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(54) **Title:** TETHERED INTERLEUKIN-2 TO ITS RECEPTOR IL-2RBETA, A PLATFORM TO ENHANCE NATURAL KILLER AND REGULATORY T CELL ACTIVITY

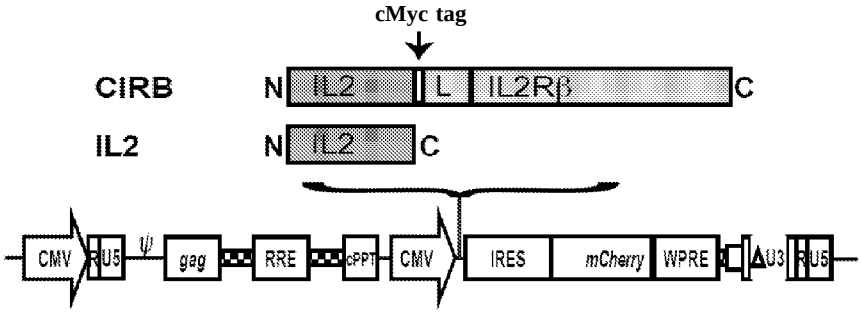


FIG. 1

(57) **Abstract:** Fusion proteins comprising IL2 and IL2Rβ (e.g., CIRB), IL2, IL2Rβ and IL2 IR (e.g., CIRB21), and/or comprising IL2, IL2Rβ, and CD28 (e.g., CIRB28); natural killer (NK) cells that express the fusion proteins and methods of use thereof, e.g., to treat subjects with cancer; and regulatory T cells (T-regs) that express a fusion protein comprising IL2, IL2Rβ, and CD28 and methods of use thereof, e.g., to treat subjects with autoimmune disease or GVHD.

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# Tethered Interleukin-2 to its Receptor IL-2RBeta, A Platform to Enhance Natural Killer and Regulatory T Cell Activity

## CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial Nos. 62/338,757, filed on May 19, 2016, and 62/471,456, filed on March 15, 2017. The entire contents of the foregoing are incorporated herein by reference.

## TECHNICAL FIELD

Described herein are Natural killer (NK) cells that express chimeric proteins comprising IL2, IL2Rβ and IL21R (e.g., CIRB21); and/or a chimera comprising IL2, IL2Rβ, and CD28 (e.g., CIRB28), and methods of use thereof, e.g., to treat subjects with cancer, GVHD, and autoimmune diseases.

## BACKGROUND

Natural killer (NK) cells are lymphocytes endowed with the innate ability to attack malignant and virus infected cells without prior exposure to specific antigens(l-3). Several interleukins, and in particular IL2, activate and expand critical immune cells such as T-cells and NK cells(4). Systemic IL2 supplementation could therefore enhance immunity in a variety of diseases ranging from cancer to viral infection. However, in cancer patients, tumor cells and their microenvironment (TME) often repress NK cells anti-tumor activity by orchestrating a multitude of escape mechanisms (5).

Clinical trials using high dose IL2 infusions have met limited success due to severe side effects that mimic sepsis(6-8), while low-dose IL2 efficacy is limited by the short half-life (less than 10 min) of IL2 *in vivo(9),* and due to depletion of low IL2 doses by T-regs and other lymphoid cells(lO). A number of strategies based on IL2 have aimed to enhance NK cytotoxicity while reducing toxicity in patients, with limited efficacy. Cultured *ex-vivo* NK cells can be activated and induced to proliferate by exposure to IL2 before transfer *in vivo. Ex-vivo* activated autologous NK cells display less anti-tumor efficacy(l 1) than NK cells from allogeneic donors(12), because self class I HLA signaling suppresses NK cytotoxicity and cytokine release(13). However, in order for allogeneic donor NK cells to be effective,

pre-transfer lymphodepletion to reduce competition for growth factors and cytokines is required(14, 15). Moreover, systemic IL2 administration is needed to sustain NK cytotoxicity after *in vivo* transfer, exposing patients to systemic side effects.

Past therapeutic efforts to express endogenous IL2 in NK cells showed limited success with micro metastatic models and were not as efficacious as NK cells stimulated with exogenous IL2( 16). Similarly, effort to express membrane-bound endogenous IL2 did not show any advantage above parental NK92 cells(17). The limited success of several immunotherapy strategies using NK cells could be explained by the failure of activated NK cells to outcompete T-regs for cytokines in the host and the immunosuppressive effect of the TME, which includes myeloid derived suppressor cells (MDSCs). Both MDSCs and T-regs mediate NK cell functions suppression either by direct contact or by secretion of TGFβi ( 18, 19).

## SUMMARY

Interleukin-2 (IL2) is an immunostimulatory cytokine for key immune cells including T cells and natural killer (NK) cells. Systemic IL2 supplementation could enhance NK-mediated immunity in a variety of diseases ranging from neoplasms to viral infection. However, its systemic use is restricted by its serious side effects and its efficacy may be limited by activation of T-regulatory (T-regs) cells. IL2 signaling is mediated through interactions with a high affinity multi-subunit receptor complex containing IL2Rot, IL2Rβ and IL2Ry. Adult NK cells may express only IL2Rβ and IL2Ry subunits and are therefore relatively insensitive to IL2. To overcome these limitations, we created a novel chimeric IL2-IL2Rp (CIRB) fusion protein of IL2 and its receptor IL2Rβ joined via a peptide linker. NK92 cells expressing CIRB ($NK92^{CIRB}$) are highly activated and expand indefinitely without exogenous IL2. They are highly cytotoxic, and were resistant to TGF-β1 and dexamethasone. Furthermore, CIRB induced substantial expression of natural cytotoxicity receptors NKP44, NKP46 and NKP30 as well as CD 16, which enhanced NK cytotoxicity with Trastuzumab via antibody dependent pathways against HER2 positive cells. When compared to an IL2 secreting NK92 cell line ($NK92^{IL2}$), $NK92^{CIRB}$ cells display superior *in vivo* anti-tumor effect and survival in mice (at least 3 weeks). This novel chimera eliminates the need for both IL2Rot and IL2Rβ expression and offers an alternative to exogenous IL2 stimulation. Collectively, the present data show that tethering IL2 to its receptor

IL2Rβ offers a new platform that may be useful in selectively activating and enhancing immune therapy.

Thus, provided herein are fusion proteins comprising interleukin 2 (IL2) fused to the N-terminus of interleukin 2 receptor beta (IL2Rβ), with an intervening linker therebetween. In some embodiments, the IL2 comprises SEQ ID NO:34, and/or the IL2Rβ comprises amino acids 27-55 1 of SEQ ID NO:35. In some embodiments, the intervening linker between IL2 and the N-terminus of IL2Rβ comprises an extracellular domain of IL2Ra. In some embodiments, the extracellular domain of IL2Ra comprises SEQ ID NO:28.

In some embodiments, the fusion protein also includes a cytoplasmic domain of IL21R at the C-terminus of IL2Rβ, optionally with an intervening linker therebetween. In some embodiments, the cytoplasmic domain of IL21R comprises amino acids 254-538 of SEQ ID NO:36.

In some embodiments, the fusion protein also includes an activation domain of CD28 at the C-terminus of the IL2Rβ portion, optionally with an intervening linker therebetween. In some embodiments, the activation domain of CD28 comprises amino acids 180 to 220 of SEQ ID NO:38.

Also provided herein are nucleic acids encoding the fusion proteins described herein, as well as expression vectors comprising the nucleic acids, preferably with one or more regulatory regions for expression of a fusion protein described herein.

Further, provided herein are isolated natural killer (NK) cells (e.g., CD3-CD56+ lymphocytes) expressing a fusion protein as described herein, preferably wherein the NK cell is CD3-CD56+ lymphocyte also expresses CD16 and optionally NKP44, NKP46 and NKP30, and the use thereof in treating cancer.

Also provided are Regulatory T cells (T-regs) expressing fusion proteins comprising interleukin 2 (IL2) fused to the N-terminus of interleukin 2 receptor beta (IL2RP), with an intervening linker therebetween, and an activation domain of CD28 at the C-terminus of the IL2Rβ portion, optionally with an intervening linker therebetween. Preferably the Tregs are CD4+CD25+, e.g., CD4+CD25+CD127- Tregs (e.g., CD4+CD25highCD127-ICOS+ for atopy Tregs or CD4+CD25+CD127-CD62L+ for GVHD), and are optionally FOXP3+ as well. Also provided is the use thereof in treating GVHD and autoimmune disease, e.g., for depleting alloreactive T cells.

Further, provided herein are methods for treating a subject, preferably a human subject, who has cancer (e.g., who has been diagnosed with cancer), comprising administering a therapeutically effective amount of natural killer (NK) cells expressing a fusion protein as described herein, preferably wherein the NK cells are CD3-CD56+ lymphocyte also expresses CD16 and optionally NKP44, NKP46 and NKP30. The NK cells can be formulated and/or administered in a physiologically acceptable composition, e.g., as described herein, e.g., formulated to be administered intravenously.

In some embodiments, the subject has a solid tumor.

In some embodiments, the methods include administering one or more of an anti-tumor monoclonal antibody or a checkpoint inhibitor.

In some embodiments, the NK cells are administered intravenously.

In some embodiments, the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

Also provided herein are methods for treating a subject who has GVHD or an autoimmune disease, comprising administering a therapeutically effective amount of regulatory T (T-reg) cells expressing fusion proteins comprising interleukin 2 (IL2) fused to the N-terminus of interleukin 2 receptor beta (IL2Rβ), with an intervening linker therebetween, and an activation domain of CD28 at the C-terminus of the IL2Rβ portion, optionally with an intervening linker therebetween. Preferably the Tregs are CD4+CD25+, e.g., CD4+CD25+CD127- Tregs (e.g., CD4+CD25highCD127-ICOS+ for atopy Tregs or CD4+CD25+CD127- CD62L+ for GVHD), and are optionally FOXP3+ as well.

In some embodiments, the T-reg cells are administered intravenously.

In some embodiments, the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

Also provided are NK cells expressing a fusion protein as described herein, for use in treating a subject, preferably a human subject, who has cancer, e.g., a solid tumor. In some embodiments the subject is also administered one or more of an anti-tumor monoclonal antibody or a checkpoint inhibitor. In some embodiments, the NK cells are formulated to be administered intravenously. In some embodiments, the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

## DESCRIPTION OF DRAWINGS

Figure 1 - Diagrams of the Human IL2 and the chimera IL2 fused with receptor IL2Rβ (CIRB) in lentiviral constructs. A Linker (L) composed of the cMyc tag (EQKLISEEDL) and a fragment of the extracellular domain of IL2 receptor alpha (EMETSQFPGEEKPQASPEGRPESETSC), joins IL2 and its receptor IL2Rβ.

Figures 2A-D - Surface detection and expression of chimera CIRB. A, detection by anti-cMyc monoclonal antibody of CIRB expression at the surface of transiently transfected HEK293 cells and B, in lentivirus transduced NK92 cells. C, CD122 expression detected using an anti-CD122 monoclonal antibody, which recognizes the native IL2Rβ (CD122) as well as the chimera. CD122 is present in $NK92^{IL2}$ cells and as expected, much higher in $NK92^{CIRB}$ due to the additional expression of ORB. Expression was higher than the background detected in the absence of antibody (Neg). D, CIRB was further detected by western blot using monoclonal anti-human IL2.

Figures 3A-C - A, cytotoxicity assays of $NK92^{IL2}$, $NK92^{CIRB}$, and parental NK92 cell lines against multiple myeloma U266GFP cells plated 24 hours prior to co-culture. Increased E/T ratios of NK cells induced more U266GFP cell death as quantified by GFP-emitted fluorescence. B, cytotoxicity assay against a panel of five human cancer cell lines: Cells were plated 24 hours prior to adding NK cell lines at E/T ratio of 2:1. While in C, cancer cells were plated only 5 hours prior to exposure to increasing E/T ratios of $NK92^{IL2}$ (β ), or $NK92^{CIRB}$ (▩) cells. Remaining cells after this time were determined using a crystal violet/alcohol-extraction assay. Data are

presented as cell number relative to NK cells-free controls, , mean + SE values for triplicate samples.

Figures 4A-C - impact of TGFβi, IL-4 and Dexamethasone on NK cells viability. A, 64x10$^3$ NK92$^{IL2}$ and B, NK92$^{CIRB}$ cells were plated with TGFβi (lOng/ml), IL-4 (lOng/ml), or Dex (luM) for 6 days of growth. Viable cells were counted using Trypan blue. Data are presented as final cell number (10$^5$ cells/ml), mean + SE values for triplicate samples. C, NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ cell lines exposed for 24 hours to TGFβi (20ng/ml **11),** Dex (0.5uM A), or no drug (UT ▩). Parental NK 92 cells were incubated with IL2 at 20IU/ml. Cancer cells (32 xlO$^3$ cells), were then added to NK cells at E/T ratio of 2:1, then incubated for 4 days. Cancer cells viability was determined using a crystal violet extraction assay. Data are presented as mean percentage cell number relative to NK cells-free controls (UT).

Figures 5A-D - NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ cell lines phenotype. A, Flow cytometry shows the increased cell surface density of CD 16 in NK92$^{IL2}$ and NK92$^{CIRB}$ and the low expression of CD25 in NK92$^{CIRB}$. B, Human primary NK cells (hNK) phenotypic expression of NKG2D, CD25 and CD 16 in comparison to NK92, NK92$^{IL2}$ and NK92$^{CIRB}$. C, direct cytotoxicity and ADCC activity mediated by effector NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ cells against HER2 positive BT474 cell line at E/T ratio of 2:1 when incubated with Trastuzumab (lug/ml). Data are presented as cell number percentage relative to NK cells-free controls, mean + SE values for triplicate samples. Statistical differences were determined by one-way Anova test ((*P<0.05). D, expression profiles of NKP30, NKP44, NKP46, Granzyme-B, Perforin- 1, TNF-a, and INF-γ in NK cells lines. Data are presented as mean + SE values for triplicate samples. Two tails t-test analysis was used to evaluate statistical differences.

Figures 6A-B - Non-irradiated NK92$^{IL2}$ and NK92$^{CIRB}$ cells evaluation *in vivo*. A, When U25 1 tumor volume reached ~160mm$^3$, non-irradiated NK92$^{IL2}$ and NK92$^{CIRB}$ cells (10$^7$ cells) were injected into mice (arrows), via the tail vein. A second injection of non-irradiated NK cells (5xl0$^6$ cells) was carried out 4 days later. Tumor sizes were monitored until 31 days post tumor implantation. B, 17 days after the last NK cells injection, animals were sacrificed and blood was collected from 3 animals in each group. Blood samples (0.5ml) were processed and analyzed by flow cytometry using human specific marker CD45 and the mCherry fluorescence marker, which is

co-expressed with IL2 or CIRB in NK92 $^{IL2}$ and NK92 $^{CIRB}$, respectively. NK92 $^{CIRB}$ cells detected (circled)

Figures 7A-B - Cell survival *in vitro* and anti-tumor efficacy of irradiated NK92 $^{IL2}$ and NK92 $^{CIRB}$ cells *in vivo.* A, NK cells were irradiated at lOGy (0.83gy for 12 min) and then plated in complete NK92 media to determine their survival using Trypan Blue every 24 hours. The survival advantage of NK92 $^{CIRB}$ cells was statistically significant at days 1 and 2 (One-way Anova test *P<0.05). B, PC-3 tumors were grown in 5 week-old male Nod/Scid mice. When tumor reached -200 $mm^3$ NK cells were irradiated with 500cGy were administered, as four weekly injections of 15xl0 $^6$ cells in 200ul per mouse, via the tail vein (arrows). After the last NK92 $^{CIRB}$ cells injection, a significant tumor growth delay of 17 days was recorded (**P<0.0 1), comparatively to the untreated group. NK92 $^{IL2}$ treated group tumors produced a tumor delay of only 7 days (*P<0.05). Statistical differences were determined using One-way Anova test.

Figure 8- Helix dominated predicted structure of the linker determined using the server(20).

Figure 9- Cell growth of NK92 $^{IL2}$ and NK92 $^{CIRB}$ cell lines - NK92 $^{IL2}$ and NK92 $^{CIRB}$ cell lines showed fast recovery after freezing and excellent survival after subjection to multiple freezing and plating in tissue culture. NK92. NK92 $^{IL2}$, NK92 $^{CIRB}$ and IL2-activated NK92 (10 IU/ml), growths were similar after six days and slightly higher for NK92 at 40IU/ml. NK92-MI cells showed the slowest growth during the same period.

Figures 10A-B- Expression of CD 16 in NK92-MI and NK92. A, Flow cytometry confirms the lack of expression of CD 16 in NK92 and NK92-MI. B, shows the lack of expression of CD 16 in NK92 activated with 100 or lOOOIU/m of glycosylated (G) and non-glycosylated IL2.

Figure 11. A schematic illustration showing signaling through JAK-STAT pathway using common signaling chain IL2Rg.

Figure 12. NK92CIRB killing of PC-3 at a ratio of 1:1, when stimulated with IL2 1 or IL2.

Figure 13. Exemplary schematic of an IL2-IL2R{5-IL2 1 chimera (CIRB2 1).

Figure 14. Graph showing the cytotoxicity of NK92CIRB and NK92CIRB2 1 vs. PC-3 cells at a ratio of 1:1.

Figure 15. Graph showing folds expression of activators and cytokines in NK92CIRB and NK92CIRB21 relative to NK92.

Figure 16. IL2Rβ receptor cytoplasmic domain with the activation regions depicted. Box 1 is required for JAK-STAT interaction with Socs 1 inhibitory motif. Y536 is required for STAT5 binding and phosphorylation.

Figure 17. IL21 receptor cytoplasmic domain with the activation regions depicted. Box 1 is required for JAK-STAT interaction with Box 2 contribution. Y519 is required for STAT3 and STATl binding and phosphorylation.

Figure 18. Exemplary schematic of an IL2-IL2Rp-CD28 chimera (CIRB28).

## DETAILED DESCRIPTION

The present compositions and methods selectively activate and expand NK cells without exogenous IL2, while maintaining NK cytotoxicity and proliferation both *in vitro* and *in vivo,* circumvent the requirement of IL2Ra and its lack of expression in NK cells, thus avoiding IL2 off-target effects, cytokine competition, and activation of down-regulating lymphoid cells like T-regs.

IL2 will bind to either low affinity receptor IL2Ra (CD25) (21) or to intermediary affinity receptor IL2Rβ (CD 122) with the common IL2Ry chain (CD 132) (22,23) and to all, to form a high affinity quaternary complex(24). Adult NK cells may express only IL2Rβ and IL2Ry subunits(25) and are, therefore, relatively insensitive to low doses of IL2, but acquire sensitivity upon IL2Rot expression(26). A recently developed IL2 "superkine" (27) that bypasses IL2Rot by binding directly and with high affinity to IL2Rβ produced better antitumor effects than wild type IL2 in mice. However, it still causes some form of pulmonary edema.

The novel chimera CIRB described herein comprises IL2 and its receptor IL2Rβ, joined by a peptide linker derived from the extracellular domain of IL2Rot. The linker was computationally determined as reasonably flexible, without adversely affecting the chimera stability which is generally inversely correlated to flexibility(28). When introduced in NK92 cells, CIRB induces indefinite cell expansion and conferred an *in vitro* cytotoxicity similar or higher than that elicited by IL2 expression. *In vivo,* the anticancer activity of $NK92^{CIRB}$ against mid-size solid tumors was substantially superior to that elicited by $NK92^{IL2}$. Additionally, CIRB confers, in contrast to IL2, substantial resilience to TGFβi, dexamethasone as well as

IL4. This advantage could be crucial in the TME where TGFβi is secreted by a variety of cells including cancer associated fibroblasts(29), and exists in a membrane bound form on T-regs to induce anergy of NK cells(30), or by MDSCs to inhibit NKG2D expression, and IFN-γ production in NK cells(3 1). Cancer cells also regularly shed tumor-derived exosomes (TDEs) containing a membrane bound form of TGFβi resulting in the down regulation of NKG2D(32), and the inhibition of IL2 signaling(33). TGFβi mediates NK inhibition by an induced microRNA (miR)- 183 which represses the co-activator/adapter DAP 12 expression, thus destabilizing several activation signals in NK cells(34). CIRB expression in NK92$^{CIRB}$ cells also provides resistance to dex while NK92$^{IL2}$ cells were eliminated. Dex impairs the function of lymphocytes in part by suppressing IL2 production from CD4+ T cells, and reducing the activation receptors NKG2D and Nkp46 in NK cells (35). Glucocorticoid hormones can interfere with macrophage activation and antigen presentation, repress the transcription of several pro-inflammatory cytokines, chemokines, cell adhesion molecules and other enzymes involved in the inflammatory response(36). The extreme sensitivity of NK92$^{IL2}$ to dex, could be explained by the previously reported destabilization of IL2 RNA(37). This RNA destabilization could potentially occur in NK92$^{IL2}$ cells but not when it is fused with IL2RJ3 RNA as in NK92$^{CIRB}$ cells.

CIRB and to a lesser degree the stable expression of IL2 allowed substantial CD 16 expression in NK92 cell line. However, exogenous recombinant IL2 was not able to mediate such expression. Similarly, NK92-MI cell line which produces and secretes IL2 was found deficient in CD 16, as previously reported(38). When combined with Trastuzumab, CD 16 expression further enhanced NK92$^{CIRB}$ and NK92$^{IL2}$ cytotoxicity by ADCC. CIRB induced substantial expression of NCRs, NKP44 (9 fold), NKP46 (1.4 fold) and NKP30 (1.7 fold) as well as a modest but significant increase in INFy. Finally, Granzyme-B expression declined substantially in NK92$^{IL2}$. Interestingly, CD25 expression declined dramatically in NK92$^{CIRB}$, as it is unnecessary in the presence of the chimera CIRB (figure 5A).

Current genetic modifications introducing CD 16 in NK cells were shown to increase NK cell mediated ADCC against multiple myeloma when combined with Elotuzumab(39). The fact that CD 16 was induced only in NK92$^{CIRB}$ and NK92$^{IL2}$ but not in NK92-MI or NK92 stimulated with IL2 could be possibly explained by the persistent IL2 signaling that somehow translates into stronger activation and growth

$_{NK92}$ $_{CI}R_{B}$ and NK92$^{IL2}$. In fact, the growth rates of both NK92$^{CIRB}$ and NK92$^{IL2}$ were 2-fold that of NK92-MI (Figure 9), suggesting a higher level of activation. Another indication of higher activation of NK92$^{CIRB}$ and NK92$^{IL2}$ is the dramatic induction of NKP44, compared to parental NK92 stimulated with IL2 for 48 hours.

Additionally, NK92$^{CIRB}$ can proliferate in vivo far longer and also have a better survival after irradiation than NK92$^{IL2}$ cells. They also surpass that of NK92-MI when exposed to similar conditions (38). *In vitro,* NK92$^{IL2}$ cells secrete sufficient IL2 to sustain their activation and proliferation. However, they may not be able to produce enough IL2 extracellular concentrations to sustain activation and proliferation *in vivo.* This could be compounded by the competition for IL2 by T-regs and other immune cells in an immune competent animal.

