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(54) Title: CLONOGENIC NATURAL KILLER (NK) CELL POPULATIONS AND METHODS OF PRODUCING AND USING
SUCH POPULATIONS

(57) Abstract: The present invention relates to novel methods for *in vitro* expansion of clonogenic natural killer (NK) cells. The methods utilize the stimulatory effects of trans-presented IL-15 in cell culture using feeder cells to expand isolated NK cell clones with a predetermined phenotypic trait of interest. The resultant clonogenic NK cell populations are homogenous, in contrast to heterogeneous, crude NK cells typically isolated and expanded from a human tissue sample. The present invention also relates to feeder cells that are genetically engineered to trans-present IL-15 involving co-expressing human IL-15 and IL-15 receptor α subunit (IL-15R α) that are capable of stimulating the expansion of NK cell clones *in vitro*. The present invention further relates to viable, functional clonogenic NK cell populations with the predetermined phenotypic trait of interest obtained via the above *in vitro* expansion methods and their use in personalized medicine to treat diseases, including leukemia, lymphoma and human hematopoietic cell transplantation (HCT), in drug screening and in basic and translational studies involving NK cells.



CLONOGENIC NATURAL KILLER (NK) CELL POPULATIONS AND METHODS OF PRODUCING AND USING SUCH POPULATIONS

STATEMENT OF RELATED APPLICATIONS

[0001] This PCT application filed April 3, 2015 claims the priority of U.S.
5 Provisional Application No. 61/974,946 filed April 03, 2014. The contents of this
provisional application are incorporated herein in their entirety for all purposes.

GOVERNMENT SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE DISCLOSURE

Technical Field

[0003] The present disclosure relates, generally, to personalized medicine and
15 the treatment of disease. More specifically, this disclosure concerns the production
and use of clonogenic natural killer (NK) cell populations for the treatment of
diseases, including cancers. Provided are methods for producing clonogenic NK cell
populations, which methods employ the *in vitro* culturing of a clonogenic NK cell in
the presence of a feeder cell expressing IL-15 on its surface. Also provided are
20 methods for the treatment of diseases, including cancers, which method comprise the
administration to a patient a composition comprising a clonogenic NK cell
population.

Description of the Related Art

[0004] Natural killer (NK) cells play a central role in innate immunity by
25 providing a powerful defense against infectious disease, including viral and bacterial
disease, and through their anti-neoplastic functions. Trinchieri *et al.*, *Adv Immunol*
47:187–376 (1989); Raulet, *Nat Immunol* 5:996–1002 (2004); Lanier, *Ann Rev*
Immunol 23:225–74 (2005).

[0005] Autoimmunity associated with immune cells, including NK cells and T and B lymphocytes, is tightly controlled through clonal deletion or anergy, a functionality that is exploited in certain immunotherapies for cancers and autoimmune diseases. Hogquist *et al.*, *Nat. Rev. Immunol.* 5:772-782 (2005) and
5 Wardemann *et al.*, *Science* 301:1374-1377 (2003). Unlike T and B cells, however, NK cells develop tolerance to normal self-tissues through a mechanism involving a “missing self-MHC-class I.” Karre *et al.*, *Nature* 319:675-678 (1986) and Ljunggren and Karre, *Immunol. Today* 11:237-244 (1990).

[0006] Both T and NK cells exert clinically-relevant cytotoxicity against cancer
10 cells. T cells recognize tumor associated peptide antigens in the context of cell surface expressed HLA class I or II molecules, whereas NK cell-mediated immune responses become non-specific with loss of HLA class I “self” recognition. Schroers *et al.*, *Exp Hematol* 32:536-546 (2004). While antigen specific donor T cells exhibit graft-versus-tumor activity against HLA matched tumors, donor NK
15 cells can mediate anti-tumor activity against HLA mismatched tumors, including leukemias.

