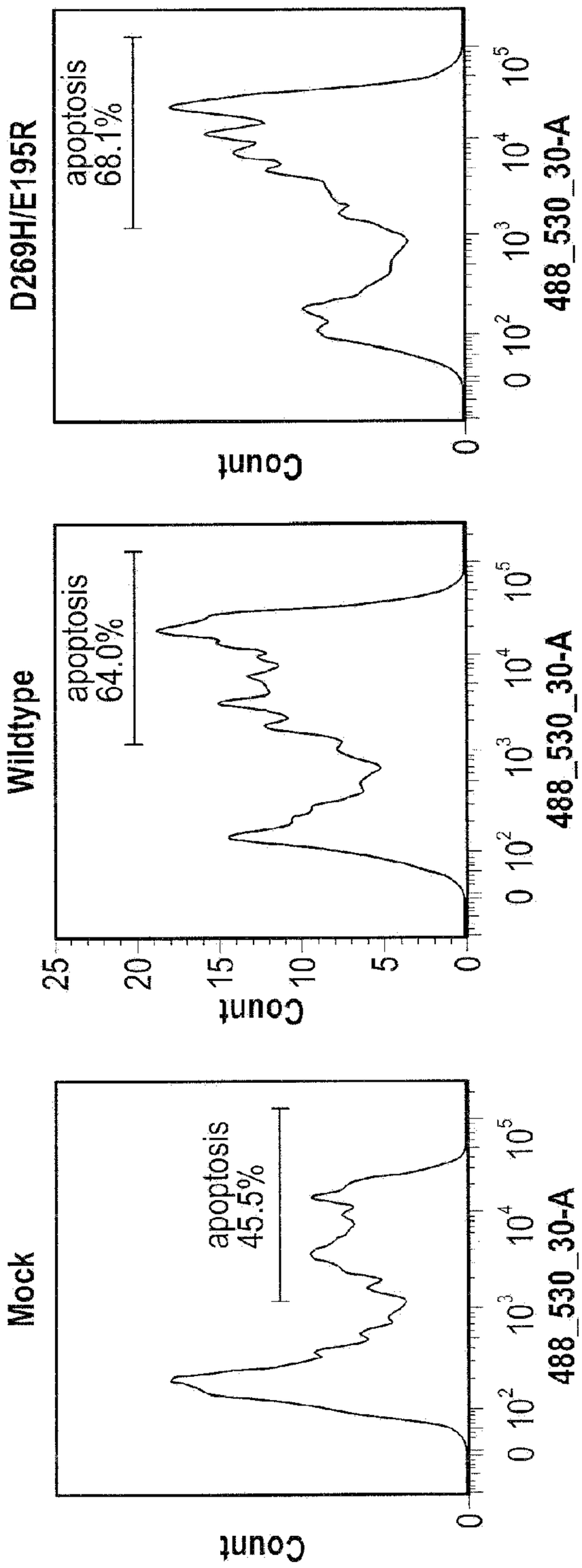

Mock
apoptosis
45.5%
Count
0
0 10^2 10^3 10^4 10^5
488_530_30-A
Wildtype
apoptosis
64.0%
Count
25
20
15
10
5
0
0 10^2 10^3 10^4 10^5
488_530_30-A
D269H/E195R
apoptosis
68.1%
Count
0
0 10^2 10^3 10^4 10^5
488_530_30-A