Thus the novel chimeras described herein comprising CIRB endow NK92 cells with very useful attributes that improve immune therapy of cancer and potentially viral infections.

Cellular immunotherapy using donor NK cells is an emerging field that could achieve significant anti-cancer effects, safely and without the risk of inducing graft-versus-host disease (GVHD). This safety feature as well as the off tumor/on target toxicity are currently hindering the success of CAR-T technology(40). Several NK cell lines (Khyg-1, NKL, NKG, NK-YS, YT, YTS and HANK-1 cells) are currently used in preclinical studies. However, only the NK92 cell line has been extensively evaluated for its safety and efficacy in clinical settings(4 1,42). NK92 cells are $CD56^{+}$, $CD3^{-}$ and $CD16^{-}$ and require IL2 for growth and activation(43). Unlike primary NK cells, NK92 cells and other NK cell lines constitute a stable and homogenous population. They are amenable to genetic modification by lentiviruses, a gene transfer platform that has shown a good safety profile for lymphocytes(44). Many encouraging advances have been achieved in NK cell-directed immunotherapy(45). However, the increasing demand for NK cells expansion *ex-vivo* requires both highly activated cells and reduced costs of cell expansion. Moreover, infused cells must have higher activation potential and possess favorable characteristics against immunosuppressors found in the TME.

The present strategy includes fusing interleukins to their receptors in the CIRB, CIRB28, and CIRB2 1 chimeras achieves better cytokine activation, with specificity, and without systemic toxicity or competition by other cellular components

of the immune system. Self-activation of NK cells provides several distinguishing features such as resilience to TGFβi or glucocorticoid hormones, substantial expression of CD16, higher survival after irradiation and a superior antitumor activity *in vivo*.

***Chimeric Proteins***

The present disclosure provides chimeras as described herein, e.g., a chimera comprising IL2 and IL2Rβ (e.g., CIRB); a chimera comprising IL2, IL2Rβ and IL21R (e.g., CIRB21); and/or a chimera comprising IL2, IL2Rβ, and CD28 (e.g., CIRB28). All of the fusion proteins described herein can be generated using standard molecular biological procedures, e.g., for manipulating and expressing recombinant DNA. See, e.g., *Current Protocols in Molecular Biology*, Ausubel, F.M. et al. (eds.) John Wiley & Sons (1995), and Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (Fourth Edition), Cold Spring Harbor Laboratory Press (June 15, 2012) and supplements thereof, and other standard laboratory manuals. The chimeras can be expressed, e.g., stably expressed, in an NK cell, e.g., a primary or cultured NK cell. The cells are then infused into a subject, e.g., a subject who has (e.g., has been diagnosed with) cancer.

Provided hereinbelow are exemplary sequences for the various domains that make up the chimeras described herein. In some embodiments, the sequences used are at least 80% identical to the exemplary sequence as defined herein. In some embodiments, the sequences are at least 85%, 90%, 95%, 99% or 100% identical.

To determine the percent identity of two sequences, the sequences are aligned for optimal comparison purposes (gaps are introduced in one or both of a first and a second amino acid or nucleic acid sequence as required for optimal alignment, and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is at least 80% (in some embodiments, about 85%, 90%, 95%, or 100% of the length of the reference sequence) is aligned. The nucleotides or residues at corresponding positions are then compared. When a position in the first sequence is occupied by the same nucleotide or residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into

account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch ((1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package, using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

*IL2-IL2Rfi (CIRB)*

The fusion proteins described herein include, *inter alia*, IL2 and IL2Rβ fused together with an intervening linker. Sequences for IL2 are known in the art; an exemplary human IL2 precursor sequence is shown in SEQ ID NO:34.

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  1 MYRMQLLSCI ALSLALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML
 61 TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE
121 TTFMCEYADE TATIVEFLNR WITFCQSIIS TLT                (SEQ ID NO: 34)
```

Amino acids 1-20 are a signal sequence, and so can be replaced by other signal sequences if desired. An exemplary nucleic acid sequence for human IL2 is

Linker sequences known in the art can be used between the various domains of the fusion protein; for example, one, two, three, four, five or more GGGS sequences can be used. In preferred embodiments, the linker between IL2 and the N-terminus of IL2Rβ comprises the extracellular domain of IL2Ra (EMETSQFPGEEKPQASPEGRPESETSC (SEQ ID NO:28). A tag, e.g., a cMyc tag (EQKLISEEDL (SEQ ID NO:29)), can also be added, e.g., between IL2 and the linker. An exemplary nucleic acid sequence encoding IL2 is available in GenBank at Acc. No. NM_000586.3 .

Sequences for IL2Rβ are also known in the art; an exemplary human IL2Rβ precursor sequence is shown in SEQ ID NO: 35.

```
  1 MAAPALSWRL PLLILLLPLA TSWASAAVNG TSQFTCFYNS PANISCVWSQ DGALQDTSCQ
 61 VHAWPDRRRW NQTCELLPVS QASWACNLIL GAPDSQKLTT VDIVTLRVLC REGVRWRVMA
121 IQDFKPFENL RLMAPISLQV VHVETHRCNI SWEISQASHY FERHLEFEAR TLSPGHTWEE
181 APLLTLKQKQ EWICLETLTP DTQYEFQVRV KPLQGEFTTW SPWSQPLAFR TKPAALGKDT
241 IPWLGHLLVG LSGAFGFIIL VYLLINCRNT GPWLKKVLKC NTPDPSKFFS QLSSEHGGDV
```

```
301 QKWLSSPFPS SSFSPGGLAP EISPLEVLER DKVTQLLLQQ DKVPEPASLS SNHSLTSCFT
361 NQGYFFFHLP DALEIEACQV YFTYDPYSEE DPDEGVAGAP TGSSPQPLQP LSGEDDAYCT
421 FPSRDDLLLF SPSLLGGPSP PSTAPGGSGA GEERMPPSLQ ERVPRDWDPQ PLGPPTPGVP
481 DLVDFQPPPE LVLREAGEEV PDAGPREGVS FPWSRPPGQG EFPALNARLP LNTDAYLSLQ
541 ELQGQDPTHL V                                          (SEQ ID NO: 35)
```

Amino acids 1-26 are a signal sequence, and are preferably deleted in the present constructs, e.g., the sequence comprises amino acids 27-55 1 of SEQ ID NO: 35. Exemplary nucleic acid sequences encoding IL2Rβ are available in GenBank at Acc. No. NM_000878.4 (Var. 1), NM_001346222. 1 (Var. 2); and NM_00 1346223 .1 (Var. 3). Variants 1, 2 and 3 encode the same protein.

*IL2-IL2R0-IL-21 (CIRB21)*

Interleukins IL4, IL7, IL9, IL15 and IL2 1 belong to the same family as IL2, and use the same common IL2Rg. They all have their own private receptors, except for IL2 and IL15, which use IL2Rβ in addition to their own alpha receptors (Figure 11). When soluble IL2, IL4, IL7, or IL2 1 were added to NK92 cells expressing the chimera NK92 $^{CIRB}$, only IL2 1 dramatically enhance cytotoxicity against PC-3 cells. Thus the entire cytoplasmic domain of IL2 1R was cloned then added Head-to-Tail to the C-terminal of IL2Rβ in the chimera CIRB. This resulted in a novel IL2-IL2Rp-IL2 1R chimera (called CIRB2 1, exemplified in Figure 13). As shown herein, it was possible to emulate the activation signals from multiple cytokines that activate NK cells via different receptors by using only one ligand and a hybrid receptor.

In some embodiments, the present constructs include the cytoplasmic domain of IL2 1R at the C-terminus of the IL2Rβ portion (optionally with an intervening linker therebetween). Sequences for IL2 1R are also known in the art; an exemplary human IL2 1R precursor sequence is shown in SEQ ID NO:36.

```
  1 MPRGWAAPLL LLLLQGGWGC PDLVCYTDYL QTVICILEMW NLHPSTLTLT WQDQYEELKD
 61 EATSCSLHRS AHNATHATYT CHMDVFHFMA DDIFSVNITD QSGNYSQECG SFLLAESIKP
121 APPFNVTVTF SGQYNISWRS DYEDPAFYML KGKLQYELQY RNRGDPWAVS PRRKLISVDS
181 RSVSLLPLEF RKDSSYELQV RAGPMPGSSY QGTWSEWSDP VIFQTQSEEL KEGWNPHLLL
241 LLLLVIVFIP AFWSLKTHPL WRLWKKIWAV PSPERFFMPL YKGCSGDFKK WVGAPFTGSS
301 LELGPWSPEV PSTLEVYSCH PPRSPAKRLQ LTELQEPAEL VESDGVPKPS FWPTAQNSGG
361 SAYSEERDRP YGLVSIDTVT VLDAEGPCTW PCSCEDDGYP ALDLDAGLEP SPGLEDPLLD
421 AGTTVLSCGC VSAGSPGLGG PLGSLLDRLK PPLADGEDWA GGLPWGGRSP GGVSESEAGS
481 PLAGLDMDTF DSGFVGSDCS SPVECDFTSP GDEGPPRSYL RQWWIPPPL  SSPGPQAS
(SEQ ID NO:36)
```

Preferably, in these embodiments the IL21R-derived domain comprises amino acids 254-538 of SEQ ID NO:36. An exemplary nucleic acid sequences encoding IL21R is available in GenBank at Acc. No. NM_021798.3.

*IL2-IL2Rfi-CD28 (CIRB28) in NK Cells*

NK cells (and others) are activated when MHC-1 molecule expression is down regulated in transformed cells (Algarra et al, Hum Immunol 2000;61(l):65-73) and during viral infection (Tortorella et al., Annu Rev Immunol 2000;18:861-92). However, the acquisition of resistance phenotype by tumor cells is often caused by the expression of inhibitory signals from MHC-1 (Kochan et al, Oncoimmunology 2013;2(1 l):e26491). HLA-G in particular is known to inhibit NK92 mediated tumor cell lysis (Lin et al., Ann Oncol 2007; 18(1 1): 1804-9). One potential solution to this problem could be the use of multiple activating signals to offset these inhibitory signals. Among the most effective co-stimulatory molecules used for T-cells are CD28 and 4-1BB. CD28 activation requires CD80 and CD86 stimulatory ligand expression on tumor cells. As a result, CD80 expression in tumors was shown to lead to their rejection (Townsend et al., Science 1993;259(5093):368-70), conversely, in $CD28^{-/-}$ mice, cellular and T cell-dependent immunity are quite deficient (Shahinian et al., Science 1993;261(5121):609-12). Therefore, low levels of CD80 are considered an escape mechanism for tumors in several cancers (Tirapu et al, Cancer Res 2006;66(4):2442-50; Hersey et al, Int J Cancer 1994;58(4):527-32; Bernsen et al, Br J Cancer 2003;88(3):424-3 1). For example, the use of a CD28 activation domain in an anti erbB2 chimeric receptor allowed the inhibition of tumor progression in vivo of a $MHC\text{-}1^{+}$ lymphoma Pegram et al., J Immunol 2008;181(5):3449-55). Although CD28 is expressed by NK92 cells (Gong et al, Leukemia 1994;8(4):652-8), its activation is not mediated by all cancers.

In some embodiments, the present constructs include the activation domain of CD28 at the C-terminus of the IL2Rβ portion (optionally with an intervening linker therebetween). Sequences for CD28 are also known in the art; an exemplary human CD28 precursor sequence is shown in SEQ ID NO:38.