[0007] Natural killer (NK) cells are one of the main components of innate immunity (Trinchieri G. *et al.* *Adv Immunol*, 1989, 47:187–376; Raulet DH. *Nat Immunol*, 2004,5:996–1002; Lanier LL. *Annu Rev Immunol*, 2005, 23:225–74). It is
20 thought that NK cells provide the body with a powerful defense against microorganisms, such as viruses and bacteria, together with their efficient action in limiting neoplastic cell growth (Trinchieri G. *et al.* *Adv Immunol*, 1989) 47:187–376). Different from T cells, which recognize tumor-associated peptide antigens presented by surface HLA class I or class II molecules, NK effector function is not
25 dependent upon MHC-restricted antigen presentation. Rather, NK function is mediated by the overall balance of signals transduced by a complex array of receptors providing inhibitory and activating signaling upon interaction with cognate ligands expressed on target cells.

[0008] Early studies demonstrated that MHC class I antigen expression by
30 normal somatic cells is critical for NK tolerance, as syngeneic MHC class I or B2-microglobulin deficient bone marrow or splenocytes transferred to wild-type mice

are promptly rejected (Karre, K., et al. 1986. Nature 319:675-678; Ljunggren, H. G., and K. Karre. 1990. Immunol. Today 11:237-244). In humans, the first inhibitory NK receptor with ligand specificity for HLA class I, originally named p58, was reported in 1993 (Moretta et al., 1993; Vitale et al., 1995).

5 [0009] HLA class I-specific human inhibitory receptors are type I transmembrane proteins belonging to the Ig superfamily, and are thus designated Killer Immunoglobulin-like Receptors (KIR). Inhibitory KIR may recruit the SH2-domain-containing tyrosine phosphatase 1 protein (SHP1) (Burshtyn et al., 1996, Immunity, 4, 77-85; Burshtyn et al., 1997, J Biol Chem, 272, 13066-72.; Campbell
10 et al., 1996, J Exp Med, 184, 93-100; Fry et al., 1996, J Exp Med, 184, 295-300) through a single or double immunoreceptor tyrosine-based inhibitory motif (ITIM) contained in their long cytoplasmic tail (denoted L, i.e. KIR2DL; KIR3DL). Of several known inhibitory KIR, 2DL1, 2DL2, 2DL3 and 3DL1 are particularly relevant for HLA class I recognition. KIR2DL1 is specific for HLA-C2 group
15 antigens (sharing the Asn⁷⁷/Lys⁸⁰ residues in the HLA-Cw heavy chain); KIR2DL2 and KIR2DL3 are specific for HLA-C1 group antigens (sharing the Ser⁷⁷/Asn⁸⁰ in the HLA-Cw heavy chain) (Bianconi et al., 1995, J Exp Med, 182, 605-9; Winter et al., 1997); and KIR3DL1 is specific for HLA-Bw4 ligands, sharing a group of sequence motifs in residues 77-83 of the heavy chain of certain HLA-B and HLA-A
20 alleles (Gumperz et al., 1995, J Exp Med, 181, 1133-44; Litwin et al., 1994, J Exp Med, 180, 537-43; Wagtmann et al., 1995, Immunity, 2, 439-49).

[0010] NK cells are central players in innate immunity particularly regarding the surveillance against malignant tumors (Vivier E, et al. Nat Immunol, 2008, 9:503-10). The role of NK cells in tumor-cells clearance is proved by haplotype-mismatched allogeneic HCT, where improved engraftment and reduced relapse rates
25 are mediated by donor derived alloreactive NK cells (Ruggeri L, et al. Blood Cells Mol Dis, 2008, 40:84-90). The triggering event of NK cell activation and killing of target cells results from a balance between activating and inhibitory signals sent by membrane receptors that either enhance or block the NK-mediated cytotoxicity
30 (Costello RT, et al. Arch Immunol Ther Exp (Warsz), 1999) 47:83-8). Inhibitory signals arise from interaction between HLA-specific inhibitory receptors, such as the killer immunoglobulin-like receptors (KIR), NK group protein 2A (NKG2A), or

immunoglobulin-like transcript 2 (ILT-2) with HLA class I molecules, whereas the absence or abnormal expression of the later molecules induces NK-cell cytotoxicity (Ljunggren HG, et al. Immunol Today, 1990, 11:237-44).