FIG. 15

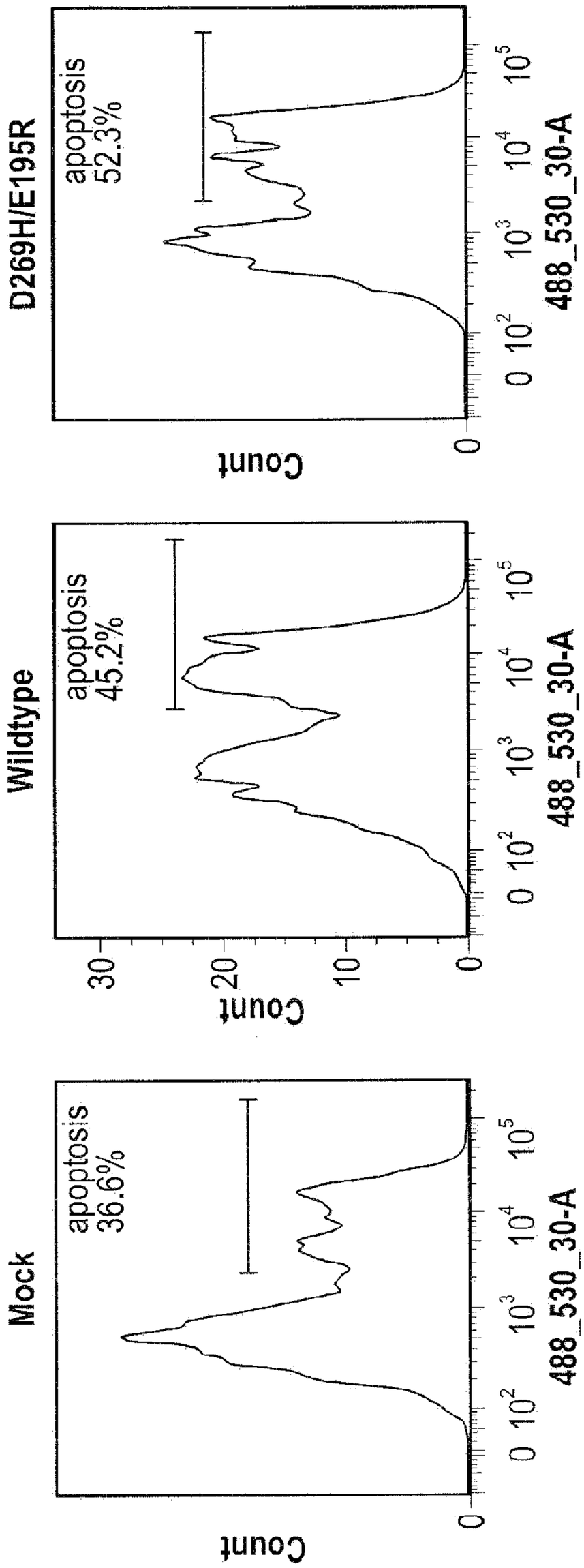

Mock
apoptosis
36.6%
Count
0
0 10^2 10^3 10^4 10^5
488_530_30-A
Wildtype
apoptosis
45.2%
Count
0
10
20
30
0 10^2 10^3 10^4 10^5
488_530_30-A
D269H/E195R
apoptosis
52.3%
Count
0
0 10^2 10^3 10^4 10^5
488_530_30-A

FIG. 16

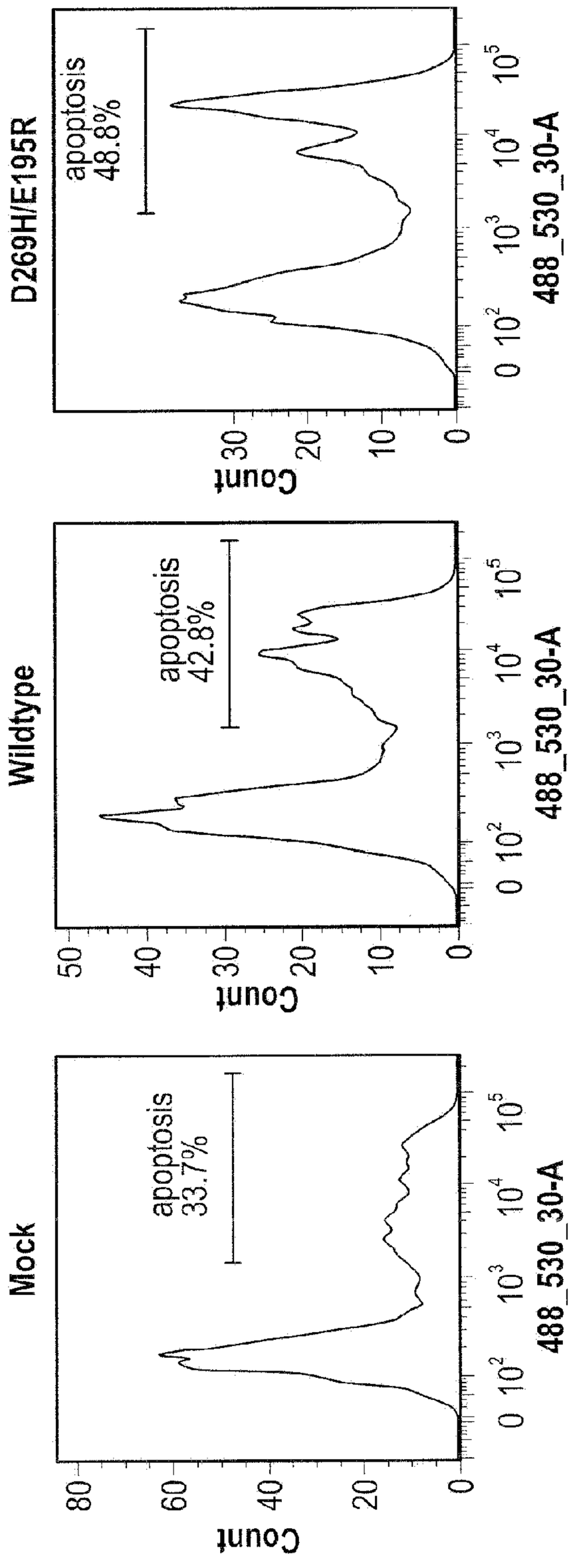

Mock
apoptosis
33.7%
Count
0
20
40
60
80
0 10^2 10^3 10^4 10^5
488_530_30-A
Wildtype
apoptosis
42.8%
Count
0
10
20
30
40
50
0 10^2 10^3 10^4 10^5
488_530_30-A
D269H/E195R
apoptosis
48.8%
Count
0
10
20
30
0 10^2 10^3 10^4 10^5
488_530_30-A