```
  1 MLRLLLALNL FPS IQVTGNK I LVKQS PMLV AYDNAWLSC  KYSYNLFSRE FRAS LHKGLD
 61 SAVEVCWYG NYSQQLQVYS KTGFNCDGKL GNESVTFYLQ NLYWQTDIY  FCKI EVMYPP
121 PYLDNEKSNG TI IHVKGKHL CPS PLFPGPS KPFWVLVWG GVLACYSLLV TVAFI I FWVR
181 SKRSRLLHSD YMNMTPRRPG PTRKHYQPYA PPRDFAAYRS                (SEQ I D NO:38)
```

Preferably, in these embodiments the CD28-derived domain comprises the intracellular domain, e.g., amino acids 180 to 220 of SEQ ID NO:38, i.e., RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 39). An exemplary nucleic acid sequence encoding CD28 is available in GenBank at Acc. No. NM_006 139.3 .

*IL2-IL2Rfi-CD28 (CIRB28) in Regulatory T cells (T-regs)*

Patients with hematological malignancies greatly benefit from allogeneic hematopoietic stem cell transplant (AHSCT). In this strategy the donor immune system will attack the patient tumor cells with curative potential in a phenomena known as graft versus tumor. Unfortunately, the donor immune cells may also attack the recipient patient healthy tissue either immediately or in the 100 days that follows and cause GVHD. This could lead to death in 15% and/or morbidity in 40 to 60% of AHSCT. Immuno-suppression is currently the standard of care to manage GVHD (Luznik and Fuchs, Immunol Res 2010;47(1-3):65-77; Storb et al., Biol Blood Marrow Transplant 2010; 16(1 Suppl): S18-27). However, T-regs cells expressing the transcription factor Forkhead box P3 (FOXP3) (Roncador et al, Eur J Immunol 2005;35(6): 1681-91; Hall et al, J Exp Med 1990; 171(1): 141-57) have been found to suppress or alleviate GVHD during AHSCT (Beres et al., J Immunol 2012; 189(1):464-74; Brunstein et al., Blood 2011; 117(3): 1061-70). The persistence of FOXP3 expression is maintained by the epigenetic demethylation of 11 CpG motifs in the conserved non-coding sequence 2 (CNS2), located in its first intron. This demethylation pattern lasts for the life span of T-regs and is protected by Ten-Eleven-Translocation DNA dioxygenase, which is recruited to CNS2 by STAT5 activated by IL2 signaling (Nair et al., Mol Cells 2016;39(12): 888-97), to protect the CpG motifs in CNS2 from re-methylation by DNA methyltransferases. Similarly, CTLA-4, an important down regulator of T-cell activation is up regulated in T-regs and is also controlled by IL2 (Wang et al, Scand J Immunol 2001;54(5):453-8; Bell et al., J Autoimmun 2015;56:66-80; Gasteiger et al., Front Immunol 2012;3: 179). T-regs are extremely responsive to IL2, due to their massive CD25 expression (Dieckmann et al, Exp Med 2001 ;193(11): 1303-10) and their ability to reach IL2 sources by chemokine receptor CCR7 (Smigiel et al, J Exp Med 2014;211(1): 121-36). However, activated T-regs have been shown to lower CD25 expression and change their IL2 signaling in favor of ICOS signaling pathway. This leads to instability of FOXP3 expression

making the transition possible from an activated and not terminally differentiated T-regs (Sharma et al., Immunity 2010;33(6):942-54) to a pro-inflammatory T-cell effector or develop into IFN-gamma- producing pro-inflammatory Thl effector cells (Zhang et al., J Immunol 2017; 198(7):2612-25; Feng et al, Gastroenterology 2011;140(7):2031-43; Takahashi et al., J Exp Med 2011;208(10):2055-67) or even Thl 7 (46). In short, T-regs long-term activation and demethylation of CNS2 as well as proliferation require both IL2 and CD28 co-stimulations (Tang and Bluestone, Immunol Rev 2006;212:217-37; Chen et al, J Immunol 2011;186(11):6329-37).

Thus the IL2-IL2Rp-CD28 chimeras described herein could have a dual use: to help NK92 cells override inhibitory signals from MHC-1+ cancer cells, and separately, to activate T-regs for the purpose of treating GVHD. As described herein, without wishing to be bound by theory, addition of the activation domain of CD28 into a novel chimera, IL2-IL2Rp-CD28 (Figure 18), combining co-stimulatory signals from IL2 and CD28 will lead to a superior NK92 activation that could help override tumor escape via MHC-1+. This chimera could also lead to proliferation of T-regs cells with long- term FOXP3 expression. This strategy could bypass the use of artificial antigen presenting cells (aAPC), dendritic cells or anti-CD3 antibody required for T-regs activation and expansion.

*Nucleic Acids and Expression Vectors*

The compositions described herein can include nucleic acid molecules encoding a chimera as described herein. Nucleic acid molecules comprising expression vectors can be used for expression of the chimeras, e.g., in an NK or T-reg cell as described herein.

A nucleic acid encoding the selected chimera can be inserted in an expression vector, to make an expression construct. A number of suitable vectors are known in the art, e.g., viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, lentivirus, herpes simplex virus-1, adenovirus-derived vectors, or recombinant bacterial or eukaryotic plasmids. For example, the expression construct can include a coding region for the chimera and one or more regulatory regions, e.g., a promoter sequence, e.g., a promoter sequence that restricts expression to a selected cell type, a conditional promoter, or a strong general promoter; an enhancer sequence; untranslated regulatory sequences, e.g., a 5'untranslated region (UTR), a 3'UTR; a polyadenylation site; and/or an insulator sequence, that direct expression of

the chimera. Such sequences are known in the art, and the skilled artisan would be able to select suitable sequences. See, e.g., *Current Protocols in Molecular Biology*, Ausubel, F.M. et al. (eds.) John Wiley & Sons (1995), and Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (Fourth Edition), Cold Spring Harbor Laboratory Press (June 15, 2012) and supplements thereof, and other standard laboratory manuals.

Expression constructs can be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the component gene to cells in vivo. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (e.g., Lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaP04 precipitation. In some embodiments, the nucleic acid is applied "naked" to a cell, i.e., is applied in a simple buffer without the use of any additional agents to enhance uptake. See, e.g., Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals.

*NK and T-reg Cells*

The present methods include expressing, either stably or transiently, a chimera described herein in an NK cell, e.g., a CD3-CD56+ lymphocyte; see Cheng et al, Cellular & Molecular Immunology (2013) 10, 230-252. The NK cell can be a primary cell, e.g., derived from the peripheral blood of a subject and proliferated ex vivo, or can be a cultured NK cell.

When primary cells are used, allogeneic NK cells are preferred, as they were not exposed to immunosuppression and should be fully active. In preferred embodiments, the cells are obtained by performing apheresis on haploidentical related donors to collect peripheral blood leukocytes, which are then depleted of CD3+ cells before optional expansion and administration. See, e.g., Davis et al., Cancer J. 2015 Nov-Dec; 21(6): 486-491 . Alternatively, the cells can be obtained from peripheral or cord blood cells, stem cells or even induced pluripotent stem cells (iPSCs); see Cheng et al., Cellular & Molecular Immunology (2013) 10, 230-252.

Cultured NK cell lines are known in the art, e.g., including NK-92, KHYG-1, NKL, NKG, NK-YS, YT, YTS and haNK-1 cells, as are methods of making new NK

cell lines. NK-92 is a cytolytic cancer cell line that was immortalized ex vivo from NK cells from the blood of a subject suffering from a non-Hodgkins lymphoma. NK-92 cells retain most of the activating receptors and cytolytic signaling pathways but lack the major inhibitory receptors displayed by normal NK cells, and do not express the Fc receptor CD16, and so cannot mediate antibody-dependent cellular cytotoxicity (ADCC). NK-92 cells are tumor-selective and non-immunogenic in humans. The NK-92 cell line is described in Gong et al, Leukemia. 8:652-8 (1994); Yan et al., Clin Cancer Res. 4:2859-68 (1998); WO 1998/49268 and U.S. 2002/0068044. NK-92 cells have been evaluated for potential therapeutic use in cancers, including hematological malignancies; see, e.g., Ljunggren and Malmberg, Nat Rev Immunol. 2007 May;7(5):329-39; Tonn et al., J Hematother Stem Cell Res. 2001 Aug; 10(4):535-44; Klingemann, Cytotherapy. 2005;7(1): 16-22; Malmberg et al., Cancer Immunol Immunother. 2008 Oct;57(10): 1541-52. haNK is an NK-92 variant cell line that expresses the high-affinity Fc receptor FcyRIIIa (158V), and is in clinical development to be combined with IgGl monoclonal antibodies (mAbs). taNKs are targeted NK-92 cells that have been transfected with a gene that expresses a chimeric antigen receptor for a given tumor antigen. KHYG-1 cells were developed the blood of a patient with aggressive NK leukemia (Yagita et al, Leukemia (2000) 14, 922-930) that is IL-2 dependent and produces granzyme M. NKL cells were established from the peripheral blood of a patient with CD3-CD16+CD56+ large granular lymphocyte (LGL) leukemia (Robertson et al., Exp Hematol. 1996 Feb;24(3):406-15). NKG cells were established from the peripheral blood of a patient with rapidly progressive non-Hodgkin's lymphoma (Cheng et al., Cell Transplant. 2011;20(11-12): 1731-46). NK-YS cells were established from a patient with a leukemic-state nasal angiocentric natural killer (NK) cell lymphoma with systemic skin infiltration (Tsuchiyama et al., Blood. 1998 Aug 15;92(4): 1374-83). YT cells, a human NK-like leukaemia cell line, was established from cells in the pericardial fluid of a patient with acute lymphoblastic lymphoma (ALL) and thymoma (Yodoi et al., J Immunol 134: 1623-1630 (1985)); Harnack et al, Anticancer Research 31(2):475-479 (2011)). YTS is a sub-clone of the NK cell leukemia line YT. All of these cell lines are commercially available. For additional information on NK cell lines, Klingermann et al., Front. Immunol. 7:91 (2016); Dahlberg et al., Front. Immunol. 6:605 (2015). The cells can be used as is, or modified, e.g., genetically modified as described in

US7618817; US8034332 (NK-92 cells secreting cytokines including IL2); US8313943 (NK-92 cells expressing CD16); WO 2015193411 (CAR-expressing nk-92 cells); and WO2016160602 (NK-92 cells expressing FcR including CD16). Additional methods for generating and manufacturing cultured NK cells are known in the art; see, e.g., Chabannon et al, Front Immunol. 2016; 7:504, which provides exemplary parameters for media, cytokines, and culture systems, *inter alia.*

The present methods also include expressing, either stably or transiently, a chimera described herein (e.g., IL2-IL2Rp-CD28 chimera) in a T-reg cell, i.e., a CD4+/CD25+ T cell. The T-reg cell can be a primary cell, e.g., derived from the peripheral blood of a subject and proliferated ex vivo, or can be a cultured T-reg cell.

When primary T-reg cells are used, ex-vivo expanded donor T-reg cells, e.g., naturally occurring regulatory T cells (nT-regs) from peripheral blood, are preferred. In preferred embodiments, the cells are obtained from peripheral blood from a donor and expanded ex-vivo using methods known in the art; see, e.g., Dieckmann et al., J. Exp. Med. 193(11):1303-1310 (2001) Chakraborty et al., Haematologica 98(4):533-537 (2013); Hippen et al., Sci Transl Med. 2011 May 18; 3(83): 83ra41; and Taylor et al, Blood. 2002;99:3493-3499. Alternatively, the cells can be obtained from umbilical cord blood (see, e.g., Brunstein et al., Blood. 2011 Jan 20; 117(3): 1061–1070).

The NK and T-reg cells should be maintained according to good manufacturing practice (GMP) in GMP facilities.