[0011] Recent studies have emphasized the potential of NK-mediated effects in recipients of allogeneic HCT. In animal models of transplantation, donor NK cells could lyse leukemic cells and host lympho-hematopoietic cells without affecting nonhematopoietic tissues, (Caligiuri M A, et al. Hematology; Am Soc Hematol Educ Program 337-353, 2004). Because NK cells are inhibited by self-HLA molecules which bind to killer immunoglobulin-like receptors (KIR), these findings have led to the clinical practice of selecting hematopoietic stem cell transplant donors with an HLA and KIR type that favors NK -cell activation and thus could be expected to promote an antileukemic effect (Cooper L J, et al. Blood 101:1637-1644, 2003). However, this approach has at least two shortcomings: first selection of the “best” donor is limited to patients who have more than one potential donor and second the capacity of graft NK cells to lyse lymphoid cells is generally low and difficult to predict. (Imai C. et al. Leukemia 18:676-684, 2004; Ito C, et al. Blood 93:315-320, 1999).

[0012] Underpinning the complexity of the role NK cells play in various disease settings is the heterogeneity of NK cell populations found *in vivo* in humans. The functional definition of NK cells, that is their ability of killing other cells without any prior stimulation, implies that different cell populations can have the functional characteristics of NK cells while also possessing diverse phenotypic traits.

[0013] Recent studies have demonstrated that NK cells are heterogeneous populations and have differential functions according to their cell surface activating or inhibitory receptors. Indeed, distinct subsets of NK cell populations have been discovered in humans and in animals. Although NK cells share some common markers, (e.g., the traditional phenotype of human circulating NK cells has been: CD3⁻CD16⁺CD56⁺), several unique and functionally different NK cell populations, based on the expression intensity of NK cell surface receptors, were noted more than 28 years ago (Lanier LL, et al. J Immunol 136: 4480-4486, 1986).

[0014] For example, CD3⁺CD56^{dim} cells, which express high levels of CD16, are more cytotoxic than CD3⁺CD56^{bright} cells, which express low or no levels of CD16 (Lanier LL, et al. J Immunol 136: 4480-4486, 1986). Mounting evidence suggests that the CD56^{bright} subset, which comprises ~10% of circulating NK cells and possesses the capacity to produce abundant cytokines (Cooper MA, et al. Trends Immunol 22: 633-640, 2001), may be of particular relevance in the early events of immune challenge by coordinating “cross-talk” between innate and adaptive arms of immunity (Fehniger TA, et al. Blood 101: 3052-3057, 2003). There is growing evidence that NK cell subsets differ in the makeup of cell surface receptors. In a comprehensive study by Hanna and colleagues (Hanna J, et al. J Immunol 173: 6547-6563, 2004), gene expression profiling of NK subsets revealed several novel functions. In the CD56^{bright} subset, 888 genes were found to be transcribed at significantly lower levels (at least two fold) when compared with CD56^{dim} cells, while 380 genes were up-regulated.

[0015] The heterogeneity of NK cells can be exploited in cell therapy. NK cells are regulated by inhibiting and activating cell surface receptors. Most inhibitory receptors recognize MHC-class I antigens, and protect healthy cells from NK cell-mediated auto-aggression. However, certain activating receptors, including the human killer cell Ig-like receptor (KIR) 2DS1, also recognize MHC-class I. This raises the question of how NK cells expressing such activating receptors are tolerized to host tissues.

[0016] NK cells have promising therapeutic applications in a variety of conditions, especially in cancer immunotherapy and HCT. Recent clinical and basic research has causally linked the expression of NK cell surface receptors to NK cell function. In the last fifteen years, growing knowledge of NK tolerance to self, cancer immuno-surveillance and licensing has been extensively applied to the field of human HCT, in an effort to identify donors protecting from leukemia relapse through NK-based alloreactivity. In the HCT setting, HLA-KIR interactions play a crucial role in mediating clinically relevant NK alloreactivity phenomena.