FIG. 17

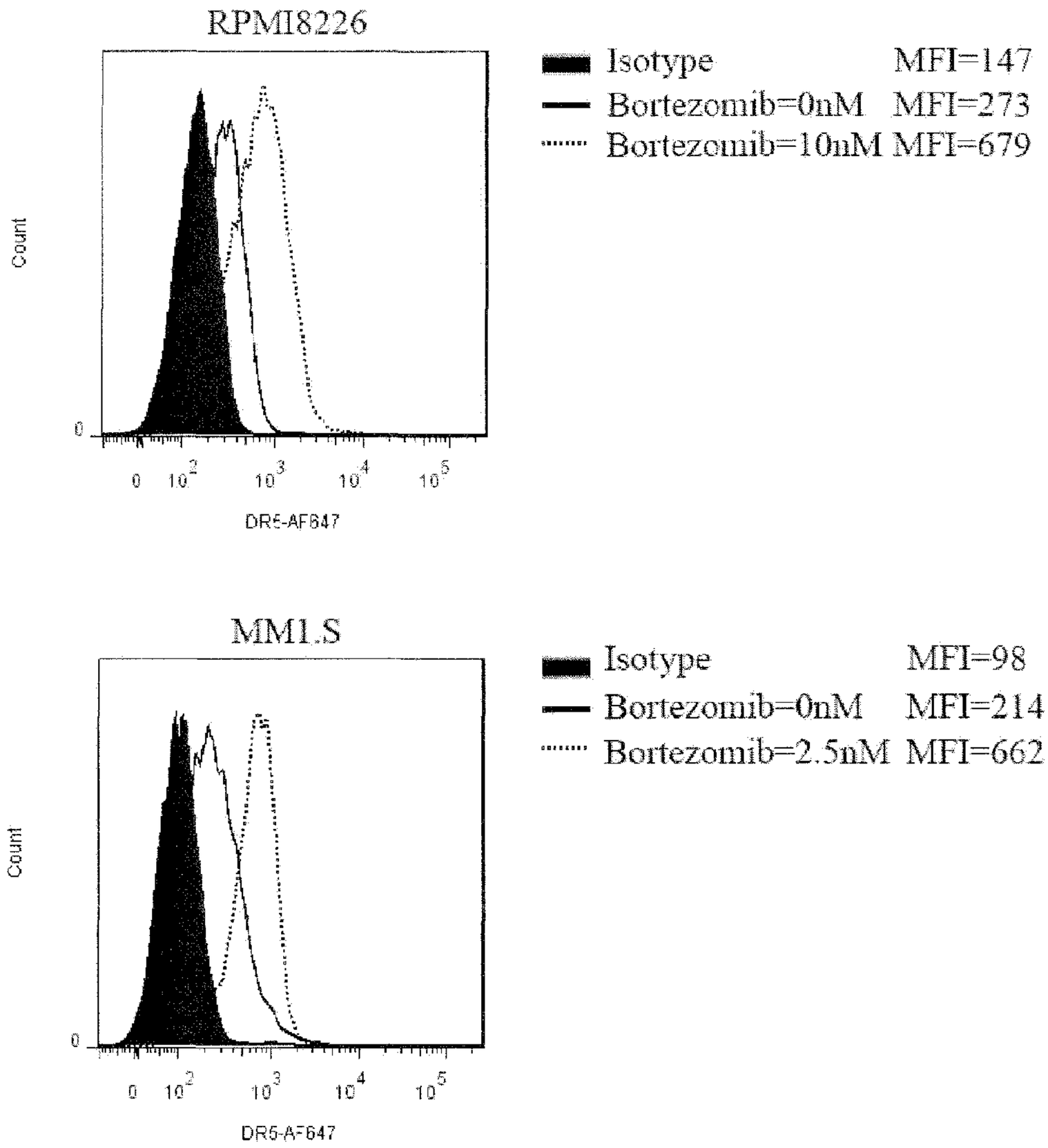

RPMI8226
Count
0
10^2
10^3
10^4
10^5
DR5-AF647
Isotype MFI=147
Bortezomib=0nM MFI=273
Bortezomib=10nM MFI=679
MM1.S
Count
0
10^2
10^3
10^4
10^5
DR5-AF647
Isotype MFI=98
Bortezomib=0nM MFI=214
Bortezomib=2.5nM MFI=662