In some embodiments, the NK or T-reg cells are engineered to include a suicide gene, e.g., that allows for the cells expressing the gene to be killed by introduction of a specific and selective agent, as a safety measure to guard against tumorigenesis. A number of suicide gene systems are known in the art, including the cytosine deaminase gene, the varicella-zoster virus thymidine kinase gene, the nitroreductase gene, the *Escherichia coli* gpt gene, the *E. coli* Deo gene (see, e.g., Yazawa et al., World J. Surg. 2002 July; 26(7):783-9), inducible caspase 9 (iCas9) (Di Stasi, N Engl J Med 365: 1673-1683 (2011) and Morgan, Molecular Therapy (2012); 20: 11-13), cytochrome P450, or the herpes simplex virus thymidine kinase (TK) gene, which can be wild-type or mutant TK gene (e.g., tk30, tk75, sr39tk). Cells expressing the TK protein can be killed using ganciclovir. See, e.g., WO 2016160602.

The NK or T-reg cells expressing a chimera as described herein, as well as any supplemental active agents for coadministration, can be incorporated into pharmaceutical compositions. Such compositions typically comprise the cells and a pharmaceutically acceptable carrier. A "pharmaceutically acceptable carrier" includes any and all solvents, antibacterial and antifungal agents, isotonic agents, and the like, compatible with pharmaceutical administration (Gennaro, 2000). Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. Supplementary active compounds can also be incorporated into the compositions.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., NK or T-reg cells as described herein) in the required amount in an appropriate solvent with one or a combination of ingredients as required; preferably the solvent is already sterilized, or the formulation can be followed by sterilization. In some embodiments, the cells are cryopreserved.

*Cancer Immunotherapy*

The methods described herein include methods for the treatment of disorders associated with abnormal apoptotic or differentiative processes, e.g., cellular proliferative disorders or cellular differentiative disorders, e.g., cancer, including both solid tumors and hematopoietic cancers. In some embodiments, the disorder is a solid tumor, e.g., breast, prostate, pancreatic, brain, hepatic, lung, kidney, skin, or colon cancer. Generally, the methods include administering a therapeutically effective amount of NK cells expressing a chimera as described herein, to a subject who is in need of, or who has been determined to be in need of, such treatment.

As used in this context, to "treat" means to ameliorate at least one symptom of the disorder associated with abnormal apoptotic or differentiative processes. For example, a treatment can result in a reduction in tumor size or growth rate. Administration of a therapeutically effective amount of a compound described herein for the treatment of a condition associated with abnormal apoptotic or differentiative processes will result in a reduction in tumor size or decreased growth rate, a reduction in risk or frequency of reoccurrence, a delay in reoccurrence, a reduction in metastasis, increased survival, and/or decreased morbidity and mortality, *inter alia.*

Examples of cellular proliferative and/or differentiative disorders include cancer, e.g., carcinoma, sarcoma, metastatic disorders or hematopoietic neoplastic disorders, e.g., leukemias. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of prostate, colon, lung, breast and liver origin.

As used herein, the terms "cancer", "hyperproliferative" and "neoplastic" refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair.

The terms "cancer" or "neoplasms" include malignancies of the various organ systems, such as affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus.

The term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. In some embodiments, the disease is renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

Additional examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Preferably, the diseases arise from poorly differentiated acute leukemias, e.g., erythroblastic leukemia and acute megakaryoblastic leukemia. Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) *Crit Rev. in Oncol./Hemotol.* 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Sternberg disease.

*GVHD and Autoimmune Disease*

The methods described herein include methods the treatment of disorders associated with abnormal immune response, e.g., graft-versus-host disease or GVHD or autoimmune disease. The methods can include administering T-regulatory cells expressing a IL2-IL2Rp-CD28 chimera as described herein to a subject who is in need thereof.

Allogeneic bone marrow transplantation (BMT) has been shown to be effective in hematologic malignancies and some solid tumors, but the high incidence of GVHD has limited the effectiveness and use of BMT. T-regs cells have shown efficacy in suppressing GVHD; see Olson et al., Blood. 2010 May 27; 115(21):4293-301; Sung and Chao, STEM CELLS TRANSLATIONAL MEDICINE, 2013;2:25-32; Dieckmann et al., J. Exp. Med. 193(11): 1303-1310 (2001) Chakraborty et al., Haematologica 98(4):533-537 (2013); Hippen et al, Sci Transl Med. 2011 May 18;

3(83): 83ra4 1; Taylor et al., Blood. 2002; 99:3493-3499; and Brunstein et al., Blood. 20 11; 117(3): 1061-1070).

Impairment of T-regs functions or resistance of effector T cells to T-regs has been reported in many autoimmune diseases such as type-1 diabetes (T1D) (Brusko et al, Diabetes 2005;54(5): 1407-14), rheumatoid arthritis (van Amelsfort et al., Arthritis Rheum 2004;50(9):2775-85), multiple sclerosis (Fletcher et al., J Immunol 2009; 183(1 1):7602- 10), systemic lupus erythematosus (Lyssuk et al,. Adv Exp Med Biol 2007;60 1:113-9) and psoriasis (Sugiyama et al., J Immunol 2005; 174(1): 164-73), as well as atopic disease (Singer et al., Front Immunol. 20 14; 5: 46). Elevated CD25 expression in T-regs makes them particularly responsive to IL2 and this was exploited for example in the case of T1D, where administration of low dose IL-2 promoted T-regs survival and protects NOD mice against diabetes (Tang et al, Immunity 2008;28(5):687-9; Grinberg-Bleyer et al, J Exp Med 20 10;207(9): 187 1-8), and an infusion of T-regs preserved beta-cell function in type 1 diabetes in children (Marek-Trzonkowska et al., Diabetes Care (20 12) 35:1817-20 10).

T-regs, e.g., CD4+CD25+, e.g., CD4+CD25+CD 127- Tregs (e.g., CD4+CD25 $^{high}$CD 127-ICOS+ for atopy Tregs or CD4+CD25+CD 127- CD62L$^{+}$ for GVHD), which are optionally FOXP3+ as well, expressing the IL2-IL2Rp-CD28 chimeras can be used to reduce alloreactive T cells that are believed to mediate GVHD and autoimmunity and damage host tissues. In these embodiments, an effective amount of T-regs cells expressing a IL2-IL2Rp-CD28 chimera as described herein is an amount sufficient to decrease numbers of alloreactive T cells and decrease the self-immune response, e.g., by reduction of donor T cell proliferation and increased T cell apoptosis. See, e.g., Singer et al, Front Immunol. 2014; 5: 46; Riley et al., Immunity. 2009 May; 30(5): 656-665 .

*Methods of Administration and Dosing*

The methods include administration, preferably by intravenous infusion, of a therapeutically effective amount of the NK cells described herein. A therapeutically effective dose can be determined empirically, e.g., based on animal experiments and clinical studies. In some embodiments, the methods include one or more infusions of at least $10^4$ and up to $1 \times 10^6$, $5 \times 10^6$, $1 \times 10^7$, $5 \times 10^7$, $1 \times 10^8$, $3 \times 10^8$, $5 \times 10^8$, $1 \times 10^9$, or $5 \times 10^9$ cells per dose, e.g., between 1 billion and 3 billion cells, or any ranges between any two of the numbers, end points inclusive. The cells can be administered

to a subject once or can be administered multiple times, e.g., once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 hours, or once every 1, 2, 3, 4, 5, 6 or 7 days, or once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks during therapy, or any ranges between any two of the numbers, end points inclusive. See, e.g., Ljunggren and Malmberg, Nat Rev Immunol. 2007 May;7(5):329-39; Tonn et al, J Hematother Stem Cell Res. 2001 Aug; 10(4):535-44; Klingemann, Cytotherapy. 2005;7(1): 16-22; Malmberg et al., Cancer Immunol Immunother. 2008 Oct;57(10): 1541-52; Cheng et al, Cellular & Molecular Immunology (2013) 10, 230-252.

In preferred embodiments, before being infused into a subject the cells are treated so that they are no longer capable of proliferating, but retain cytotoxic activity. One way of achieving this state is by γ irradiation, e.g., with 500 to 1000 cGy, or with 500, 1000, 2000, or 3000 cGy. Gamma irradiation of NK-92 cells at doses of between about 750 and 1000 Grays, e.g., 750, 800, 850, 900 and 950 Grays, is considered to be sufficient for this purpose. Additional forms of radiation, including, for example, ultraviolet radiation, may be employed. Suitable sources to use for this purpose include, for example, a 137Cs source (Cis-US, Bedford, Mass.; Gammacell 40, Atomic Energy of Canada Ltd., Canada). Alternatively, the cells may include a suicide gene as described above.

In some embodiments, before NK cell infusion, the subjects can be treated with a preparatory chemotherapy regimen, e.g., high cyclophosphamide and fludarabine (Hi-Cy [60 mg/kg × 2 days]/Flu [25 mg/m$^2$ × 5 days]), low cyclophosphamide (750 mg/m$^2$) and methylprednisone (1000 mg/m$^2$) or fludarabine alone (25 mg/m$^2$ × 5 days), and or with total body irradiation, e.g., a dose of 200-500, e.g., 400 cGy, radiation.

*Combination Therapies: Checkpoint Inhibitors and Anti-tumor monoclonal mAbs*

In some embodiments, the chimera-expressing NK cells described herein are administered as part of a therapeutic regimen that includes administration of one or more checkpoint blocking agents and/or anti-tumor antibodies. The NK cells can be administered concurrently, e.g., substantially simultaneously or sequentially, with the checkpoint blocking agents and/or anti-tumor antibodies, e.g., within 48, 24, 12, 6, 5,

4, 3, 2, or 1 hour, or within 45, 30, 20, or 15 minutes of administration of the checkpoint blocking agents and/or anti-tumor antibodies.

The term "antibody" as used herein refers to an immunoglobulin molecule or immunologically active portion thereof, i.e., an antigen-binding portion. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')2 fragments, which retain the ability to bind antigen. Such fragments can be obtained commercially, or using methods known in the art. For example, F(ab)2 fragments can be generated by treating the antibody with an enzyme such as pepsin, a non-specific endopeptidase that normally produces one F(ab)2 fragment and numerous small peptides of the Fc portion. The resulting F(ab)2 fragment is composed of two disulfide-connected Fab units. The Fc fragment is extensively degraded and can be separated from the F(ab)2 by dialysis, gel filtration or ion exchange chromatography. F(ab) fragments can be generated using papain, a non-specific thiol-endopeptidase that digests IgG molecules, in the presence of a reducing agent, into three fragments of similar size: two Fab fragments and one Fc fragment. When Fc fragments are of interest, papain is the enzyme of choice because it yields a 50,00 Dalton Fc fragment; to isolate the F(ab) fragments, the Fc fragments can be removed, e.g., by affinity purification using protein A/G. A number of kits are available commercially for generating F(ab) fragments, including the ImmunoPure IgGl Fab and F(ab')2 Preparation Kit (Pierce Biotechnology, Rockford, IL). In addition, commercially available services for generating antigen-binding fragments can be used, e.g., Bio Express, West Lebanon, NH.