[0017] Based on *KIR* gene content, multiple KIR haplotypes are identified, and categorized into two distinct groups, A and B. Group A haplotypes contain genes

- exclusively encoding inhibitory receptors and the activating receptor *KIR2DS4*, while group B haplotypes contain genes encoding both inhibitory and activating receptors. Products of functional KIR genes are type I transmembrane receptors with two (KIR2D) or three (KIR3D) highly homologous, extracellular immunoglobulin domains (Colonna et al., 1995, Science, 268, 405-8; D'Andrea et al., 1995, J Immunol, 155, 2306-10; Wagtmann et al., 1995, Immunity, 2, 439-49). Due to their clonal distribution in the NK repertoire, an individual NK cell may express one or more KIR (Moretta et al., 1990, J Exp Med, 172, 1589-98; Valiante et al., 1997, Immunity, 7, 739-51).
- 10 [0018] Inhibitory KIR may recruit the SH2-domain-containing tyrosine phosphatase 1 protein (SHP1) (Burshtyn et al., 1996, Immunity, 4, 77-85; Burshtyn et al., 1997, J Biol Chem, 272, 13066-72.; Campbell et al., 1996, J Exp Med, 184, 93-100; Fry et al., 1996, J Exp Med, 184, 295-300) through a single or double immunoreceptor tyrosine-based inhibitory motif (ITIM) contained in their long cytoplasmic tail (denoted L, i.e. KIR2DL; KIR3DL). Of several known inhibitory KIR, 2DL1, 2DL2, 2DL3 and 3DL1 are particularly relevant for HLA class I recognition. KIR2DL1 is specific for HLA-C2 group antigens (sharing the Asn⁷⁷/Lys⁸⁰ residues in the HLA-Cw heavy chain); KIR2DL2 and KIR2DL3 are specific for HLA-C1 group antigens (sharing the Ser⁷⁷/Asn⁸⁰ in the HLA-Cw heavy chain) (Biassoni et al., 1995, J Exp Med, 182, 605-9; Winter et al., 1997); and KIR3DL1 is specific for HLA-Bw4 ligands, sharing a group of sequence motifs in residues 77-83 of the heavy chain of certain HLA-B and HLA-A alleles (Gumperz et al., 1995, J Exp Med, 181, 1133-44; Litwin et al., 1994, J Exp Med, 180, 537-43; Wagtmann et al., 1995, Immunity, 2, 439-49).
- 25 [0019] KIR mediating activating signaling have also been identified. Unlike inhibitory KIR, they possess truncated portions that transduce activating signals via tyrosine phosphorylation of DAP12 and other proteins (Biassoni et al., 1996, J Exp Med, 183, 645-50; Campbell et al., 1998, Eur J Immunol, 28, 599-609.; Olcese et al., 1997, J Immunol, 158, 5083-6). Couples of cognate activating and inhibitory KIR, sharing almost complete homology (95-99%) in their extracellular domains, are recognized. Thus, activating KIR2DS1, KIR2DS2 and KIR3DS1 are, respectively, cognate receptors for the HLA class I-specific inhibitory KIR2DL1,
- 30

KIR2DL2 and KIR3DL1. Counter-intuitively, the identification of natural ligands for activating receptors remains largely elusive. Ligands for activating KIR2DS2 and KIR3DS1 receptors have not been identified. While it cannot be excluded that these receptors are specific for HLA class I/peptide complexes, current evidence
 5 indicates that they may not affect NK function by generating activating signaling when HLA-C1 and HLA-Bw4 are self-ligands. Unique among activating KIR, 2DS1 recognizes HLA-C2 group antigens, similar to its inhibitory homologue 2DL1.