Fig. 18

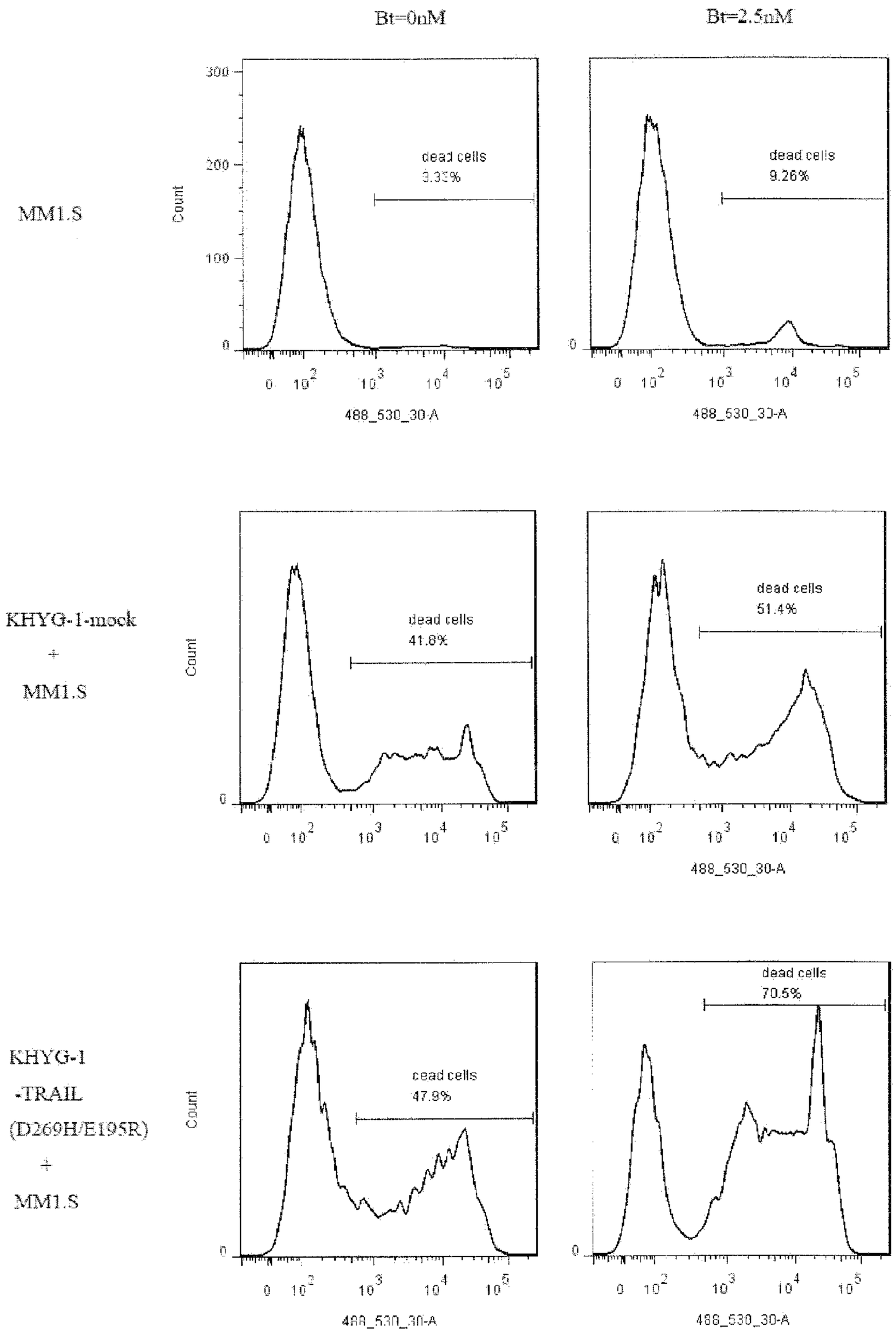

Bt=0nM
Bt=2.5nM
MM1.S
KHYG-1-mock
+
MM1.S
KHYG-1
-TRAIL
(D269H/E195R)
+
MM1.S
Count
dead cells
3.38%
dead cells
9.26%
dead cells
41.8%
dead cells
51.4%
cead cells
47.9%
dead cells
70.5%
488_530_30-A