The antibody can be a polyclonal, monoclonal, recombinant, e.g., a chimeric, de-immunized or humanized, fully human, non-human, e.g., murine, or single chain antibody. In some embodiments the antibody has effector function and can fix complement. In some embodiments, the antibody has reduced or no ability to bind an Fc receptor. For example, the antibody can be an isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor, e.g., it has a mutagenized or deleted Fc receptor binding region. The antibody can be coupled to a toxin or imaging agent.

Therapeutic anti-tumor antibodies are also known in the art and include human, humanized and chimeric antibodies that bind to tumor antigens. The antibodies are typically monoclonal and can be, e.g., naked, conjugated, or bispecific.

Specific examples include alemtuzumab, rituxumab, trastuzumab, ibritumomab, gemtuzumab, brentuximab, adotranstuzumab, blinatunomab, daratumumab and elotuzumab; abciximab; adalimumab; alefacept; basiliximab; belimumab; bezlotoxumab; canakinumab; certolizumab pegol; cetuximab; daclizumab; denosumab; efalizumab; elotuzumab; golimumab; inflectra; ipilimumab; ixekizumab; natalizumab; nivolumab; obinutuzumab; olaratumab; omalizumab; palivizumab; panitumumab; pembrolizumab; tocilizumab; secukinumab; and ustekinumab. A number of antibodies against cancer-related antigens are known; exemplary antibodies are described in Tables 2-3 (Ross et al., Am J Clin Pathol 119(4):472-485, 2003). The method can be used, e.g., to treat a subject who has a cancer that the anti-tumor antibody has been approved to treat (e.g., NK cells in combination with trastuzumab for a subject who has breast cancer, with berntuximab in a subject who has Hodgkin lymphoma, with daratumumab in a subject who has multiple myeloma, or with elotuzumab in a subject who has multiple myeloma).

Checkpoint blocking agents are known in the art and include antibodies directed to CTLA-4 (e.g., ipilimumab, tremelimumab); PD-1 (e.g., nivolumab, pembrolizumab, BGB-A317); PD-Ll (e.g., atezolizumab, avelumab and durvalumab).

## EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

### MATERIALS AND METHODS

The following materials and methods were used in the Examples below.

**Reagents** - Dexamethasone (Dex), chloroquine and Matrigel (cat# 126-2.5) and human glycosylated IL2 were from Sigma-Aldrich Co. Horse serum (HS), DMEM/F12 medium, Lipofectamine 2000 and TRIzol were from Life Technologies. Fetal bovine serum (FBS) was from Atlanta Biologicals. RPMI 1640 was from LONZA. Smartscribe and Blueprint Onestep RT-PCR Takara kit were from Clontech Laboratories; Platinum SYBR Green qPCR was from Invitrogen, PfuUltra DNA polymerase was from Stratagene. Human TGFβi was from Antigenix America Inc. IL2 was obtained from MGH-DF/HCC Recombinant Protein Core (Boston, MA). Human IL-4 was from Shenandoah Biotechnology Inc. Anti-HER2 (Trastuzumab), humanized Antibody was from BioVision Inc.

**Plasmids** - packaging plasmid pVSV was from Clontech Laboratories, pCMV-dR8.2 dvpr was from Addgene (plasmid # 8455). Lentiviral vectors CSCW-GFP and CSCW-mCherry were from MGH Vector Core (Boston, MA).

**Cells** - HEK293T, NK92, NK92-MI U25 1, PC-3, HepG2, MDA-MB-23 1, Panc-1, U25 1, BT474 and U266 cells were from ATCC. U266-GFP-Luc and NK92-mCherry-Luc cells were generated by lentiviral transduction using CSCW-GFP and CSCW-mCherry lentiviral vectors, respectively. Tumor cell lines PC-3, U251, U266, Panc-1, BT474 and MDA-MB-23 1 were cultured in complete RPMI1640 medium. HEK293T and HepG2 cells were cultured in complete DMEM/F12. NK92 and derived cell lines were cultured in RPMI1640 with 10% heat-inactivated FBS, and 10% heat-inactivated horse serum, supplemented with 0.2 mM I-Inositol, 0.02 mM folic acid, 0.1 mM 2-β mercaptoethanol, 2 mM L-glutamine, add 100 IU/ml IL2. All cell lines and assay cultures were maintained at 37 °C and 5% C02.

**Chimera CIRB construction** - IL2 cDNA was amplified from human brain total RNA by RTPCR using Forward primer 5'-TGCAGGATCCACTCACAGTAACCTCAACTCC-3' (SEQ ID NO:l) and reverse primer 5'-TGCACTCGAGAGTGAAACCATTTTAGAGCC-3 ' (SEQ ID NO: 2) and cloned in BamHI-XhoI in pCDNA4- TO. To build the CIRB chimera we first constructed a chimera from IL2 and the extracellular domain of its receptor IL2Ra, which was amplified by RT-PCR from NK92 total RNA using forward oligo 5'-GGATTACCTTTTGTCAAAGCATCATCTCAACACTGACTGAGCAGAAGCTC ATTTCGGAAGAAGACCTTGAAATGGAGACCAGTCAGTTTCCAGG-3 ' (SEQ ID NO:3), bridging IL2 C-terminal (12 amino acids before the stop codon), and contains the cMyc Tag, the sequence between amino acids 187-194 of IL2Ra as well as and the non-coding 3' sequence of IL2 plasmid. This primer was used with reverse oligo 5'-CCTGATATGTTTTAAGTGGGAAGCACTTAATTATCAGATTGT TCTTCTACTCTTCCTCTGTCTCC -3' (SEQ ID NO: 4). The amplified fragment was used, as an oligo to mutagenize IL2 wild type resulting in an IL2-IL2Ra chimera. To build CIRB final chimera construct, the IL2 receptor alpha chimera was used to amplify IL2 with a C-terminal cMyc tag followed by only the extra cellular domain of IL2Ra then followed by the N-terminal fragment of IL2Rβ using Forward 5'-TGCAGGATCCACTCACAGTAACCTCAACTCC-3' (SEQ ID NO:5) and reverse 5'-GGGAAGTGCCATTCACCGCGCAGGAAGTCTCACTCTCAGGA-3 '(SEQ ID

NO:6). This later introduces the N-terminal end of IL2Rβ. The product was then re-amplified using the same forward primer and reverse 5'-GGCTCTCGAGTTGTAG AAGCATGTGAACTGGGAAGTGCC ATTCACCGC-3' (SEQ ID NO: 7). An Xbal site in IL2 was first removed by mutagenesis using primers forward 5'-CATCTTCAGTGCCTAGAAGAAGAACTC-3 ' (SEQ ID NO:8) and reverse 5'-GAGTTCTTCTTCTAGGCACTGAAGATG-3 ' (SEQ ID NO: 9). IL2Rβ was then amplified using forward 5'-TTCCCAGTTCACATGCTTCTACAAGTCGA CAGCCAACATCTCCTG-3' (SEQ ID NO: 10) and reverse 5'- AGCTTCTAGACTC GAGTTATCACACCAAGTGAGTTGGGTCCTGACCCTGG -3' (SEQ ID NO: 11). Next the fragment IL2-cMyc-IL2Ra was open Xho-Xbal and IL2Rβ was added as Sall-Xbal fragment to form the final chimera CIRB. Both IL2 and CIRB were transferred from pcDNA4-TO using Spel (blunt end) and Xhol to CSCW-mcherry lentiviral vector digested with BamHI (blunt end) and Xhol. All constructs were sequenced and verified for Lentivirus integrity.

**Lentivirus production and transduction** - HEK293T cells were transfected using Lipofectamine 2000 with 2.4 μg DNA of pVSV, pCMVdr8.2dvrp and the lentiviral construct CSCW-GFP or CSCW-mCherry vectors expressing either CIRB or IL2 constructs, using the ratios 1: 0.4: 1, respectively, with 25uM chloroquine. 6 hours post-transfection media was changed and cells were incubated for 36 hours prior to collecting and filtering lentiviral supernatant through a 0.45um syringe filter. Viral titers were determined by serial dilutions and counting of mCherry expressing HEK293T cells. NK92 cells were infected by spinoculation at 1800g for 45min at an optimal multiplicity of infection (MOI) of 46 lentiviral particles per cell in a 2ml-Eppendorf tube containing $2xl0^{5}$ cells. Infected cells were then plated in a 6-well plate supplemented with lOOIU/ml of IL2. Two days later, media was changed but without IL2. NK92 cell lines expressing IL2 ($NK92^{IL2}$) or CIRB ($NK92^{CIRB}$) were then permanently weaned of exogenous IL2.

$_{N\ K\ 9}2_{CI^{R}B\ growt}\ h_{\ was}$ compared to $NK92^{IL2}$, parental NK92 and IL2-independent NK92-MI cell line. $60xl0^{3}$ cells were cultured in 6 well plates for 3days after which 3ml fresh media were added on top of old media for another 3days. Viable cells were counted by Trypan blue exclusion using a Bio-Rad TC20™ automated cell counter. Protein expression was detected by western blot on total cell lysate obtained by sonication.

**Flow cytometry** - NK cell markers expression was verified using mouse anti-Human antibodies to CD45-APC-CY7, CD25-FITC, CD 16-PE, CD3-PECY7, CD56-PAC BLUE and CD 122-PE, purchased from BD Biosciences, San Jose, CA. Antibodies to NKG2D-APC were from BioLegend, DAPI from Invitrogen, mouse anti-cMyc: sureLight APC was from Columbia Biosciences. Cells were sorted at MGH Flow Cytometry Core facility using a BD 5 laser SORP FACS Vantage SE Diva system (BD Biosciences) using argon-ion laser excitation (633 nm and 35mW for excitation). Intact cells were gated using the forward and sideward scatter from 488 nm excitation and 320 mW. Data acquisition was carried out by analyzing $10^5$ events/sample, using CellQuest Software (BD Biosciences). FACS data were analyzed using FlowJo Software (Tree Star, Inc.). Human primary NK cells were extracted from peripheral blood of healthy donors using the Rosettesep™ human enrichment kit (StemCell technologies), following the manufacturer protocol.

**Cytotoxic activity of NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ cells** - To assess the cytolytic effects of NK92$^{IL2}$ and NK92$^{CIRB}$, compared to the parental NK92 cell line (pre-stimulated for 24 hours with IL2, lOOIU/ml). 8xl0$^3$ U266GFP cells (selected for firm adherence), were plated in triplicate in 96 well plates. 24 hours later NK cells lines were added at effector/target cells ratios (E/T) of 1/8, 1/4, 1/2, 1/1 and 2/1. After two days of co-culture NK cells were suspended to allow further killing of U266GFP cells. After a total 4 days co-culture, U266GFP cell survival was evaluated using a SpectraMax® M2 Microplate fluorescence reader (Molecular Devices) with excitation at 485nm and emission at 515nm.

Another set of experiments evaluated the anti-cancer effect of NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ on a panel of five cancer cell lines, U251GM, PC-3, Panc-1, MDA-MB-231 and HepG2 cells. 32xl0$^3$ cells for each cancer cell line, were first plated in a 24-well plate for either 24 hours prior to adding NK92 (pre-stimulated with IL2, lOOIU/ml), NK92$^{IL2}$ or NK92$^{CIRB}$ at E/T ratio of 2/1 target cancer cell (Figure 3B), or only 5 hours (Figure 3C) before adding NK92$^{IL2}$ or NK92$^{CIRB}$ at E/T ratios of 0/1, 1/1, 2/1, and 3/1 for each cancer cell line. Co-cultured cells were then incubated for 4 days. Cell viability of cancer cells after this time was determined using a 0.1% crystal violet in al0% alcohol solution followed by extraction using 70% ethanol and reading absorbance at 595nm.

**ADCC of NK92$^{IL2}$ and NK92$^{CIRB}$ against Her2 positive breast cancer cell line BT474 -**

Target Breast cancer BT474 (8xl0 $^{3}$ cells) was plated in 96-well plates. 24 hours later cells were incubated for 20min at room temperature with lug/ml Trastuzumab before the addition of effector cells at an E/T ratio of 2:1. After 3 days of incubation, viability of cancer cells was determined using crystal violet/alcohol-extraction assay described earlier.

**Impact of pre-exposure to immunosuppressors on NK cells cytotoxicity and viability** - NK92, NK92 $^{IL2}$ and NK92 $^{CIRB}$ (64xl0 $^{3}$ cells), were plated and exposed for 24 hours to TGFβi (20ng/ml), 11-4 (20ng/ml), or Dex (0.5uM). During this time parental NK92 cells were incubated with IL2 at 20IU/ml. Cancer cells U251GM, PC-3, Panc-1, MDA-MB-23 1 and HepG2 cells (32 xlO $^{3}$ cells) were then added to NK cells at an E/T ratio of 2:1. Co-cultured cells were then incubated for 4 days. Cell viability of cancer cells after this time was determined using crystal violet/alcohol-extraction assay.

To determine the impact of immunosuppressors TGFβi, IL-4 and Dex on the growth of NK92 $^{IL2}$ and NK92 $^{CIRB}$ (30xl0 $^{3}$ cells), were plated in triplicate in a 12-well plate, grown under TGFβi (lOng/ml), IL-4 (lOng/ml), or Dex (luM) for 3days, then refreshed for another 3 days of growth under the same conditions for a total of six days. After that time cells viability was determined with Trypan blue.