[0020] Other NK cell activating receptors include: the natural cytotoxicity receptors (NCR) NKp46 (Mandelboim et al., 2001, *Nature* 409, 1055-1060; Pessino
 10 et al., 1998, *J Exp Med* 188, 953-960; Sivori et al., 1997, *J Exp Med* 186, 1129-1136), NKp44 (Arnon et al., 2001, *Eur J Immunol* 31, 2680-2689; Vitale et al., 1998, *J Exp Med* 187, 2065-2072) and NKp30 (Brandt et al., 2009, *J Exp Med* 206, 1495-1503; Pende et al., 1999, *J Exp Med* 190, 1505-1516; Pogge von Strandmann et al., 2007, *Immunity* 27, 965-974); NKG2D (Bauer et al., 1999, *Science* 285, 727-
 15 729; Cosman et al., 2001, *Immunity* 14, 123-133); the FcγRIII receptor (CD16) (Lanier et al., 1983, *J Immunol* 131, 1789-1796; Mandelboim et al., 1999, *Proc Natl Acad Sci U S A* 96, 5640-5644; Perussia et al., 1983, *J Immunol* 130, 2142-2148); 2B4 (Brown et al., 1998, *J Exp Med* 188, 2083-2090; Garni-Wagner et al., 1993, *J Immunol* 151, 60-70); the C-type lectin CD94/NKG2C receptor (Braud et al., 1998,
 20 *Nature* 391, 795-799; Lanier et al., 1998, *Immunity* 8, 693-701); the DNAX accessory molecule 1 (DNAM-1) (Bottino et al., 2003, *J Exp Med* 198, 557-567; Shibuya et al., 1996, *Immunity* 4, 573-581); the SLAM family member CRACC (Boles et al., 2001, *Immunogenetics* 52, 302-307; Kumaresan et al., 2002, *Mol Immunol* 39, 1-8); the CD2 superfamily NTB-A receptor (Bottino et al., 2001, *J Exp*
 25 *Med* 194, 235-246; Falco et al., 2004, *Eur J Immunol* 34, 1663-1672; Flaig et al., 2004, *J Immunol* 172, 6524-6527); and the killer cell lectin-like receptor subfamily F, member 1 (KLRF1) (Vitale et al., 2001, *Eur J Immunol* 31, 233-242; Welte et al., 2006, *Nat Immunol* 7, 1334-1342).

[0021] Ligands for several NK inhibitory and activating receptors, while yet
 30 partially undefined, have been found to be commonly expressed by virally infected, or transformed cells. For example, ligands for the activating human NKG2D receptor are stress-induced proteins MHC (HLA) class I chain-related (MIC) A and

B and the UL16 binding protein (ULBP) 1 to 6 (Raulet, 2003, *Nat Rev Immunol* 3, 781-790; Zafirova et al., 2011, *Cell Mol Life Sci* 68, 3519-3529). Overall, NK-mediated killing of aberrant cells is thought to occur whenever prevalent activating signaling is generated from NK receptors interacting with cognate ligands on target cells (Lanier, 2005, *Annu Rev Immunol* 23, 225-274).

[0022] In the last fifteen years, growing knowledge of NK tolerance to self, cancer immunosurveillance and functional licensing has been extensively applied to the field of hematopoietic stem cell transplantation (HCT), in an effort to identify donors protecting from leukemia relapse through NK-based alloreactivity. Most studies investigating NK effects on transplantation outcome are rationally based upon one of the following three models: the missing-self recognition paradigm, the missing ligand model and the activating receptor-based NK cell alloreactivity. This section (Chouaib et al., 2014, *Front Immunol* 5, 95) addresses the mechanisms known to be responsible for NK cell alloreactivity, as well as their clinical impact in the HCT setting.

[0023] Inhibitory KIR interactions with cognate HLA class I ligands play a critical role in NK cell education and tolerance to self. In normal individuals, NK cells commonly possess KIR repertoires including one or more KIR with ligand specificity for self-HLA class I ligands (Valiante et al., 1997, *Immunity*, 7, 739-51). It is generally believed that such NK cells are rendered functionally competent, or licensed, by continuous signaling generated by inhibitory KIR upon interaction with self-HLA class I antigens (Anfossi et al., 2006, *Immunity*, 25, 331-42; Jonsson et al., 2009, *Adv Immunol*, 101, 27-79). HLA class I is critical to maintaining NK cell tolerance to self, and targets failing to express sufficient levels of HLA class I ligands are promptly cleared by NK-mediated cytotoxicity. This phenomenon, known as missing-self recognition, was first postulated in a report by Kärre et al., describing that lack of MHC class I (H2) antigen expression rendered mice lymphoma cells highly sensitive to NK-mediated rejection (Karre et al., 1986, *Nature*, 319, 675-8).