Fig. 19

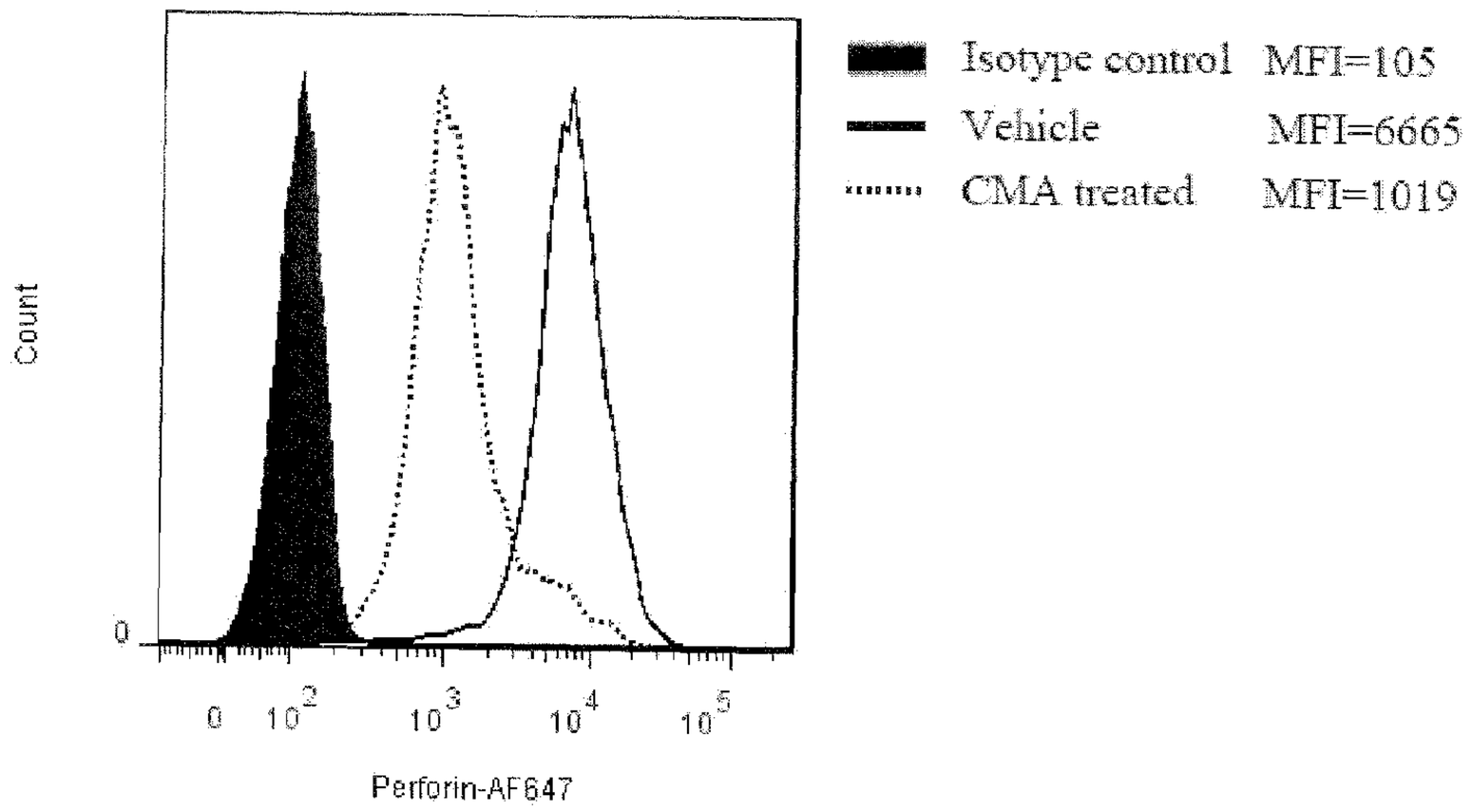

Isotype control MFI=105
Vehicle MFI=6665
CMA treated MFI=1019
Count
0
0 10^2 10^3 10^4 10^5
Perforin-AF647