**Tumor Growth Delay Experiments** - 5 week-old (24-25g) male NOD.Cg-Prkdcscid I12rgtml Wjl/SzJ immunodeficient mice were purchased from Jackson Laboratory and were housed in the MGH center for comparative medicine. These animals are devoid of B and T cells as well as NK cells, thus offer a suitable platform to evaluate the therapeutic efficacy of modified NK cells. U25 1 or PC-3 cells were suspended in serum-free RPMI containing 20% Matrigel and injected sub-cutaneously (s.c.) as 4xl0 $^{6}$ cells for PC-3 or 3xl0 $^{6}$ cells for U25 1 in a volume of 0.5 ml using a 0.5-inch 29-gauge needle and a 1 ml insulin syringe. Tumor areas (length x width) were measured twice a week using Vernier calipers (Manostat Corp., Switzerland) and tumor volumes were calculated based on: Volume = π/6 (length x width) $^{3/2}$. Treatment with NK92 $^{CIRB}$ or NK92 $^{IL2}$ was initiated when the average tumor volume reached -200 $mm^3$ for PC-3 or ~160$mm^3$ for U25 1. For animals bearing PC-3 tumors freshly prepared NK cells were suspended in PBS irradiated with 500cGy and

administered as 4 weekly injections (15xl0 $^{6}$ cells in 200ul per mouse), via the tail vein. For animals bearing U25 1 cells NK92$^{CIRB}$ or NK92$^{IL2}$ were not irradiated.

**Detection of NK92$^{CIRB}$ and NK92$^{IL2}$ in peripheral blood** - U25 IMG tumor cells were grown s.c. in Nod/scid mice. When tumor size reached ~ 160mm$^{3}$, non-irradiated NK92$^{IL2}$ and NK92$^{CIRB}$ cells (10$^{7}$ cells in 200ul per mouse), were injected, via the tail vein. A second injection of non-irradiated NK cells (5xl0 $^{6}$ cells) was carried out 4 days later. 17 days later, animals were killed and cardiac blood was collected from 3 animals in each group. Heparinized blood was processed and analyzed by flow cytometry using human specific anti-CD45 and mCherry fluorescent protein, which is exclusively co-expressed with IL2 or CIRB via an internal ribosome entry site.

**Survival of irradiated NK92$^{CIRB}$ and NK92$^{IL2}$ cells**- After irradiation at lOGy (0.83Gy for 12 min), NK92$^{CIRB}$ and NK92$^{IL2}$ were then cultured and their survival was determined, using Trypan Blue every 24 hours for 3 days.

**Expression profiles of cytotoxicity effectors in NK92, NK92$^{IL2}$ and NK92$^{CIRB}$** - Expression profiles of natural cytotoxicity receptors NKP30, NKP44, NKP46, cytolytic enzymes Perforin- 1 and Granzyme-B, and cytokines TNFa and IFN-γ were quantified by qRT-PCR using the primers listed in Table 1. Results were analyzed using comparative CT (ΔΔOT) method and are presented as RNA folds relative to NK92 parental cell line after normalization to the GAPDH RNA content of each sample.

**Table 1. Primers used for Expression profiles of cytotoxicity effectors in NK92, NK92$^{IL2}$ and NK92$^{CIRB}$**

| Gene name | Forward primer sequence 5' to 3' | SEQ ID NO: | Reverse primer sequence 5' to 3' | SEQ ID NO: | Size bp |
|---|---|---|---|---|---|
| NKp30 | GCTGGTGGTGGAGAAA GAAC | 12 | GGACCTTTCCAGGT CAGACA | 13 | 144 |
| NKp44 | TCACAGCCACAGAACT CCAC | 14 | CCTGAGCTCCATCA TGGTTT | 15 | 262 |
| NKp46 | TGCCGTCTAGACACTG CAAC | 16 | CCAAAACATCGGTA TGTCCC | 17 | 146 |
| Perforin- 1 | CGCCTACCTCAGGCTTA TCTC | 18 | CCTCGACAGTCAGG CAGTC | 19 | 155 |
| Granzyme-B | CCCTGGGAAAACACTC ACACA | 20 | CACAACTCAATGGT ACTGTCGT | 2 1 | 108 |
| TNFalpha | CCCAGGGACCTCTCTCT AATCA | 22 | AGCTGCCCCTCAGC TTGAG | 23 | 115 |
| INFgamma | TCGGTAACTGACTTGA | 24 | TCCTTTTTCG CTTC C | 25 | 100 |

| Gene name | Forward primer sequence 5' to 3' | SEQ ID NO: | Reverse primer sequence 5' to 3' | SEQ ID NO: | Size bp |
|---|---|---|---|---|---|
| | ATGTCCA | | CTGTTTT | | |
| GAPDH | ATGGGGAAGGTGAAGG TCG | 26 | GGGGTCATTGATGG CAACAATA | 27 | 108 |

**Statistical Analysis** - Statistical significance of differences was determined by two-tailed Student's test, a one-way ANOVA, paired Tukey's Multiple Comparison test. All tests included comparisons to untreated samples or as indicated in the text. Statistical significance is indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.001. Analyses were performed using Prism software version 6 (GraphPad Software).

**Example 1. Design and construction of the CIRB chimera**

The quaternary crystal structure of IL2 and its receptors complex (20) shows that the C-terminal end of IL2 and the N-terminal residue of IL2Rβ are separated by 41A. For a linker between IL2 and the N-terminus of IL2Rβ we choose the extracellular domain of IL2Ra (EMETSQFPGEEKPQASPEGRPESETSC (SEQ ID NO:28)). A cMyc tag (EQKLISEEDL (SEQ ID NO:29)) was added between IL2 and the linker. The fully mature receptor IL2Rβ protein coding sequence (without signal peptide) was placed after the linker to yield the full chimera CIRB (Figure 1). Both CIRB and IL2 were cloned in a lentiviral vector co-expressing mCherry. The linker fold was predicted computationally to be a helix-dominated structure (Figures 5A-D). Linker flexibility was assessed using the computational method of Karplus and Shultz method (47) which, indicates better than average flexibility (1 or greater on a 0 to 2 scale) at all the peptide linkages.

The resulting sequences are shown below.

Nucleotide Sequence of IL2-IL2RP:

**ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAAC**
**AGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTACTG**
**CTGGATTTACAGATGATTTTGAATGGAATTAATAAT TACAAGAATCCCAAACTCACC**
**AGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTGAAACATCTT**
**CAGTGCCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC**
**AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTG**
**GAACTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAGCAACC**
**ATTGTAGAATTTCTGAACAGATGGATTACCTTTTGT CAAAGCATCATCTCAACACTG**
**ACTGAGCAGAAGCTCATTTCGGAAGAAGACCTTGAAATGGAGACCAGTCAGTTTCCA**
**GGTGAAGAGAAGCCTCAGGCAAGCCCCGAAGGCCGTCCTGAGAGTGAGACTTCCTGC**
**GCGGTGAATGGCACTTCCCAGTTCACATGCTTCTACAACTCGCGAGCCAACATCTCC**

TGTGTCTGGAGCCAAGATGGGGCTCTGCAGGACACTTCCTGCCAAGTCCATGCCTGG
CCGGACAGACGGCGGTGGAACCAAACCTGTGAGCTGCTCCCCGTGAGTCAAGCATCC
TGGGCCTGCAACCTGATCCTCGGAGCCCCAGATTCTCAGAAACTGACCACAGTTGAC
ATCGTCACCCTGAGGGTGCTGTGTCGTGAGGGGGTGCGATGGAGGGTGATGGCCATC
CAGGACTTCAAGCCCTTTGAGAACCTTCGCCTGATGGCCCCCATCTCCCTCCAAGTT
GTCCACGTGGAGACCCACAGATGCAACATAAGCTGGGAAATCTCCCAAGCCTCCCAC
TACTTTGAAAGACACCTGGAGTTCGAGGCCCGGACGCTGTCCCCAGGCCACACCTGG
GAGGAGGCCCCCCTGCTGACTCTCAAGCAGAAGCAGGAATGGATCTGCCTGGAGACG
CTCACCCCAGACACCCAGTATGAGTTTCAGGTGCGGGTCAAGCCTCTGCAAGGCGAG
TTCACGACCTGGAGCCCCTGGAGCCAGCCCCTGGCCTTCAGGACAAAGCCTGCAGCC
CTTGGGAAGGACACCATTCCGTGGCTCGGCCACCTCCTCGTGGGTCTCAGCGGGGCT
TTTGGCTTCATCATCTTAGTGTACTTGCTGATCAACTGCAGGAACACCGGGCCATGG
CTGAAGAAGGTCCTGAAGTGTAACACCCCAGACCCCTCGAAGTTCTTTTCCCAGCTG
AGCTCAGAGCATGGAGGAGACGTCCAGAAGTGGCTCTCTTCGCCCTTCCCCTCATCG
TCCTTCAGCCCTGGCGGCCTGGCACCTGAGATCTCGCCACTAGAAGTGCTGGAGAGG
GACAAGGTGACGCAGCTGCTCCTGCAGCAGGACAAGGTGCCTGAGCCCGCATCCTTA
AGCAGCAACCACTCGCTGACCAGCTGCTTCACCAACCAGGGTTACTTCTTCTTCCAC
CTCCCGGATGCCTTGGAGATAGAGGCCTGCCAGGTGTACTTTACTTACGACCCCTAC
TCAGAGGAAGACCCTGATGAGGGTGTGGCCGGGGCACCCACAGGGTCTTCCCCCCAA
CCCCTGCAGCCTCTGTCAGGGGAGGACGACGCCTACTGCACCTTCCCCTCCAGGGAT
GACCTGCTGCTCTTCTCCCCCAGTCTCCTCGGTGGCCCCAGCCCCCCAAGCACTGCC
CCTGGGGGCAGTGGGGCCGGTGAAGAGAGGATGCCCCCTTCTTTGCAAGAAAGAGTC
CCCAGAGACTGGGACCCCCAGCCCCTGGGGCCTCCCACCCCAGGAGTCCCAGACCTG
GTGGATTTTCAGCCACCCCCTGAGCTGGTGCTGCGAGAGGCTGGGGAGGAGGTCCCT
GACGCTGGCCCCAGGGAGGGAGTCAGTTTCCCCTGGTCCAGGCCTCCTGGGCAGGGG
GAGTTCAGGGCCCTTAATGCTCGCCTGCCCCTGAACACTGATGCCTACTTGTCCCTC
CAAGAACTCCAGGGTCAGGACCCAACTCACTTGGTGTGA (SEQ ID NO:30)

Protein Sequence of IL2-IL2RP

MYRMQLLSCIALSLALVTNSAPTS SSTKKTQLQLEHLLLDLQMILNGINNYKNPKLT
RMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI SNINVIVL
ELKGSETT FMCEYADETATIVE FLNRWITFCQSIISTLTEQKL ISEEDLEMETSQ FP
GEEKPQAS PEGRPESET SCAVNGTSQFTC FYNSRANI SCVWSQDGALQDT SCQVHAW
PDRRRWNQTCELLPVSQASWACNL ILGAPDSQKLTTVDIVTLRVLCREGVRWRVMAI
QDFKPFENLRLMAPI SLQVVHVETHRCNI SWEISQASHY FERHLE FEARTLS PGHTW
EEAPLLTLKQKQEWICLETLTPDTQYE FQVRVKPLQGE FTTWS PWSQPLAFRTKPAA
LGKDTIPWLGHLLVGLSGAFGFI ILVYLL INCRNTGPWLKKVLKCNT PDPSKFFSQL
SSEHGGDVQKWLSSPFPSSS FSPGGLAPEISPLEVLERDKVTQLLLQQDKVPEPASL
SSNHSLTSCFTNQGY FFFHLPDALE IEACQVY FTYDPYSEEDPDEGVAGAPTGSS PQ
PLQPLSGEDDAYCT FPSRDDLLLFSPSLLGGPSPPSTAPGGSGAGEERMPPSLQERV
PRDWDPQPLGPPT PGVPDLVDFQPPPELVLREAGEEVPDAGPREGVS FPWSRPPGQG
EFRALNARLPLNTDAYLSLQELQGQDPTHLV* (SEQ ID NO:31)

**Example 2. Cell surface expression of the chimera CIRB**

After Lentivirus-mediated stable expression with an identical MOI of 46, $NK92^{IL2}$ and $NK92^{CIRB}$ cell lines acquire IL2 independence and proliferate indefinitely. Both cell lines showed similar growth during a 6-days period and faster

than NK92-MI another IL2-independent cell line (Figs. 6A-B), with robust survival after subjection to multiple freezing and plating cycles in culture, comparatively to the parental NK92 cell line and NK92-MI. Using an anti-cMyc monoclonal antibody, we next examined the expression of CIRB at the cell surface of transiently transfected HEK293 cells (Figure 2A) and stable NK92$^{CIRB}$ cells (Figure 2B). We found clear evidence of the surface expression of cMyc in NK92$^{CIRB}$ but not in NK92$^{IL2}$ cells. CIRB expression was further confirmed using an anti-CD 122 monoclonal antibody, which recognizes the native IL2Rβ as well as the chimera shows that the endogenous CD 122 is present in NK92$^{IL2}$ cells, as expected, but at levels lower than in NK92$^{CIRB}$ cell line, which express both IL2Rβ and CIRB. The expression of the full-length chimera CIRB was further detected by western blot using monoclonal anti-human IL2. Figure 2D shows a full-length size of 95 kDa, which is higher than the predicted size of 80kDa and could be due to post-translational glycosylation.

**Example 3. Cytotoxicity of parental NK-92 and modified cell lines**

We compared cytotoxicity of NK92, NK92$^{IL2}$ and NK92$^{CIRB}$ cells against an adherent multiple myeloma cell line U266GFP. Figure 3A shows that the parental NK92 cell line, although pre-stimulated with lOOIU/ml of IL2, was far less cytotoxic than NK92$^{IL2}$ or NK92$^{CIRB}$. Of note, IL2 was absent during the four days of co-culture. NK92$^{IL2}$ and NK92$^{CIRB}$ cells showed equivalent cytotoxicity toward U266GFP suggesting comparable levels of activation *in vitro.*

We further compared anti-cancer activity of NK92$^{IL2}$ and NK92$^{CIRB}$ cells in a panel of five human cancer cell lines. In one experiment (Figure 3B) cells were plated 24 hours prior to adding NK cell lines. The cytotoxicity of NK92$^{IL2}$ and NK92$^{CIRB}$ cells were generally equivalent with a slight edge to NK92$^{CIRB}$. NK92 cell were inferior to the other two NK lines. In other experiments, cancer cells were plated only 5 hours prior to adding NK cells (Figure 3C) the cytotoxicity of NK cell lines became more pronounced than at 24 hours post plating. Under these conditions, the cytotoxicity of all NK cell lines was greater than in cells plated for 24 hours and NK92$^{CIRB}$ showed more cytotoxicity than NK92$^{IL2}$ cells, at most E/T ratios. This difference was more evident with the most resistant cancer cell lines U25 1GM and Panc-1.

**Example 4. NK92 $^{CIRB}$ resistance to TGFpi, IL4 and Dexamethasone immunosuppression**

Transforming growth factor TGFβ1 is an immunosuppressor overexpressed in the TME and is known to inhibit NK cells functions by destabilizing several activation signals in NK cells (34). The glucocorticoid dexamethasone impairs the function of lymphocytes in part by suppressing IL2 production from CD4+ T cells (35). IL-4 was reported to inhibit the proliferation of NK cells (48). We tested the effects of these immunosuppressors in both NK92$^{IL2}$ and NK92$^{CIRB}$ lines by culturing cells for 6 days in the presence of TGFβi (lOng/ml), IL4 (lOng/ml), or dex (luM). Figure 4A shows that NK92$^{IL2}$ cells did not survive the exposure to dex, and their proliferation was inhibited strongly by TGFβi and to some extent by IL4. In contrast, the proliferation of NK92$^{CIRB}$ cells was not significantly affected by TGFβi or IL4 and was only weakly inhibited by dex (Figure 4B).