[0024] NK cells from donor-derived hematopoietic progenitor cells quickly reconstitute in HCT recipients (Anasetti et al., 1989, *N Engl J Med*, 320, 197-204; Kalwak et al., 2003, *Transplant Proc*, 35, 1551-5). In the HCT setting, HLA class I

ligands of donor origin are believed to drive functional licensing. Reconstituted NK cells expressing one KIR for HLA class I present in the donor display stronger in vitro responsiveness than NK cells expressing one KIR for HLA class I present in the recipient, but absent in the donor (Haas et al., 2011, *Blood*, 117, 1021-9).

5 Likewise, in recipients of HLA-mismatched donor or umbilical cord blood grafts, INF-gamma production has been found to only occur in the subset of reconstituted NK cells expressing KIR for donor self-ligands (Foley et al., 2011, *Blood*, 118, 2784-92). This donor HLA-based NK education model implies, that the size of licensed donor NK cell is shaped by the frequency of inhibitory KIR positive NK

10 cells combined with the presence of cognate HLA class I ligands in the donor. Thus, KIR2DL1^{pos} NK cells would acquire functional competence if donor is *HLA-C2*; KIR2DL2-3^{pos} NK cells if donor is *HLA-C1*; and KIR3DL1^{pos} NK cells if donor is positive for *HLA-A* or *-B* alleles possessing the Bw4 motif.

[0025] In HLA-mismatched HCT, *HLA-C* allele groups (*C1* or *C2*), and/or the

15 Bw4 epitope may be present in the donor and absent in the recipient. In this situation, the repertoire of licensed donor NK cells may include NK clones mediating missing self allorecognition against host tissues. For example, KIR2DL1^{pos}/KIR2DL2-3^{neg}/KIR3DL1^{neg} clones from a *HLA-C2* positive donor may display allorecognition of missing self in a *HLA-C2* negative recipient. Recognition

20 of missing self-HLA class I may improve the outcome of HCT. T-cell depleted haplotype-mismatched grafts from NK alloreactive donors mediate strong graft-versus-leukemia (GvL) effects in AML recipients, allowing for lower risk of relapse and better survival (Ruggeri et al., 2002 *Science*, 295, 2097-100; Ruggeri et al., 2007 *Blood*, 110, 433-40).

25 [0026] While in haplotype-mismatched grafts the effect of KIR ligand incompatibility is well established, studies on mismatched unrelated HCT reported conflicting results. In this setting, Giebel et al. showed that donor NK cell alloreactivity improves recipient survival (Giebel et al., 2003 *Blood*, 102, 814-9). A protective effect of KIR ligand incompatibility on post-transplantation relapse was

30 later confirmed in myeloid malignancies (Beelen et al., 2005 *Blood*, 105, 2594-600) and multiple myeloma (Kroger et al., 2005 *Transplantation*, 82, 1024-30). However, most studies failed to show a beneficial effect of donor NK cell alloreactivity on the

outcome of mismatched unrelated HCT (Bornhauser et al., 2004 *Blood*, 103, 2860-1; author reply 2862; Davies et al., 2002 *Blood*, 100, 3825-7; Farag et al., 2006 *Biol Blood Marrow Transplant*, 12, 876-84; Lowe et al., 2003 *Br J Haematol*, 123, 323-6).

5 [0027] Similarly, studies exploring the clinical impact of NK alloreactivity mediated by KIR ligand mismatch in umbilical cord blood grafts have yielded variable results (Brunstein et al., 2009 *Blood*, 113, 5628-34; Willemze et al., 2009 *Leukemia*, 23, 492-500). These inconsistent observations are possibly influenced by complex variables, including donor KIR genotype (Kroger et al., 2006), disease
10 category, type of conditioning, T cell depletion and exposure to immunosuppressive agents for control of graft-versus-host disease (GvHD). For example, T cells may dominate alloreactive phenomena in mismatched unrelated HCT and counteract the clinical benefit of NK alloreactivity (Lowe et al., 2003 *Br J Haematol*, 123, 323-6). Accordingly, *in vivo* T cell depletion with antithymocyte globulin (ATG) has been
15 shown (Giebel et al., 2003 *Blood*, 102, 814-9; Kroger et al., 2005 *Transplantation*, 82, 1024-30.; Yabe et al., 2008 *Biol Blood Marrow Transplant*, 14, 75-87) to enhance the favorable impact of NK cell alloreactivity on HCT outcome.