Fig. 20

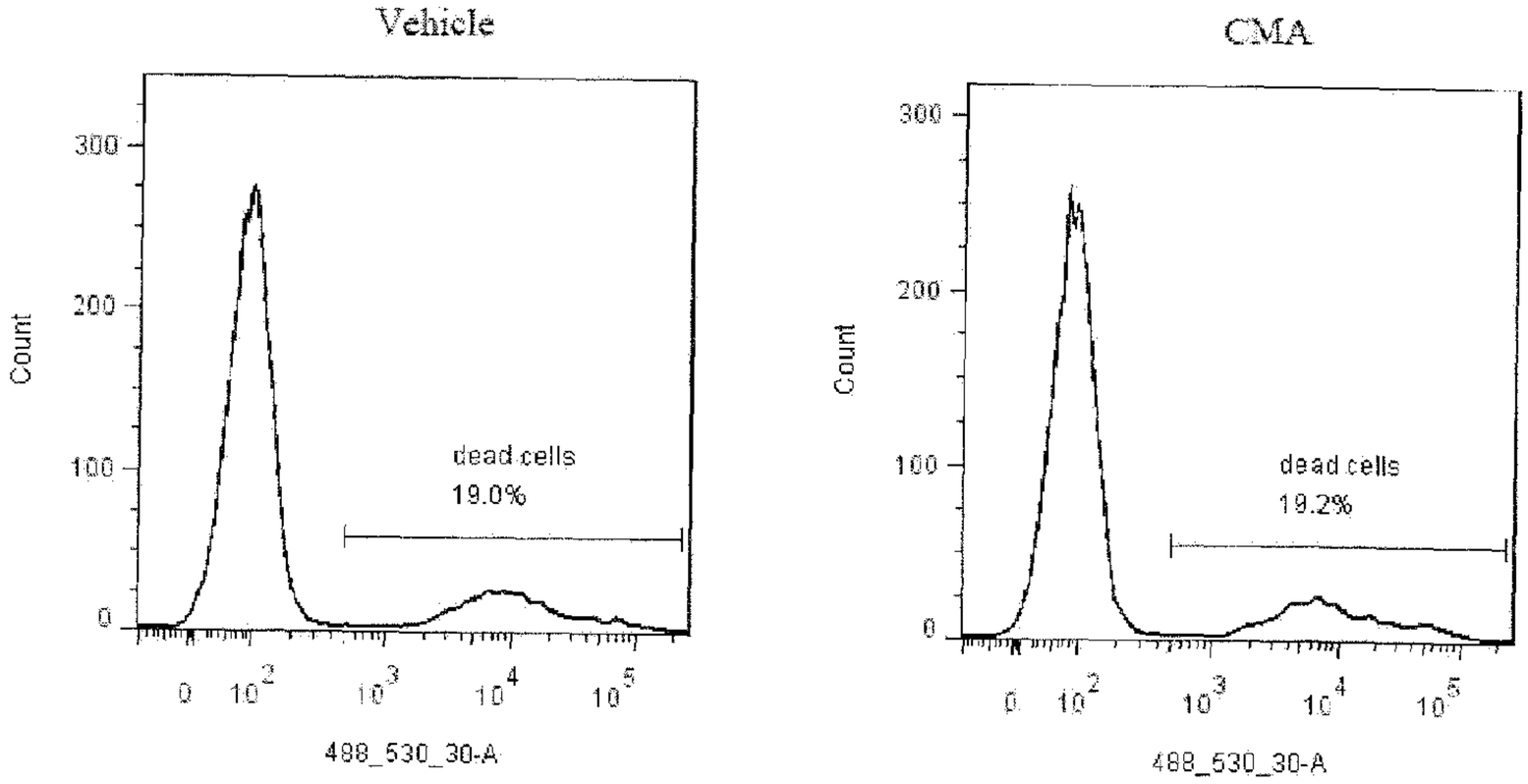

Vehicle
CMA
Count
300
200
100
0
dead cells
19.0%
dead cells
19.2%
0 10^2 10^3 10^4 10^5
488_530_30-A