The effects of TGFβi and dex pre-treatments on NK cell cytotoxicity were then evaluated using the panel of five cancer cell lines at an E/T ratio of 2:1 (Figure 4C). NK cells affected more killing in these experimental conditions since cancer cells were added to already plated NK92 cells and are more vulnerable if not already attached. Additionally, the parental NK92 cells were plated with IL2 in the media (20IU/ml) and are therefore more active than in other experiments. NK92$^{CIRB}$ cytotoxicity against MDA-MB-23 1, PC-3 and HepG2 was not affected. Dex severely reduced cytotoxicity of NK92$^{IL2}$ cells towards all cancer cell lines. Similarly, TGFβi significantly reduced NK92$^{IL2}$ cytotoxicity against most cancer cell lines except for MDA-MB-23 1 and HepG2. Surprisingly, NK92 cells also showed resistance to dex inhibition in MDA-MB-23 1 and HepG2. Overall immunosuppression of NK92$^{CIRB}$ cells was weaker than in the NK92 and NK92$^{IL2}$ lines and was also dependent on the target cancer cell line.

**Example 5. CD16 is substantially induced by endogenous expression of the chimera CIRB**

In accordance with the original characterization of NK92 cells (43), NK92 cells are CD56$^{+}$, CD3$^{-}$, CD16\ CD25$^{+}$, CD45$^{+}$ and NKg2D$^{-}$ (Figure 5A), In comparison, NK92 $^{IL2}$ cells, NK92$^{CIRB}$ cells, and freshly isolated human NK (hNK) cells display different patterns of marker expression. Unlike NK92, the cell lines NK92$^{IL2}$, NK92$^{CIRB}$, and hNK cells are all CD16$^{+}$, with expression levels of hNK >

$_{NK92}$ $_{CI}R_B$ > **NK92**$^{IL2}$ (Figure 5B). We also examined the IL2-independent NK92-MI, and in accordance with a previous report (38), did not find any expression of CD16 (Figure 10A). We also found no CD16 expression in NK92 cells treated with glycosylated or non-glycosylated IL2 (Figure 10B). Human NK (hNK) cells express much higher levels of CD16 and NKG2D (Figure 5B), than all NK92 cell lines and are virtually CD25-negative, while NK92$^{CIRB}$ cells expressed lower amounts of CD25 than both IL2-stimulated NK92 or NK92$^{IL2}$. Of the NK92 cell lines, NK92$^{CIRB}$ expression for CD25 and CD16 were most similar to hNK.

**Example 6. ADCC of NK92$^{IL2}$ and NK92$^{CIRB}$ against HER2 positive breast cancer cell line BT474**

We next examined the impact of CD16 expression on ADCC using Trastuzumab against an HER2 positive breast cancer cell line BT474. Figure 5C shows that in the absence Trastuzumab and when using an E/T ratio of 2:1, BT474 cells were not affected by the direct cytotoxicity of NK cells. However, in the presence of lug/ml Trastuzumab both NK92$^{CIRB}$ andNK92$^{IL2}$ exerted substantial cytotoxicity of about 60% and 50%, respectively. Trastuzumab alone at lug/ml did not affect significantly the survival of BT474. Similarly, parental NK92 did not provoke any significant cytotoxicity against BT474 in the presence of Tratstuzumab.

**Example 7. Expression profiles of cytotoxicity effectors in NK92, NK92$^{IL2}$ and NK92$^{CIRB}$**

qPCR analysis of NK92$^{CIRB}$ cells revealed dramatic increases of natural cytotoxicity receptor (NCRs), NKP30 (1.7 fold), NKP44 (9 fold) and NKP46 (1.4 fold), compared to NK92$^{IL2}$ and parental NK92 stimulated with IL2 for 48 hours. In NK92$^{IL2}$, NKP44 expression also increased (3.3 fold). While Perforin-1 expression was similar in all cell lines, Granzyme-B expression declined and TNF-a increased in NK92$^{IL2}$ and both declined marginally while IFN-gamma increased in NK92$^{CIRB}$.

**Example 8. In vivo detection of circulating NK92^2 and NK92$^{CIRB}$ cells**

The Survival and systemic circulation of NK92$^{IL2}$ and NK92$^{CIRB}$ *in vivo,* were evaluated in the context of tumor-bearing animals. U25 IMG tumor cells were grown s.c. in Nod/scid mice. When tumor size reached an average of 160mm$^3$, animals received, within 4 days, two injections of non-irradiated NK92$^{IL2}$ and NK92$^{CIRB}$ cells via the tail vein. Figure 6A shows that within 24 hours of the first injection of live

$NK92^{CIRB}$ cells, rapid tumor volume regression of 46% was observed, while $NK92^{IL2}$ cells caused 35% reduction. In contrast, tumors continued to grow in untreated animals to reach a maximal limit size nearing $200mm^3$ before regressing. This size-dependent limited growth was previously shown to be due to the poor angiogenesis of these tumors, which can be improved by VEGF expression (49). Tumor regression for $NK92^{CIRB}$-treated group continued after the second injection while the tumors in $NK92^{IL2}$-treated animals resumed growth and did not respond until day 18. Three weeks later, the untreated and $NK92^{IL2}$ groups showed a similar tumor size. In comparison the $NK92^{CIRB}$-treated group displayed a significant tumor volume reduction of 86%. 17days post-NK cells injections, blood (0.5ml) was collected from the three groups of mice and analyzed with cytometry for circulating cells expressing both mCherry, and human CD45. Figure 6B shows that circulating NK cells can only be detected in the $NK92^{CIRB}$-treated group. This result suggests that CIRB expression but not IL2 secretion endows NK cells with the ability to persist in tumor bearing animals.

**Example 9. Survival of irradiated $NK92^{IL2}$ and $NK92^{CIRB}$ cells**

NK92 cell line was isolated from an aggressive non-Hodgkin lymphoma patient (43). Therefore, FDA requires NK92 cells irradiation between 5 and lOGy prior to infusion to prevent proliferation. Under these conditions irradiated NK92 cells viability declines dramatically within 2 days. $NK92^{IL2}$ and $NK92^{CIRB}$ were irradiated at lOGy (0.83gy for 12 min) and then plated in complete NK92 media to determine their survival using Trypan Blue every 24 hours. Figure 7A shows that 24hours post-irradiation, 57% of $NK92^{CIRB}$ cells and 45% of $NK92^{IL2}$ cells survive. The survival $NK92^{CIRB}$ advantage was statistically significant at days 1 and 2 (*$P<0.05$).

**Example 10. Anti-tumor efficacy of irradiated $NK92^{IL2}$ and $NK92^{CIRB}$ cells**

Prostate cancer cell line PC-3 (50) is androgen receptor and PSA negative and forms very aggressive tumors when grown in Nod/Scid mice. When tumor volumes reached -200 $mm^3$ (day 28), irradiated NK cells (500cGy) were administered as 4 weekly injections via the tail vein. Figure 7B shows that the growth of PC-3 tumors in the $NK92^{CIRB}$-treated group was slowed after the first injection. After the last $NK92^{CIRB}$ cells injection, a significant tumor growth delay of about 17 days was

recorded in the period between 1st and 4th NK92$^{CIRB}$ cells injections **(**P<0.01)**, comparatively to the untreated group. In contrast, the NK92$^{IL2}$-treated group tumors produced only a tumor delay of 7 days from the untreated tumors group **(*P<0.05).**

**Example 11. Design, construction, and testing of a CIRB21 chimera**

Interleukins IL4, IL7, IL9, IL15 and IL21 belong to the same family as IL2, and use the same common IL2Rg. They all have their own private receptors, except for IL2 and IL15, which use IL2Rβ in addition to their own alpha receptors (Figure 11). We examined the impact of added cytokines (IL2, IL4, IL7, and IL21) on the cytotoxicity of NK92 cells expressing the chimera NK92$^{CIRB}$, against **PC-3** cells. We found that only IL21was able to enhance dramatically their cytotoxicity (Figure 12, shows only IL21 and IL2 impact). This prompted us to ask whether, if IL2 and IL21 use the same IL2Rg, it would be possible to combine the signaling of both in one chimeric cytokine receptor. To answer that, the entire cytoplasmic domain of IL21R was cloned then added Head-to-Tail to the C-terminal of IL2Rβ in the chimera CIRB. This resulted in a novel IL2-IL2Rp-IL21R chimera (called CIRB21, exemplified in Figure 13), which was then introduced in NK92 cells to yield NK92$^{CIRB21}$.

Nucleotide Sequence of IL2-IL2Rp-IL21R (CIRB21)

**ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAAC**
**AGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTACTG**
**CTGGATTTACAGATGATTTTGAATGGAATTAATTACAAGAATCCCAAACTCACC**
**AGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTGAAACATCTT**
**CAGTGCCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC**
**AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTG**
**GAACTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAGCAACC**
**ATTGTAGAATTTCTGAACAGATGGATTACCTTTTGT CAAAGCATCATCTCAACACTG**
**ACTGAGCAGAAGCTCATTTCGGAAGAAGACCTTGAAATGGAGACCAGTCAGTTTCCA**
**GGTGAAGAGAAGCCTCAGGCAAGCCCCGAAGGCCGTCCTGAGAGTGAGACTTCCTGC**
**GCGGTGAATGGCACTTCCCAGTTCACATGCTTCTACAACTCGCGAGCCAACATCTCC**
**TGTGTCTGGAGCCAAGATGGGGCTCTGCAGGACACTTCCTGCCAAGTCCATGCCTGG**
**CCGGACAGACGGCGGTGGAACCAAACCTGTGAGCTGCTCCCCGTGAGTCAAGCATCC**
**TGGGCCTGCAACCTGATCCTCGGAGCCCCAGATTCTCAGAAACTGACCACAGTTGAC**
**ATCGTCACCCTGAGGGTGCTGTGTCGTGAGGGGGTGCGATGGAGGGTGATGGCCATC**
**CAGGACTTCAAGCCCTTTGAGAACCTTCGCCTGATGGCCCCCATCTCCCTCCAAGTT**
**GTCCACGTGGAGACCCACAGATGCAACATAAGCTGGGAAATCTCCCAAGCCTCCCAC**
**TACTTTGAAAGACACCTGGAGTTCGAGGCCCGGACGCTGTCCCCAGGCCACACCTGG**
**GAGGAGGCCCCCCTGCTGACTCTCAAGCAGAAGCAGGAATGGATCTGCCTGGAGACG**
**CTCACCCCAGACACCCAGTATGAGTTTCAGGTGCGGGTCAAGCCTCTGCAAGGCGAG**
**TTCACGACCTGGAGCCCCTGGAGCCAGCCCCTGGCCTTCAGGACAAAGCCTGCAGCC**
**CTTGGGAAGGACACCATTCCGTGGCTCGGCCACCTCCTCGTGGGTCTCAGCGGGGCT**
**TTTGGCTTCATCATCTTAGTGTACTTGCTGATCAACTGCAGGAACACCGGGCCATGG**
**CTGAAGAAGGTCCTGAAGTGTAACACCCCAGACCCCTCGAAGTTCTTTTCCCAGCTG**

AGCTCAGAGCATGGAGGAGACGTCCAGAAGTGGCTCTCTTCGCCCTTCCCCTCATCG
TCCTTCAGCCCTGGCGGCCTGGCACCTGAGATCTCGCCACTAGAAGTGCTGGAGAGG
GACAAGGTGACGCAGCTGCTCCTGCAGCAGGACAAGGTGCCTGAGCCCGCATCCTTA
AGCAGCAACCACTCGCTGACCAGCTGCTTCACCAACCAGGGTTACTTCTTCTTCCAC
CTCCCGGATGCCTTGGAGATAGAGGCCTGCCAGGTGTACTTTACTTACGACCCCTAC
TCAGAGGAAGACCCTGATGAGGGTGTGGCCGGGGCACCCACAGGGTCTTCCCCCCAA
CCCCTGCAGCCTCTGTCAGGGGAGGACGACGCCTACTGCACCTTCCCCTCCAGGGAT
GACCTGCTGCTCTTCTCCCCCAGTCTCCTCGGTGGCCCCAGCCCCCCAAGCACTGCC
CCTGGGGGCAGTGGGGCCGGTGAAGAGAGGATGCCCCCTTCTTTGCAAGAAAGAGTC
CCCAGAGACTGGGACCCCCAGCCCCTGGGGCCTCCCACCCCAGGAGTCCCAGACCTG
GTGGATTTTCAGCCACCCCCTGAGCTGGTGCTGCGAGAGGCTGGGGAGGAGGTCCCT
GACGCTGGCCCCAGGGAGGGAGTCAGTTTCCCCTGGTCCAGGCCTCCTGGGCAGGGG
GAGTTCAGGGCCCTTAATGCTCGCCTGCCCCTGAACACTGATGCCTACTTGTCCCTC
CAAGAACTCCAGGGTCAGGACCCAACTCACTTGGTGAGCCTGAAGACCCATCCATTG
TGGAGGCTATGGAAGAAGATATGGGCCGTCCCCAGCCCTGAGCGGTTCTTCATGCCC
CTGTACAAGGGCTGCAGCGGAGACTTCAAGAAATGGGTGGGTGCACCCTTCACTGGC
TCCAGCCTGGAGCTGGGACCCTGGAGCCCAGAGGTGCCCTCCACCCTGGAGGTGTAC
AGCTGCCACCCACCACGGAGCCCGGCCAAGAGGCTGCAGCTCACGGAGCTACAAGAA
CCAGCAGAGCTGGTGGAGTCTGACGGTGTGCCCAAGCCCAGCTTCTGGCCGACAGCC
CAGAACTCGGGGGGCTCAGCTTACAGTGAGGAGAGGGATCGGCCATACGGCCTGGTG
TCCATTGACACAGTGACTGTGCTAGATGCAGAGGGGCCATGCACCTGGCCCTGCAGC
TGTGAGGATGACGGCTACCCAGCCCTGGACCTGGATGCTGGCCTGGAGCCCAGCCCA
GGCCTAGAGGACCCACTCTTGGATGCAGGGACCACAGTCCTGTCCTGTGGCTGTGTC
TCAGCTGGCAGCCCTGGGCTAGGAGGGCCCCTGGGAAGCCTCCTGGACAGACTAAAG
CCACCCCTTGCAGATGGGGAGGACTGGGCTGGGGGACTGCCCTGGGGTGGCCGGTCA
CCTGGAGGGGTCTCAGAGAGTGAGGCGGGCTCACCCCTGGCCGGCCTGGATATGGAC
ACGTTTGACAGTGGCTTTGTGGGCTCTGACTGCAGCAGCCCTGTGGAGTGTGACTTC
ACCAGCCCCGGGGACGAAGGACCCCCCCGGAGCTACCTCCGCCAGTGGGTGGTCATT
CCTCCGCCACTTTCGAGCCCTGGACCCCAGGCCAGCTAA (SEQ ID NO:32)

Protein Sequence of IL2-IL2Rp-IL2 1R (CIRB2 1)

MYRMQLLSCIALSLALVTNSAPTS SSTKKTQLQLEHLLLDLQMILNGINNYKNPKLT
RMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI SNINVIVL
ELKGSETT FMCEYADETATIVE FLNRWITFCQSIISTLTEQKL ISEEDLEMETSQ FP
GEEKPQAS PEGRPESET SCAVNGTSQFTC FYNSRANI SCVWSQDGALQDT SCQVHAW
PDRRRWNQTCELLPVSQASWACNL ILGAPDSQKLTTVDIVTLRVLCREGVRWRVMAI
QDFKPFENLRLMAPI SLQVVHVETHRCNI SWEISQASHY FERHLE FEARTLS PGHTW
EEAPLLTLKQKQEWICLETLTPDTQYE FQVRVKPLQGE FTTWS PWSQPLAFRTKPAA
LGKDTI PWLGHLLVGLSGAFGFI ILVYLL INCRNTGPWLKKVLKCNT PDPSKFFSQL
SSEHGGDVQKWLSSP FPSSS FSPGGLAPE I S PLEVLERDKVTQLLLQQDKVPEPASL
SSNHSLTSCFTNQGY FFFHLPDALE I EACQVY FTYDPYSEEDPDEGVAGAPTGSS PQ
PLQPLSGEDDAYCT FPSRDDLLLFSPSLLGGPSPPSTAPGGSGAGEERMPPSLQERV
PRDWDPQPLGPPT PGVPDLVDFQPPPELVLREAGEEVPDAGPREGVS FPWSRPPGQG
EFRALNARLPLNTDAYLSLQELQGQDPTHLVSLKTHPLWRLWKKIWAVPS PERFFMP
LYKGCSGDFKKWVGAPFTGS SLELGPWSPEVPSTLEVYSCHPPRS PAKRLQLTELQE
PAELVE SDGVPKPS FWPTAQNSGGSAYSEERDRPYGLVS IDTVTVLDAEGPCTWPCS
CEDDGY PALDLDAGLEPSPGLEDPLLDAGTTVLSCGCVSAGSPGLGGPLGSLLDRLK
PPLADGEDWAGGLPWGGRSPGGVSESEAGSPLAGLDMDT FDSGFVGSDCS SPVECDF
TSPGDEGPPRSYLRQWVVI PPPLS SPGPQAS * (SEQ ID NO:33)

Figure 14 shows that the new hybrid receptor confers substantial cytotoxicity against PC-3 cancer cells that is 5 fold better than the original chimera CIRB and also produces CD 16. They also had slower growth, which could be enhanced by exogenous IL2 addition to the media.

While IL2 has been shown to mainly activate STAT5 (5 1), IL2 1 preferentially activates STAT3 (52,53) and STATl (54). It has been shown that this activation leads to Interferon-gamma (IFN-g) production (55) and this might explain the dramatic enhancement in cancer cell killing we saw (Figure 14). RNA expression analysis by qPCR revealed the remarkable extent of activation driven by the hybrid receptor CIRB2 1 compared to CIRB. Figure 15 shows that IFN-g, Granzyme-B and Perforin- 1 were increased in CIRB2 1 by 5, 6 and 3 fold, respectively, above the levels in CIRB and NK92. These data confirm that this novel platform of IL2 signaling through hybrid receptors holds a therapeutic promise for superior NK cells activation.

However, IL-2 1 signaling also induces the transcription of many other genes (reviewed in (56)) including suppressor of cytokine signaling 1 (SOCS 1) and SOCS3 proteins, which down regulate the JAK-STAT pathway and inhibit signaling by IL2 (57,58). This inhibition could be behind the slower growth of NK92 expressing the chimera CIRB2 1.

It was first hypothesized that in the chimera CIRB2 1, the activation of STAT3 mediated by IL2 1R may be in conflict with the activation of STAT5 mediated by IL2Rβ, resulting in slower NK92CIRB2 1 cell growth. STAT3 is a major byproduct of IL2 1R signaling and its transcriptional activity could be behind the down regulation of JAK-STAT signaling mediated by IL2 in our cell line. Among STAT3 potent and selective inhibitors, 5, 15-Diphenylporphyrin (5, 15-DPP) acts in the nanomolar range and prevents STAT3 nuclear translocation. However, when we inhibited the dimerization of STAT3 using specific with 5, 15-DPP, we did not improve NK92CIRB2 1 cell growth. This result suggests either the absence of STAT3 homodimers and the possible heterodimerization of STATl and STAT3 or a predominance of STATl homodimers. STATl and STAT3 have usually opposing biological effects. While STAT3 is an oncogene (59,60), STATl acts as a tumor suppressor (61,62). STATl phosphorylation can be mediated by IFNg (63), which is highly produced in NK92CIRB2 1. Therefore, it is possible that the slower growth of NK92CIRB2 1 cells is caused by STATl tumor suppressor activity.

Three methodologies are used to restore faster growth of NK92CIRB21 without affecting their current dramatic cytotoxicity. First, it is possible that the current configuration of CIRB2 1 is sterically unfavorable to IL2Rβ due to lack of a spacer between the two cytoplasmic domains of IL2Rβ and IL2 1R in the exemplary construct. Therefore, the CIRB2 1 chimera is modified to add a flexible linker, e.g., a (GGGS)n linker, between IL2Rβ and IL2 1R. This is done using high fidelity PCR using the method of overlapping extension. The impact of the linker is examined by stable expression of the resulting chimera in NK92 cells. Second, if the addition of a linker does not restore NK92 cells growth then the Socs 1 motif, which down regulates the JAK-STAT pathway and inhibits signaling by IL2 (57,58), is removed from the receptor IL2Rβ (Figure 16). This is achieved by site mutagenic high fidelity PCR using primers sense 5'-GCAGCAACCACTCGCTGACCGCCTGCCAGGTGTACTTTAC-3 ' (SEQ ID NO:34) and reverse 5'-GTAAAGTACACCTGGCAGGCGGTCAGCGAGTGG TTGCTGC-3 ' (SEQ ID NO:35). We also weaken the signal transduction of IL2 1R by deleting Box-2 region of IL2 1R cytoplasmic domain (Figure 17). Box-1, and to a lesser degree Box-2, are both involved in the signal transduction of IL2 1 ((64)). Separately, we also remove Box-2 along with the region comprised between tyrosine Y3 17 and Y399, which was shown to contribute marginally to the overall strength of the IL2 1 signaling (53). Third, if these genetic modifications do not restore NK92 cells growth, then the DNA shuffling of the cytoplasmic domains of IL2Rβ and IL2 1R may yield a novel chimera which enables faster growth of NK cells.

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## OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. For example, although human cells and sequences are exemplified herein, e.g., for use in treating human subjects, sequences and NK cells from other species can also be used. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A fusion protein comprising interleukin 2 (IL2) fused to the N-terminus of interleukin 2 receptor beta (IL2Rβ), with an intervening linker.

2. The fusion protein of claim 1, wherein:
the IL2 comprises SEQ ID NO:34, and/or
the IL2Rβ comprises amino acids 27-55 1 of SEQ ID NO:35.

3. The fusion protein of claim 1, wherein the intervening linker between IL2 and the N-terminus of IL2Rβ comprises an extracellular domain of IL2Ra.

4. The fusion protein of claim 3, wherein the extracellular domain of IL2Ra comprises SEQ ID NO:28.

5. The fusion protein of claim 1, further comprising a cytoplasmic domain of IL21R at the C-terminus of IL2Rβ, optionally with an intervening linker therebetween.

6. The fusion protein of claim 5, wherein the cytoplasmic domain of IL2 1R comprises amino acids 254-538 of SEQ ID NO:36.

7. The fusion protein of claim 1, further comprising an activation domain of CD28 at the C-terminus of the IL2Rβ portion, optionally with an intervening linker therebetween.

8. The fusion protein of claim 7, wherein the activation domain of CD28 comprises amino acids 180 to 220 of SEQ ID NO:38.

9. A nucleic acid encoding the fusion protein of claims 1-8.

10. An expression vector comprising the nucleic acid of claim 9, with one or more regulatory regions for expression of a fusion protein of claims 1-8.

11. An isolated natural killer (NK) cell expressing a fusion protein of claims 1-8, preferably wherein the NK cell also expresses CD16 and optionally NKP44, NKP46 and NKP30.

12. A method of treating a subject, preferably a human subject, who has cancer, the method comprising administering a therapeutically effective amount of natural killer (NK) cells expressing a fusion protein of claims 1-8 to a subject in need thereof.

13. The method of claim 12, wherein the subject has a solid tumor.

14. The method of claim 12, further comprising administering one or more of an anti-tumor monoclonal antibody or a checkpoint inhibitor.

15. The method of claim 12, wherein the NK cells are administered intravenously.

16. The method of claim 12, wherein the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

17. A method of treating a subject who has GVHD or an autoimmune disease, the method comprising administering a therapeutically effective amount of regulatory T (T-reg) cells expressing a fusion protein of claims 7 or 8 to a subject in need thereof.

18. The method of claim 17, wherein the T-reg cells are administered intravenously.

19. The method of claim 17, wherein the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

20. Natural killer (NK) cells expressing a fusion protein of claims 1-8 for use in treating a subject, preferably a human subject, who has cancer.

21. The NK cells for the use of claim 20, wherein the subject has a solid tumor.

22. The NK cells for the use of claim 20, for use in a method that includes administering one or more of an anti-tumor monoclonal antibody or a checkpoint inhibitor.

23. The NK cells for the use of claim 20, wherein the NK cells are formulated to be administered intravenously.

24. The NK cells for the use of claim 20, wherein the NK cells are subjected to 500 to 1000 cGy of gamma irradiation prior to being administered.

25. Regulatory T (T-reg) cells expressing a fusion protein of claims 7 or 8, for use in treating a subject who has GVHD or an autoimmune disease.

26. The T-reg cells for the use of claim 25, wherein the T-reg cells are formulated to be administered intravenously.

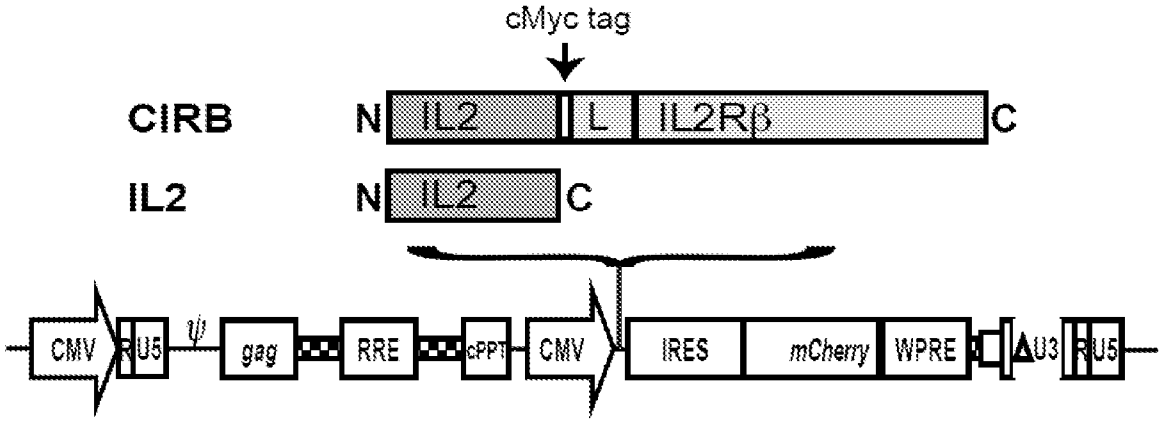


FIG. 1

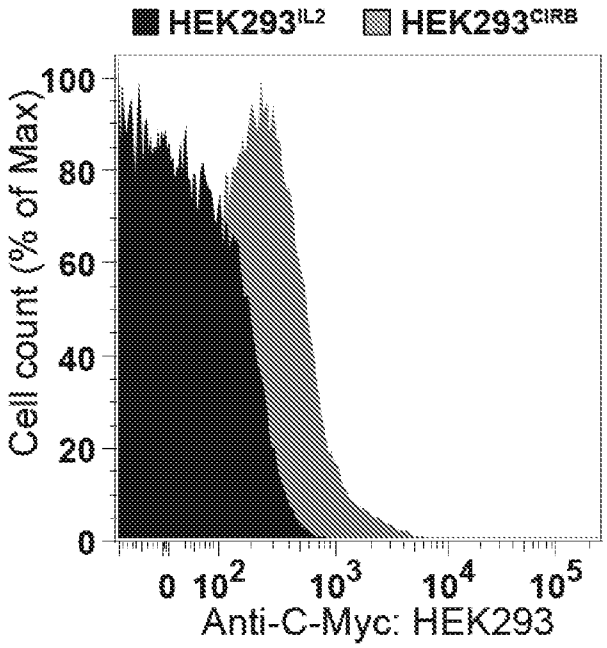


FIG. 2A

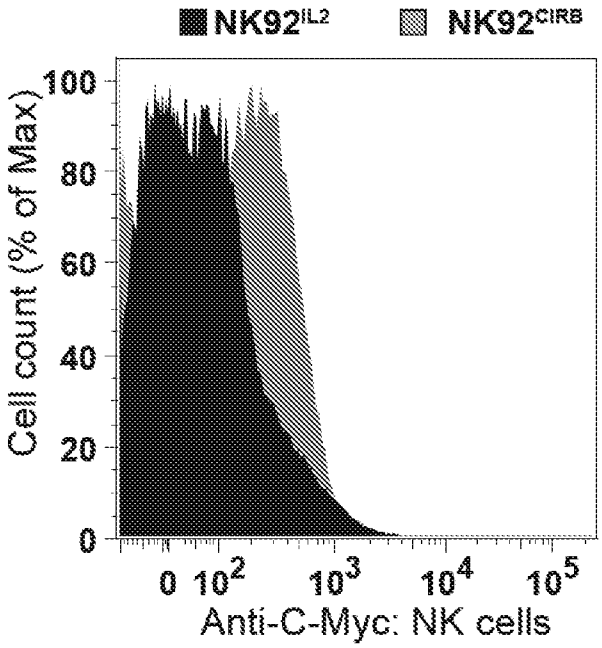


FIG. 2B

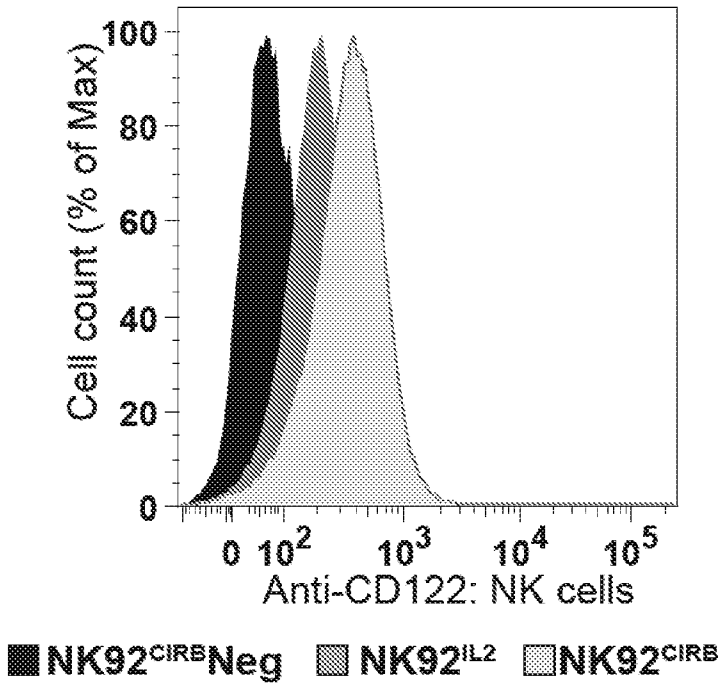
Cell count (% of Max)
100
80
60
40
20
0
0 10^2 10^3 10^4 10^5
Anti-CD122: NK cells
NK92CIRBNeg
NK92IL2
NK92CIRB

FIG. 2C


Kda
170
130
95
NK92CIRB
NK92IL2
NK92
MW

FIG. 2D

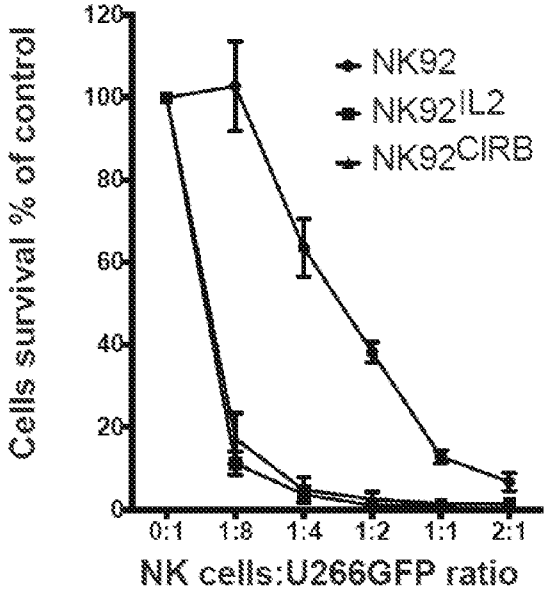
Cells survival % of control
120
100
80
60
40
20
0
NK92
NK92IL2
NK92CIRB
0:1
1:8
1:4
1:2
1:1
2:1
NK cells:U266GFP ratio

*FIG. 3A*

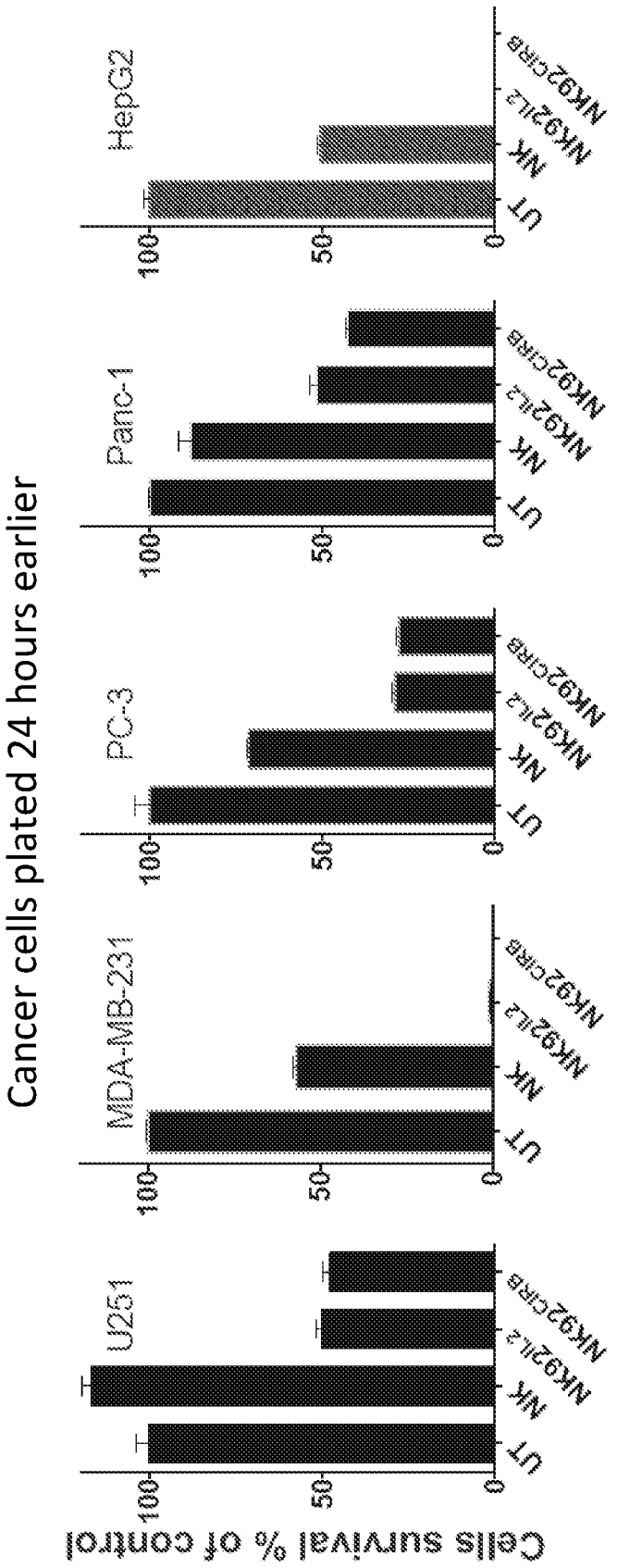
Cancer cells plated 24 hours earlier
Cells survival % of control
U251
MDA-MB-231
PC-3
Panc-1
HepG2
UT
NK
NK92IL2
NK92CIRB
0
50
100

FIG. 3B

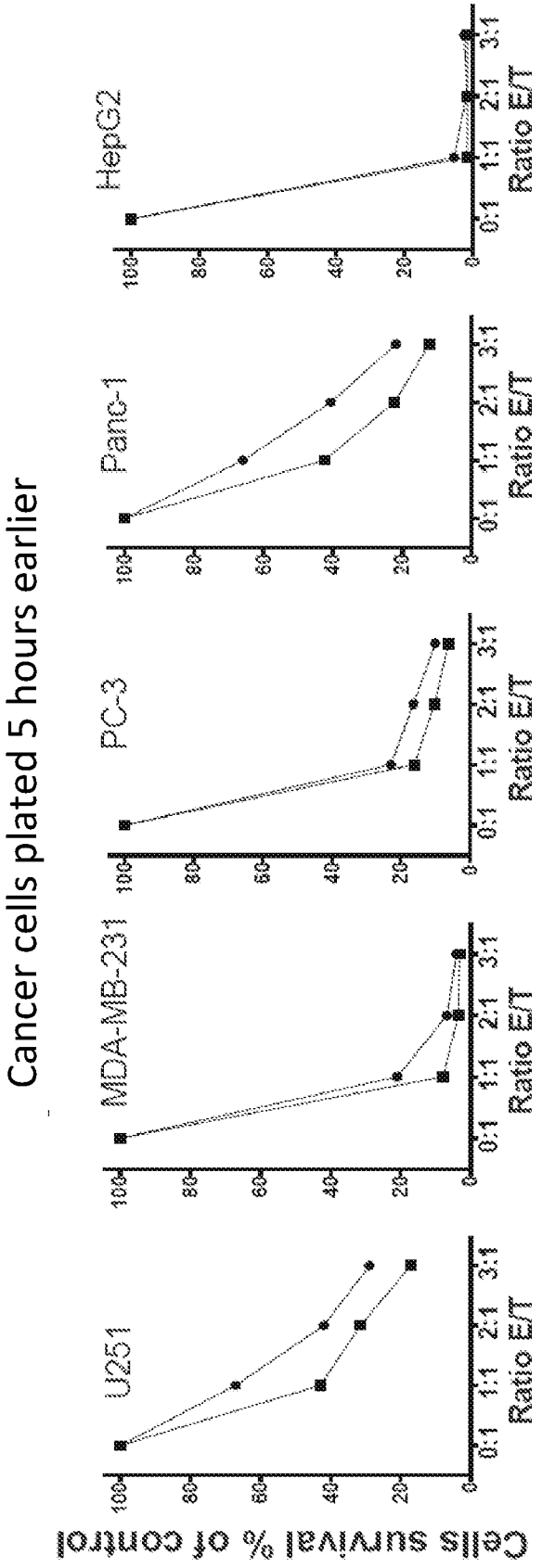
Cancer cells plated 5 hours earlier
Cells survival % of control
U251
MDA-MB-231
PC-3
Panc-1
HepG2
Ratio E/T
0:1
1:1
2:1
3:1
0
20
40
60
80
100
NK92IL2
NK92CIRB

FIG. 3C

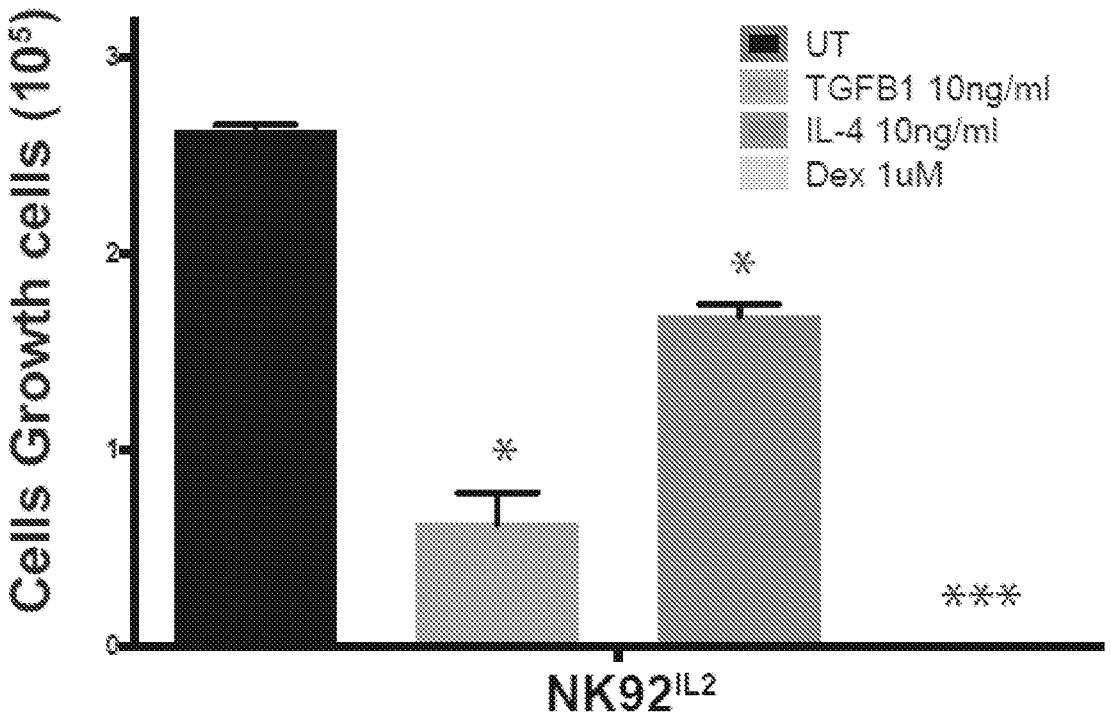
Cells Growth cells ($10^5$)
UT
TGFB1 10ng/ml
IL-4 10ng/ml
Dex 1uM
*
*
***
NK92IL2

FIG. 4A

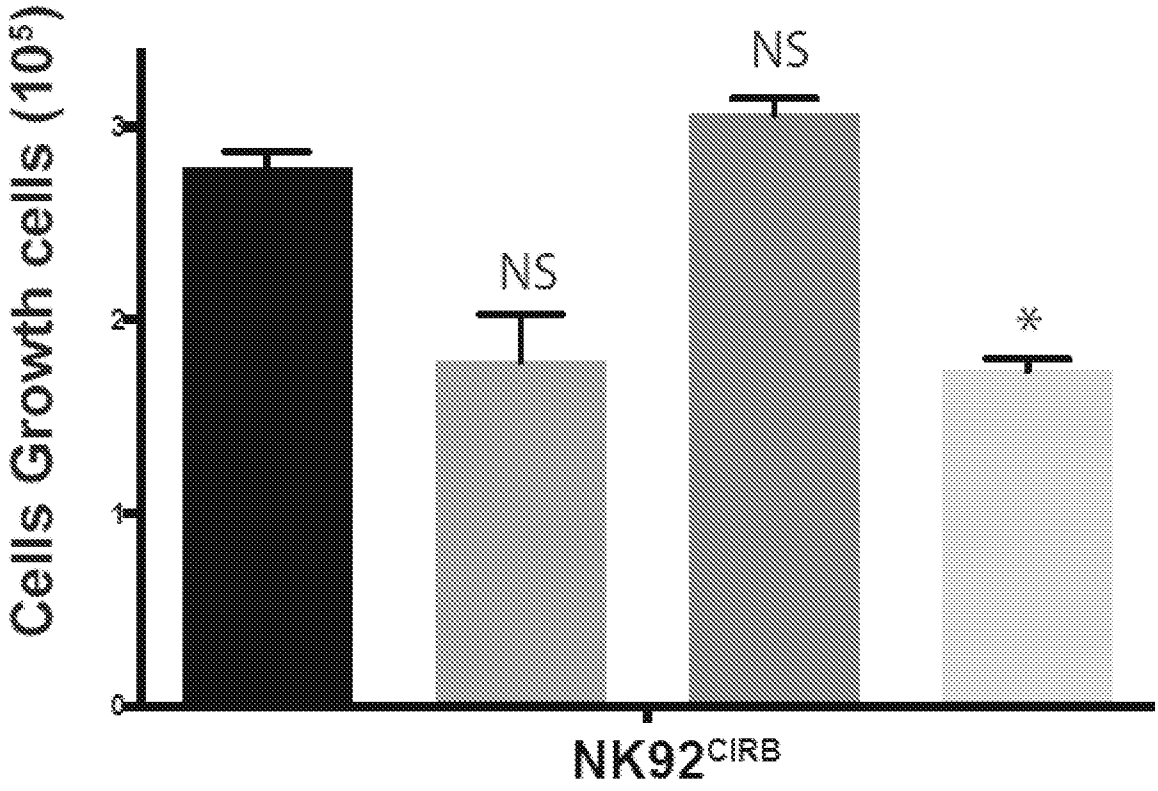
Cells Growth cells ($10^5$)
NS
NS
*
NK92CIRB

FIG. 4B

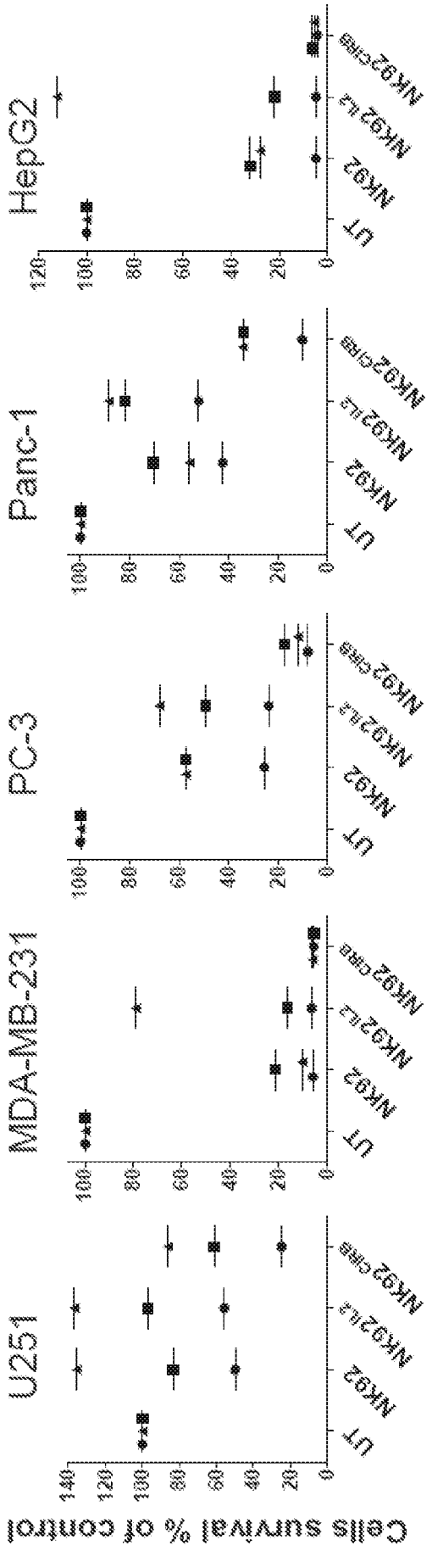
Cells survival % of control
U251
MDA-MB-231
PC-3
Panc-1
HepG2
UT
NK92
NK92IL2
NK92CIRB
TGFB1 (20ng/ml ■), Dex (0.5uM ▲) no drug (UT●)

FIG. 4C

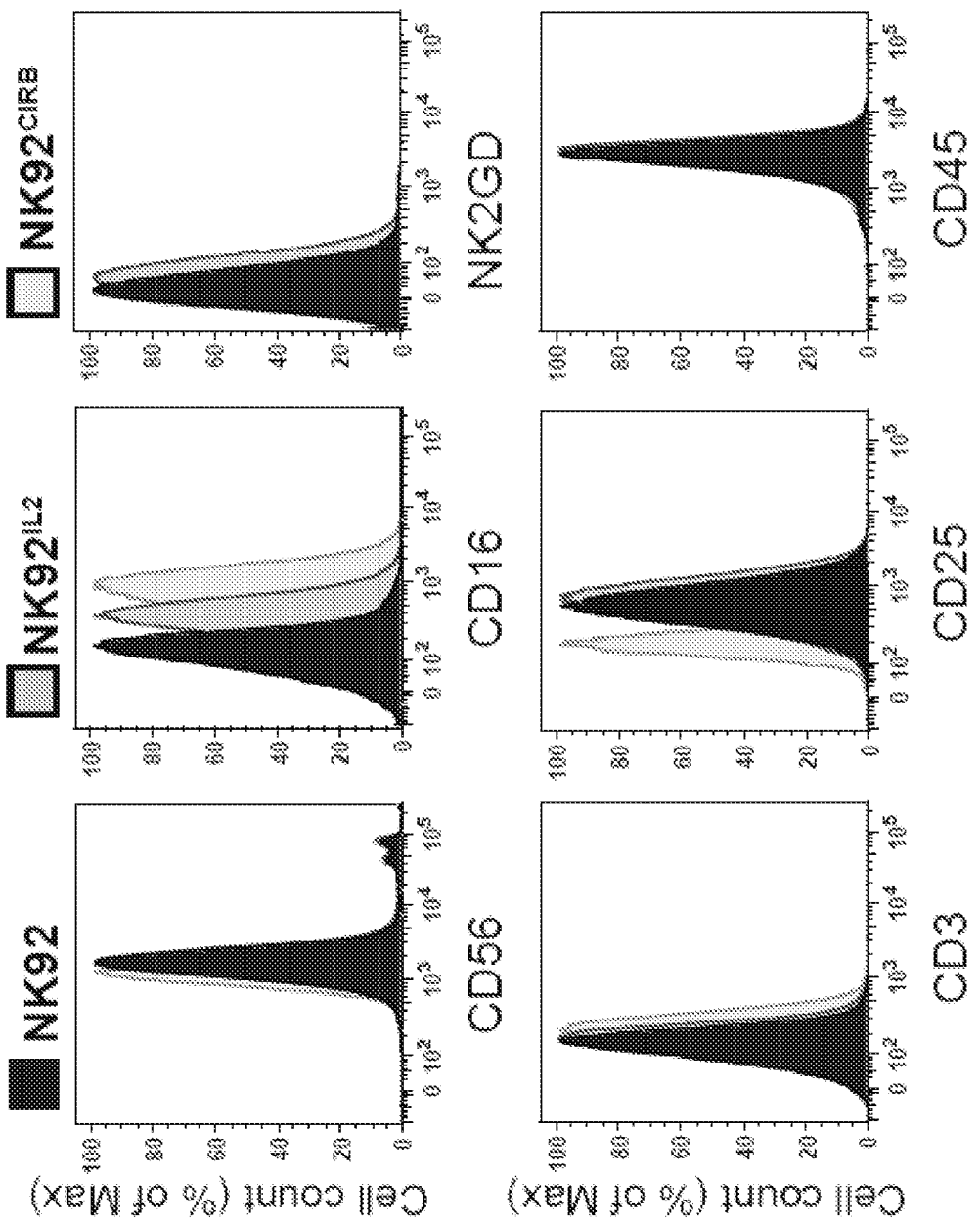
NK92
NK92IL2
NK92CIRB
Cell count (% of Max)
CD56
CD16
NK2GD
CD3
CD25
CD45

FIG. 5A

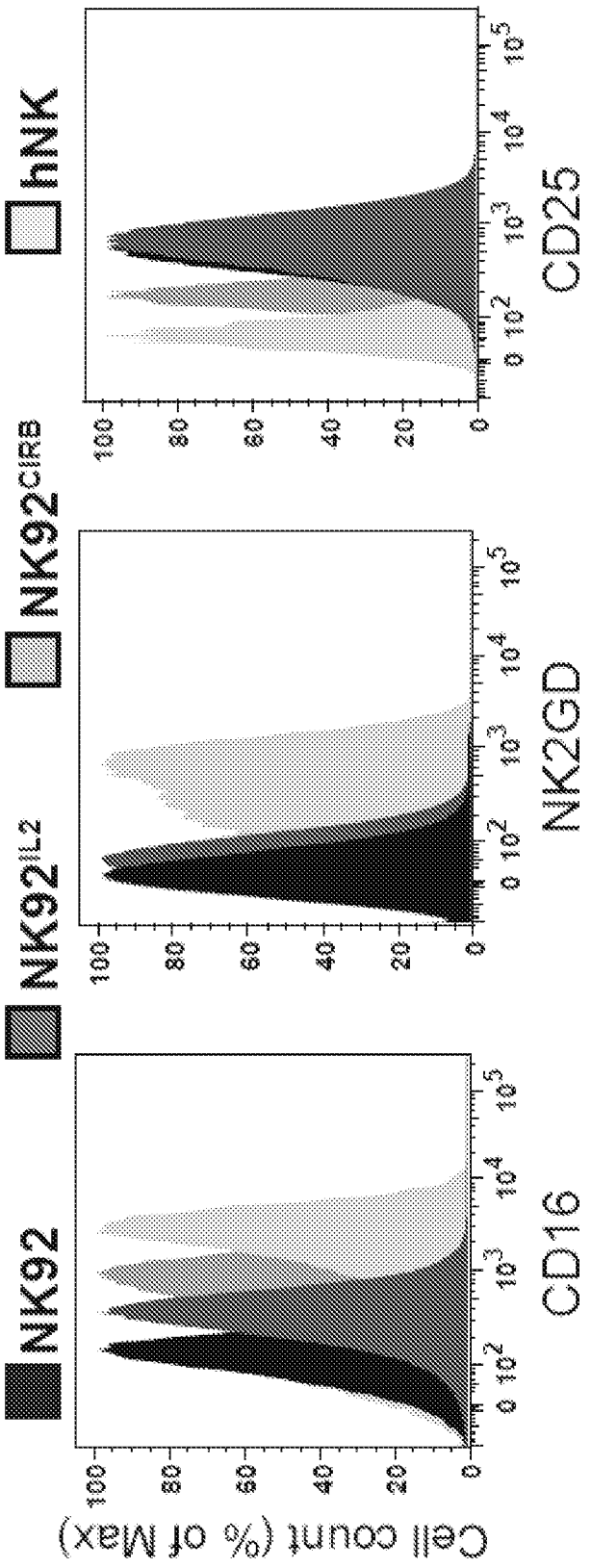


FIG. 5B

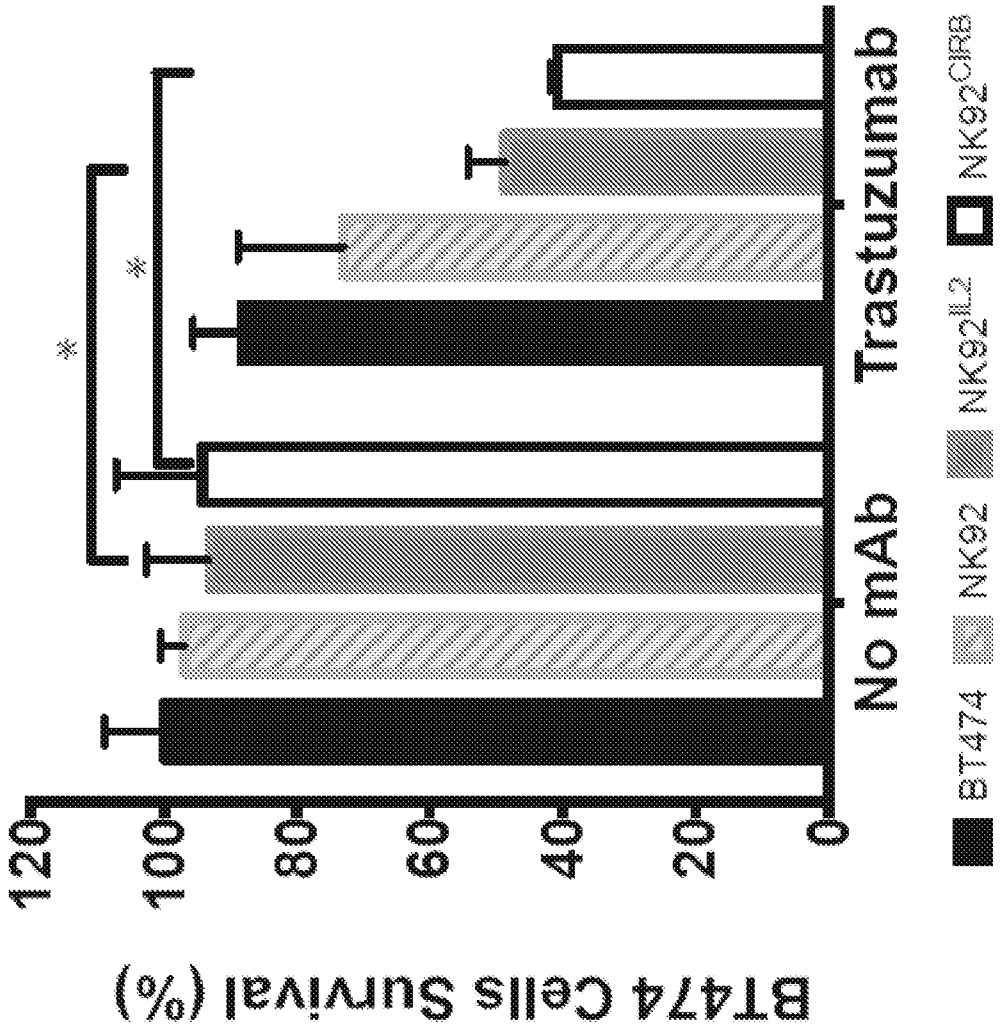
BT474 Cells Survival (%)
120
100
80
60
40
20
0
No mAb
Trastuzumab
BT474
NK92
NK92IL2
NK92CD16
*
*

FIG. 5C

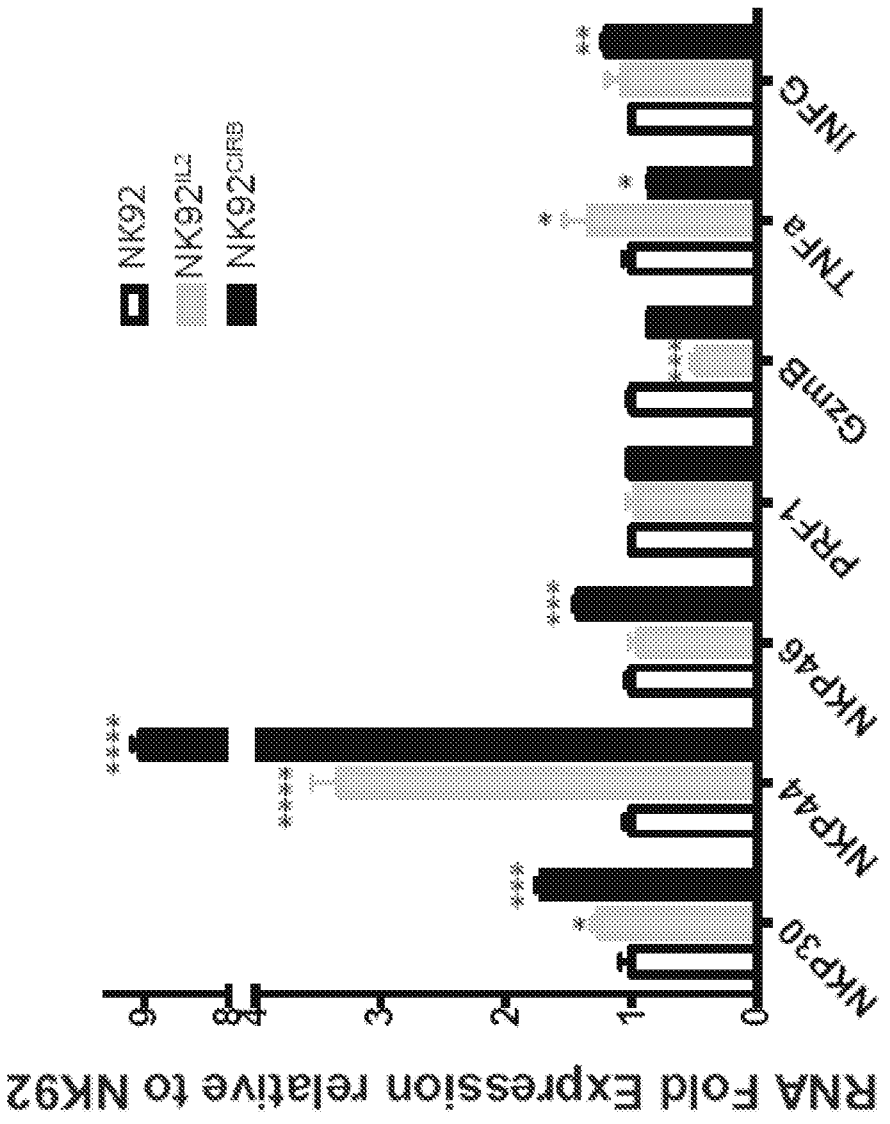
RNA Fold Expression relative to NK92
0
1
2
3
4
5
NK92
NK92IL2
NK92CIRB
NKP30
NKP44
NKP46
PRF1
GzmB
TNFa
INFG

FIG. 5D

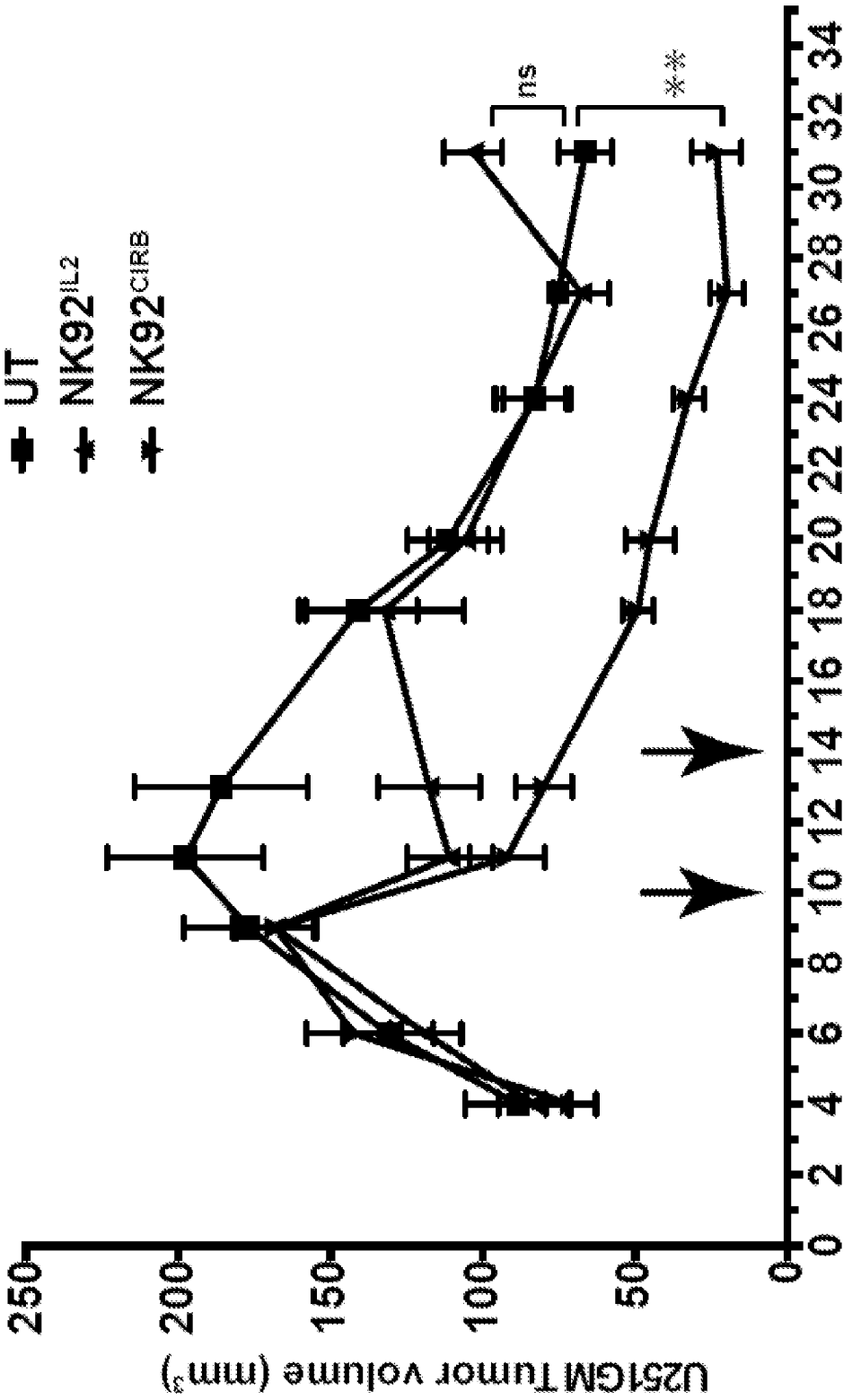
UT
NK92IL2
NK92CIRB
ns
**
U251GM Tumor volume (mm3)
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Days post inoculation

*FIG. 6A*

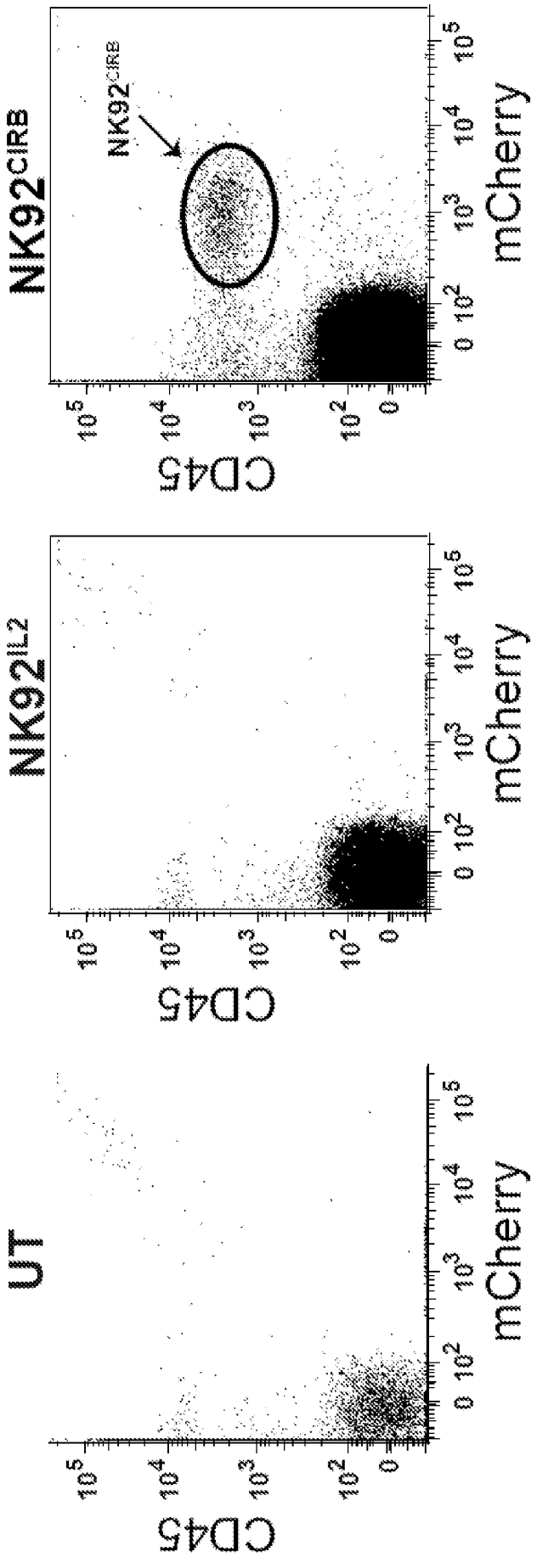


FIG. 6B

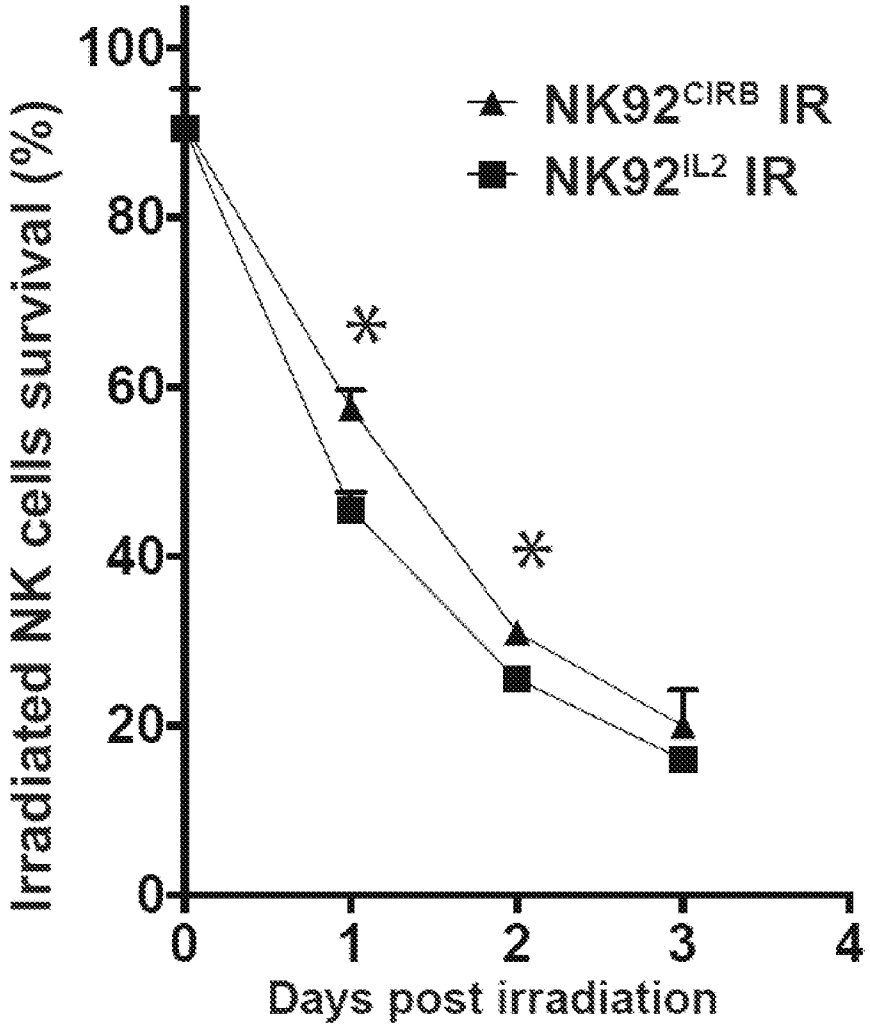
Irradiated NK cells survival (%)
100
80
60
40
20
0
0
1
2
3
4
Days post irradiation
NK92CIRB IR
NK92IL2 IR
*
*

FIG. 7A

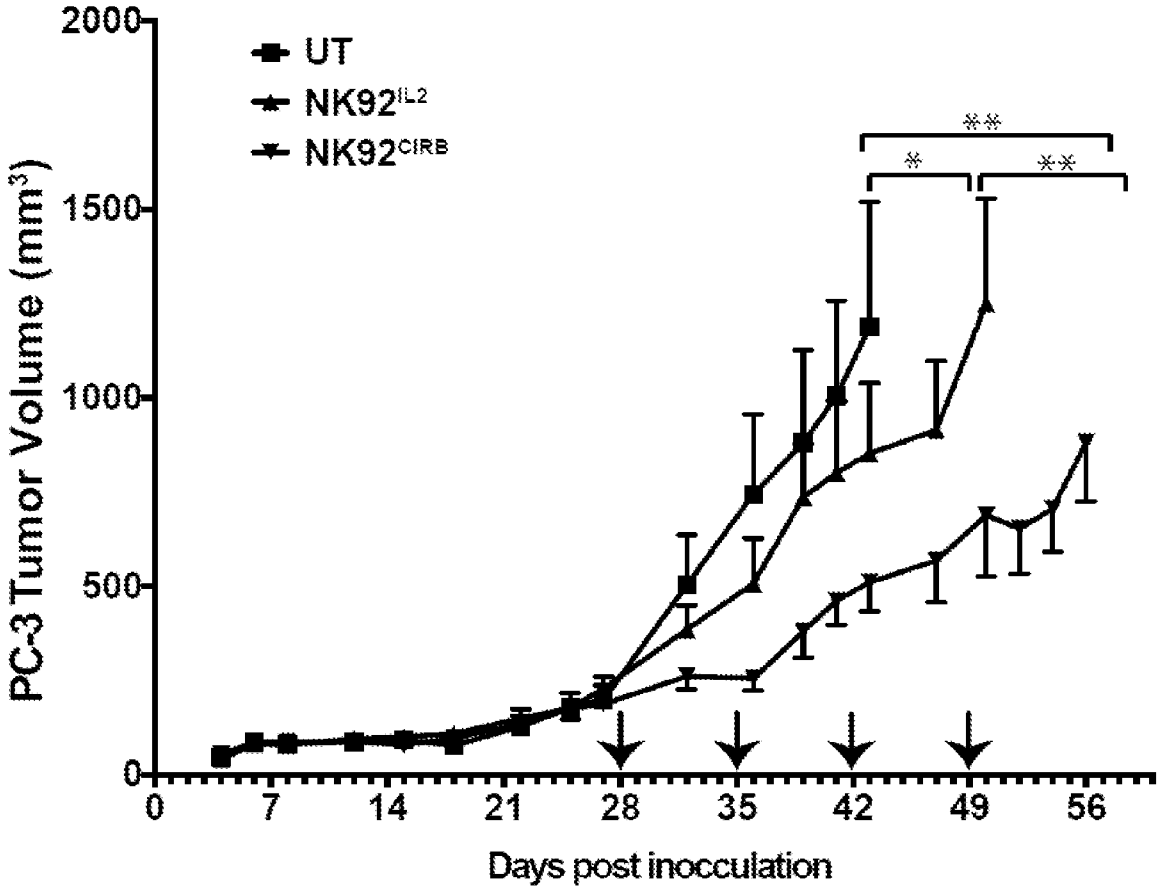
2000
1500
1000
500
0
PC-3 Tumor Volume (mm3)
UT
NK92IL2
NK92CIRB
**
*
**
0
7
14
21
28
35
42
49
56
Days post inocculation

FIG. 7B

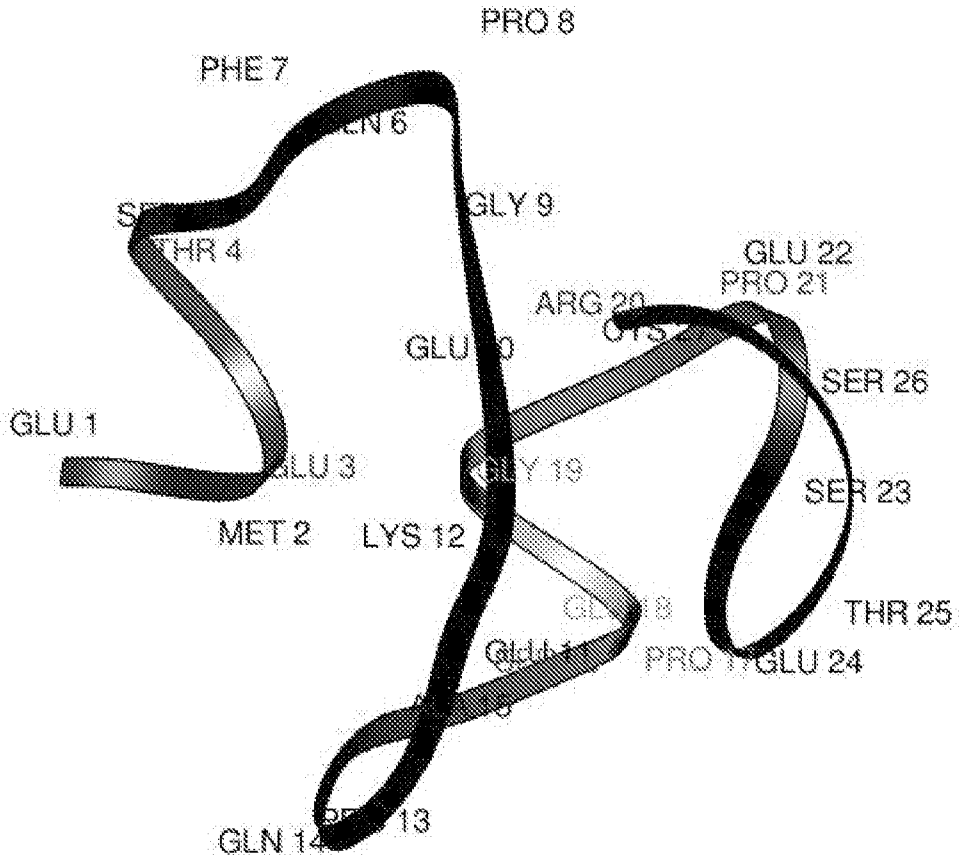
PRO 8
PHE 7
GLY 9
THR 4
GLU 22
PRO 21
SER 26
GLU 1
SER 23
MET 2
LYS 12
THR 25
PRO
GLU 24

*FIG. 8*

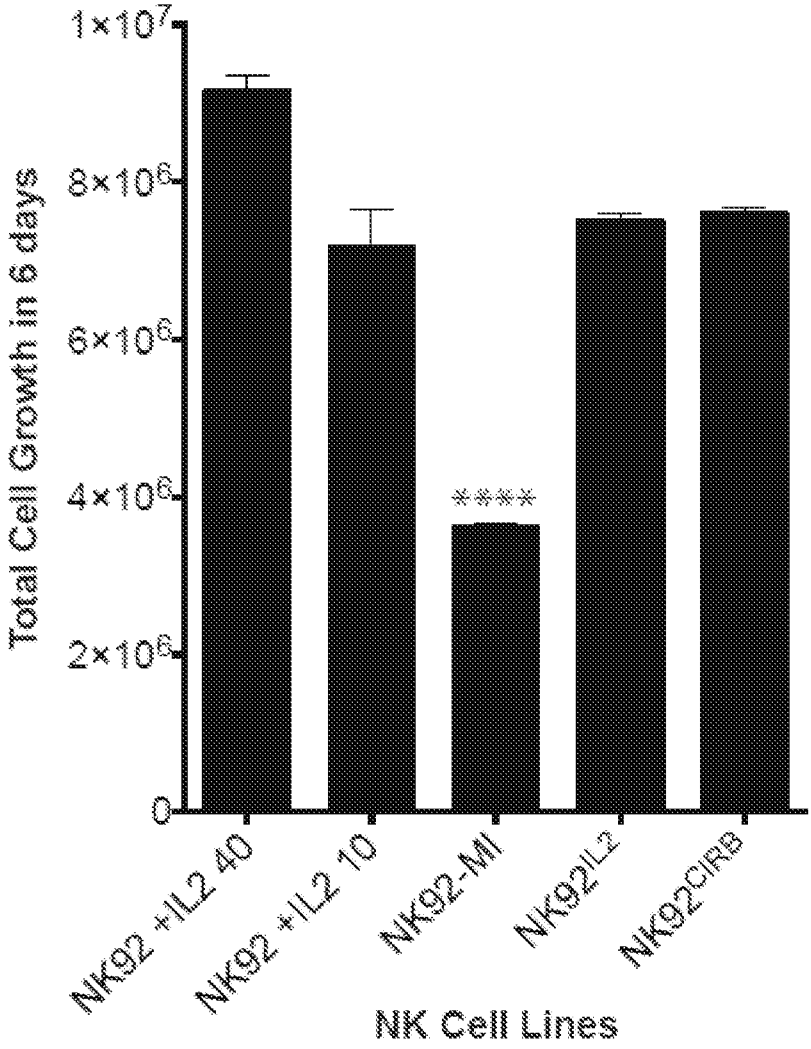
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
Total Cell Growth in 6 days
****
NK92 +IL2 40
NK92 +IL2 10
NK92-MI
NK92^IL2
NK92^CIRB
NK Cell Lines

*FIG. 9*

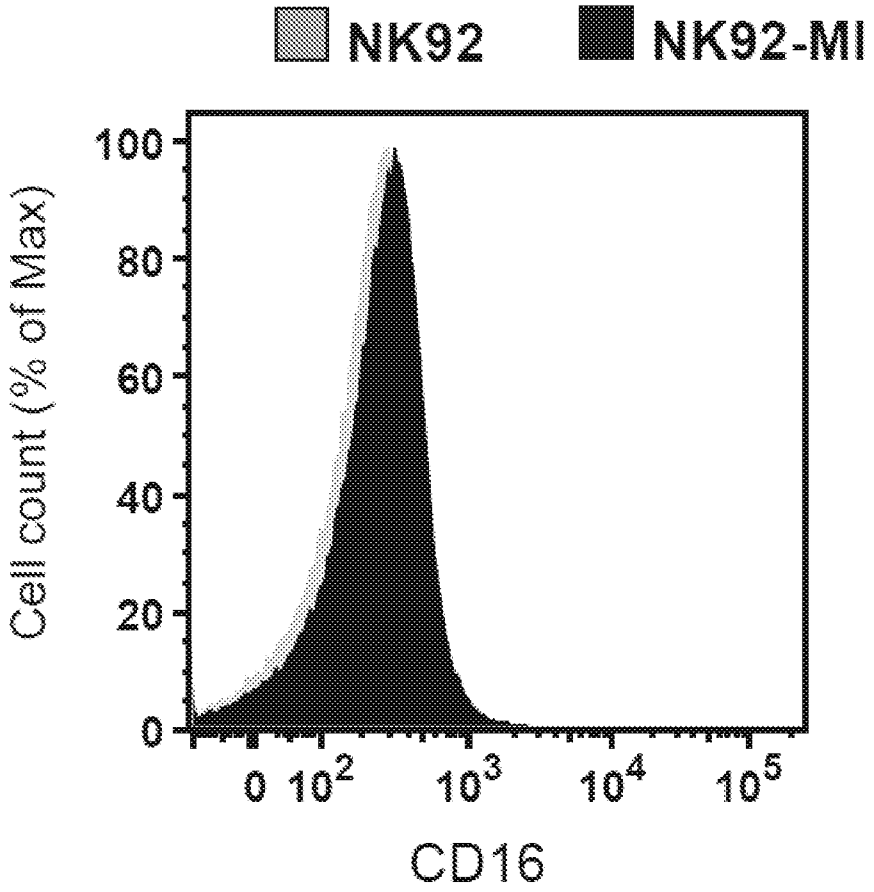


FIG. 10A

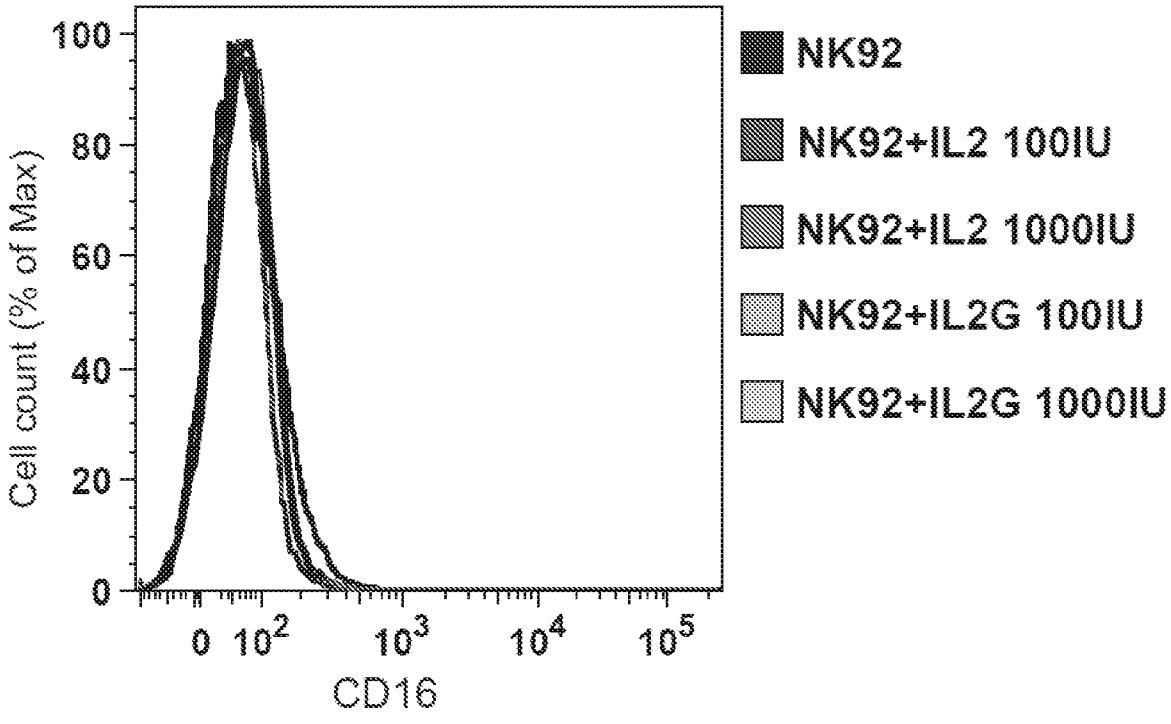


FIG. 10B

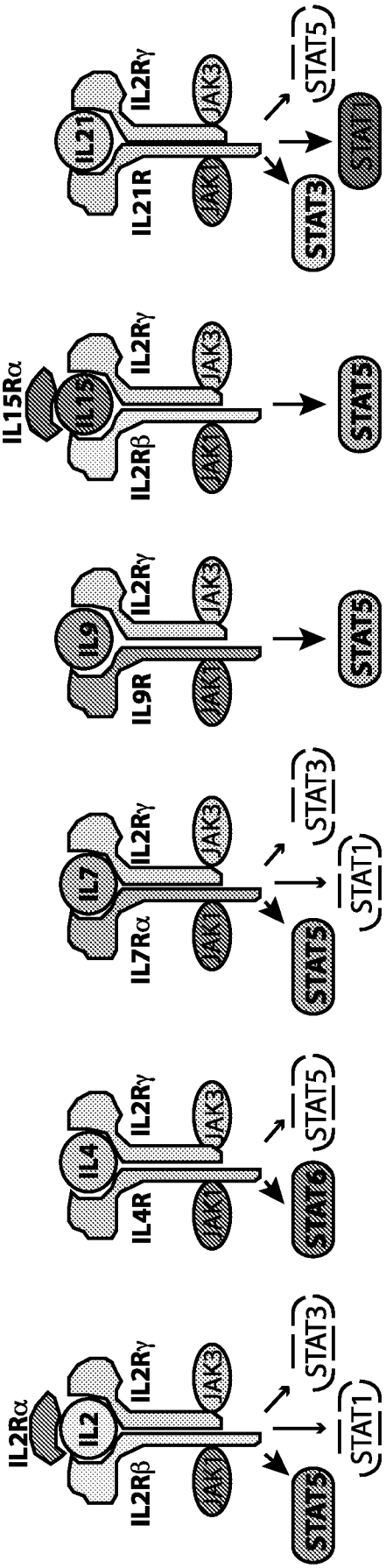
IL2Rα
IL2
IL2Rβ
IL2Rγ
JAK3
JAK1
STAT5
STAT3
STAT1
IL4
IL4R
STAT6
STAT5
IL7
IL7Rα
STAT5
STAT3
STAT1
IL9
IL9R
STAT5
IL15Rα
IL15
IL2Rβ
STAT5
IL21
IL21R
STAT3
STAT1
STAT5

FIG. 11

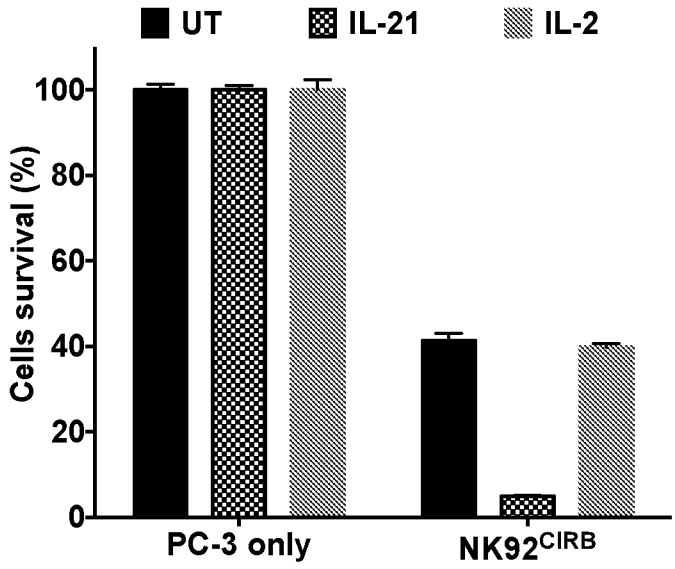
UT
IL-21
IL-2
Cells survival (%)
100
80
60
40
20
0
PC-3 only
NK92CIRB

FIG. 12

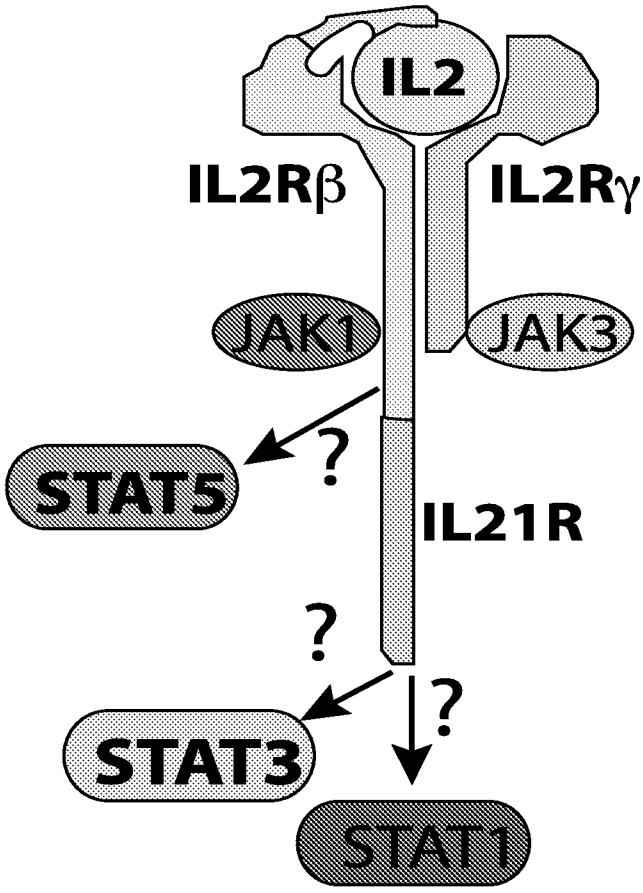
IL2
IL2Rβ
IL2Rγ
JAK1
JAK3
STAT5
?
IL21R
?
?
STAT3
STAT1

FIG. 13

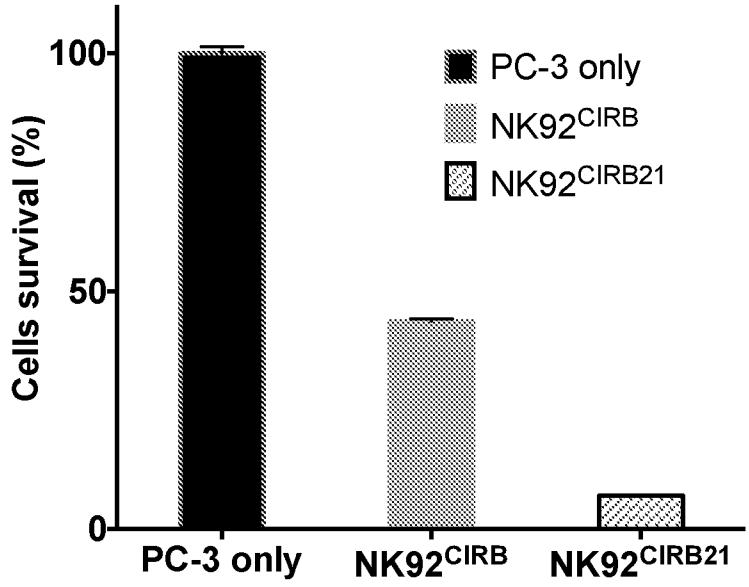
100
50
0
Cells survival (%)
PC-3 only
NK92CIRB
NK92CIRB21
PC-3 only
NK92CIRB
NK92CIRB21

FIG. 14

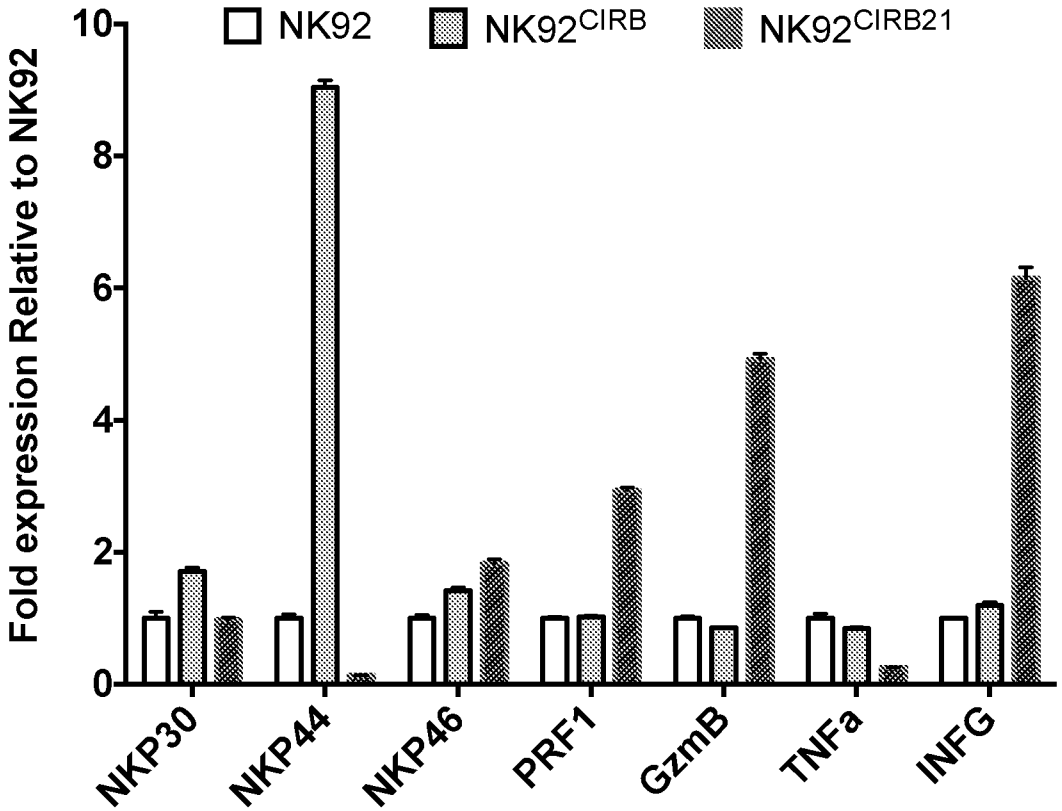
10
8
6
4
2
0
Fold expression Relative to NK92
NK92
NK92CIRB
NK92CIRB21
NKP30
NKP44
NKP46
PRF1
GzmB
TNFa
INFG

FIG. 15

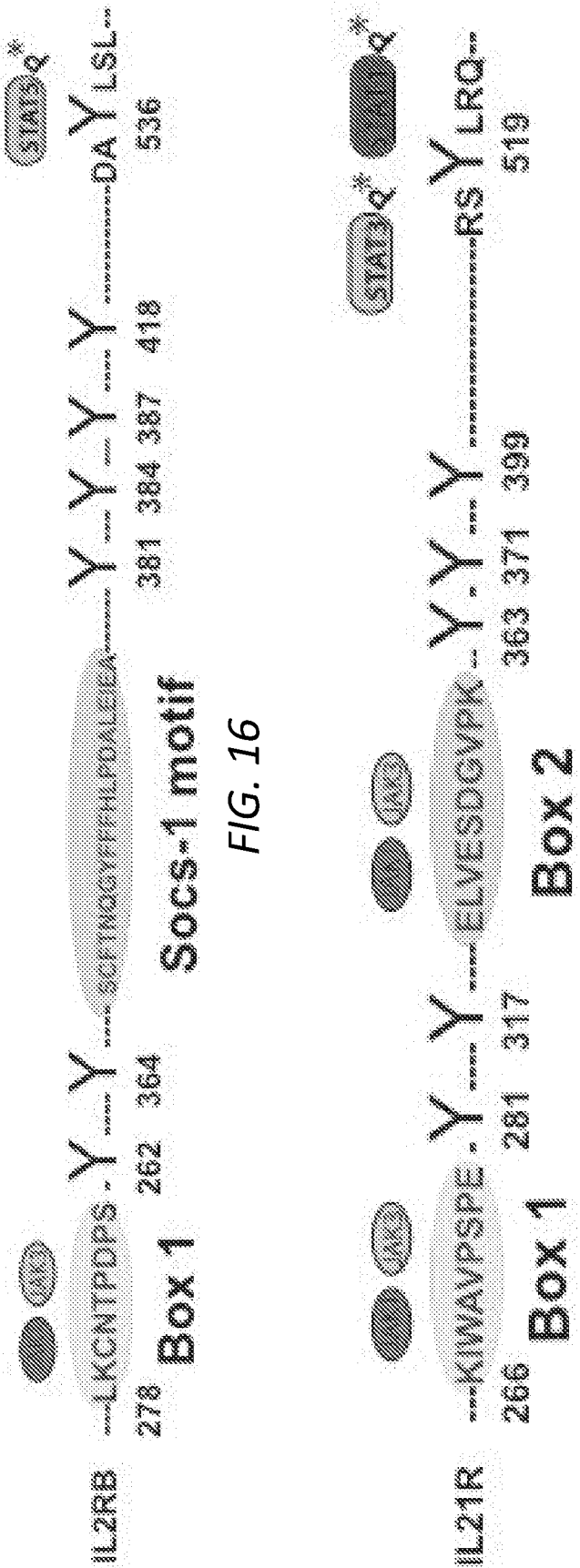


FIG. 16




FIG. 17

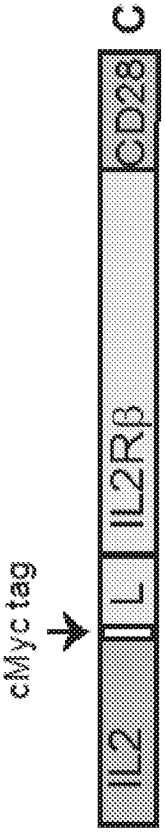


FIG. 18