Fig. 21

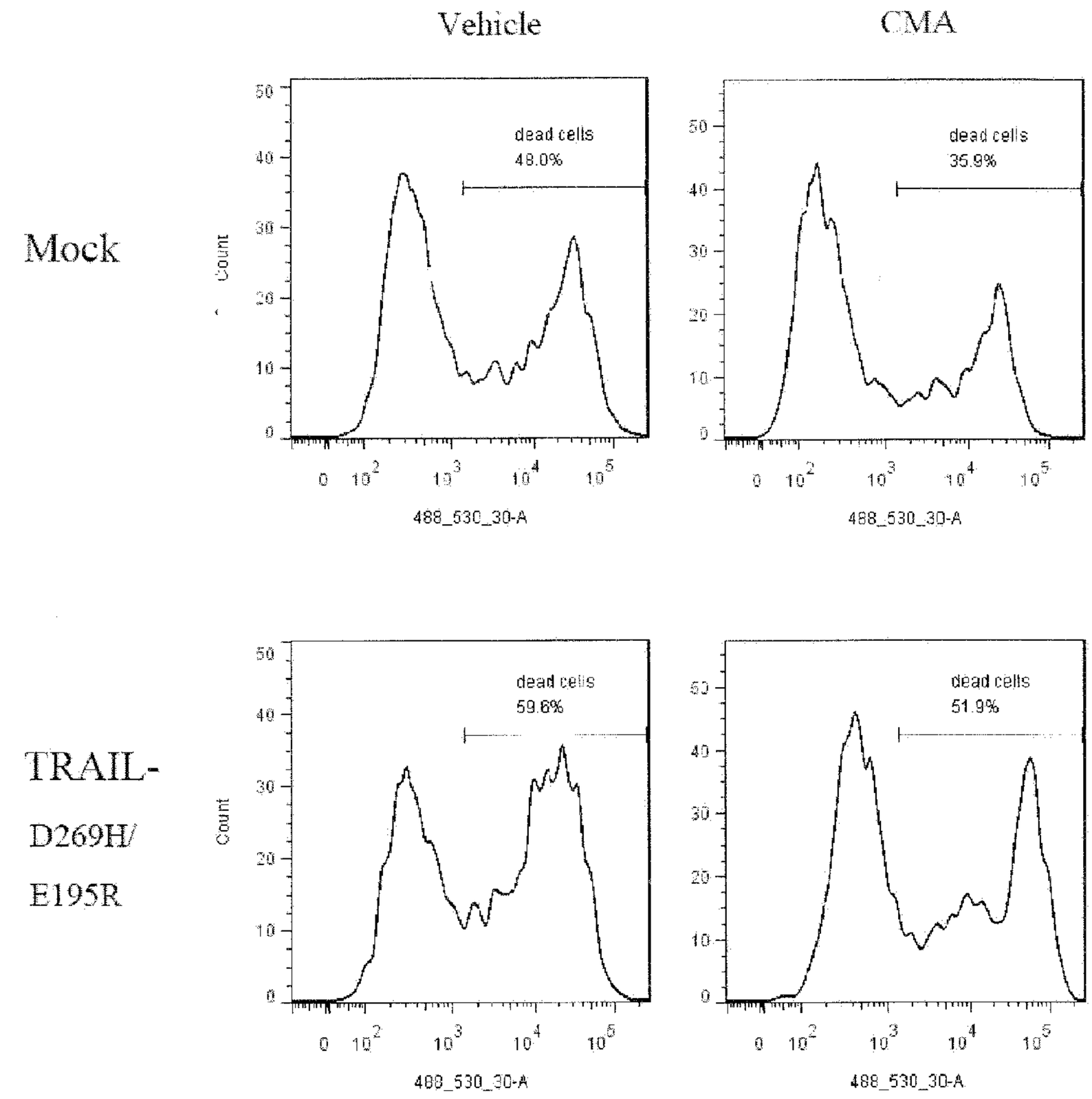

Vehicle
CMA
Mock
TRAIL-
D269H/
E195R
dead cells
48.0%
dead cells
35.9%
dead cells
59.6%
dead cells
51.9%
Count
488_530_30-A
488_530_30-A
488_530_30-A
488_530_30-A

Fig. 22

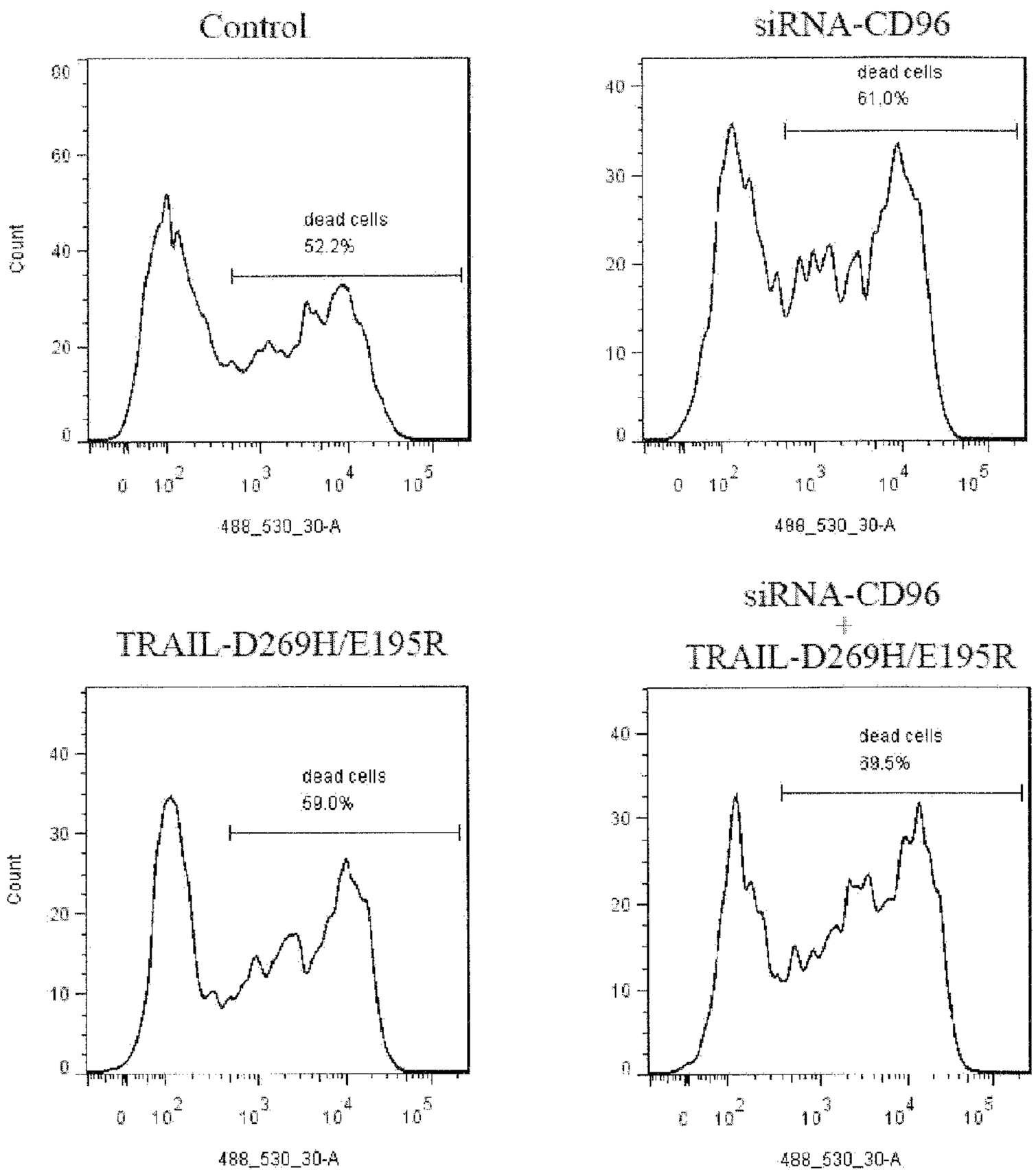

Control
siRNA-CD96
TRAIL-D269H/E195R
siRNA-CD96
+
TRAIL-D269H/E195R
dead cells
52.2%
dead cells
61.0%
dead cells
59.0%
dead cells
59.5%
Count
488_530_30-A

Fig. 23

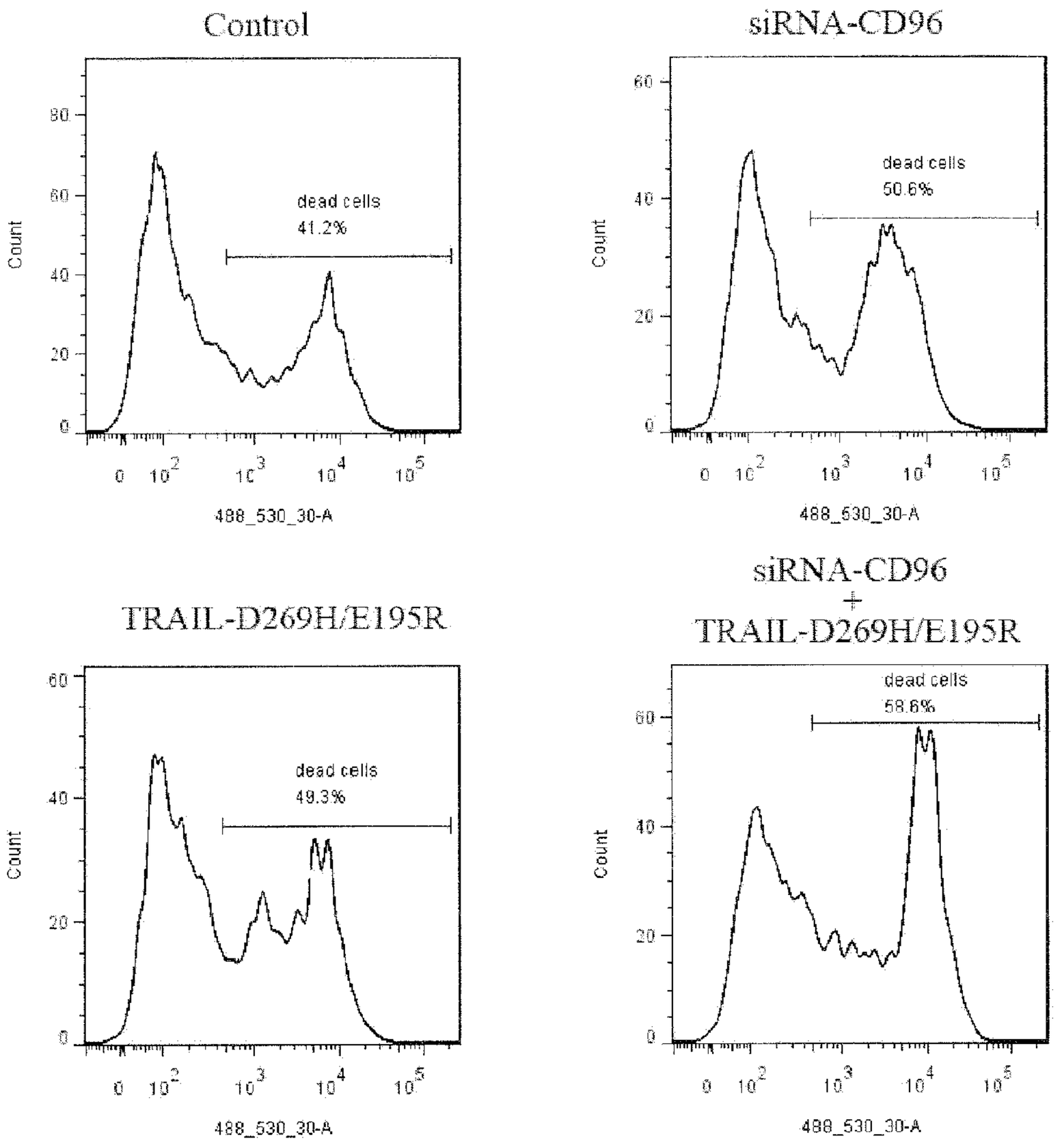

Control
dead cells
41.2%
Count
488_530_30-A
siRNA-CD96
dead cells
50.6%
Count
488_530_30-A
TRAIL-D269H/E195R
dead cells
49.3%
Count
488_530_30-A
siRNA-CD96
+
TRAIL-D269H/E195R
dead cells
58.6%
Count
488_530_30-A

Fig. 24