[0028] KIR and HLA genes map to different chromosomes and segregate independently according to a Mendelian inheritance pattern. Therefore, certain
20 individuals may have *KIR* genes in the absence of HLA/KIR ligand groups (Dupont et al., 2004 *Curr Opin Immunol*, 16, 634-43). KIR receptors are clonally distributed on NK cell surface, allowing for the possibility, that subpopulations of NK cells exclusively express KIR with ligand specificity for non-self-HLA class I ligands. These NK cells are not classically licensed by self-HLA class I ligands during their
25 development, and are believed to be hyporesponsive to stimulation in physiologic conditions. However, this non-licensed status may be transiently suspended during post-transplantation immune reconstitution, and effector functions could indeed be mediated by donor NK cells expressing KIR with ligand specificity for non-self-HLA class I. An important implication of the missing ligand model is that NK
30 alloreactivity would be observed even in the absence of donor/recipient KIR ligand mismatch, a necessary condition for missing self-mediated NK alloreactivity.

[0029] Several studies exploring the effect of the missing ligand model on HCT outcome indicate, that donors possessing inhibitory KIR but not the corresponding HLA class I ligand do mediate beneficial NK effects in HLA-identical siblings or HLA-matched unrelated recipients (Clausen et al., 2007 *Clin Exp Immunol*, 148, 520-8; Hsu et al., 2005 *Blood*, 105, 4878-84; Miller et al., 2007 *Blood*, 109, 5058-61; Sobecks et al., 2007 *Bone Marrow Transplant*, 39, 417-24). Hsu et al. originally explored a cohort of 178 subjects receiving a T-cell depleted graft from a HLA-identical sibling. In patients with AML and myelodysplastic syndromes (MDS), lack of one or more HLA ligand for donor KIR resulted in lower relapse and better survival (Hsu et al., 2005 *Blood*, 105, 4878-84).

[0030] Following the identification of beneficial NK effects in the HLA-matched setting, the function of classically non-licensed NK cells has been directly explored in the context of post-transplantation reconstitution. In T-cell depleted grafts from HLA-identical siblings, NK cells expressing KIR for non-self-HLA display strong IFN γ production and cytotoxicity to target stimulation during the first trimester post-transplantation (Yu et al., 2009 *Blood*, 113, 3875-84). These findings have not been confirmed in a cohort of recipients of T cell replete grafts from HLA-identical siblings. Here, reconstituted NK cells expressing KIR for non-self HLA ligands maintained tolerance to self. Moreover, lack of self-HLA ligands for donor inhibitory KIR was found to have no effect on HCT outcome (Bjorklund et al., 2010, *Blood*, 115, 2686-94). Presence or absence of T cells in the graft may differentially affect self-tolerance of non-licensed donor NK cells post-transplantation. Regardless, the interpretation of these conflicting results demands further studies on tolerance to self of donor NK cells reconstituting in the HLA-identical host.

[0031] Because ligands for most activating KIR are currently unknown, studies reporting associations between activating KIR and HCT outcome are not generally supported by the identification of an underlying immunological background mechanistically explaining the observed NK-mediated alloreactivity. In a cohort of 65 graft recipients from HLA-identical siblings, donors with genotypes containing both *KIR2DS1* and *KIR2DS2* genes provided protection from relapse (Verheyden et al., 2005 *Leukemia*, 19, 1446-51). Donor activating KIR was later found to control

CMV reactivation post-transplantation. Recipients of T cell replete grafts were found to have a remarkable reduction of the incidence of CMV reactivation, if donor possessed more than one activating *KIR* genes (Cook et al., 2006). Confirmation of the protective effect against CMV reactivation by donor activating *KIR* was
5 concomitantly reported by another group (Chen et al., 2006) *Bone Marrow Transplant*, 38, 437-44.

[0032] In 2009, Cooley et al. investigated the effect of different donor *KIR* haplotypes in 448 AML recipients of unrelated T cell replete HCT. Recipients of *KIR* B/x grafts (i.e., homozygous or heterozygous for *KIR* B group haplotypes)
10 displayed a higher 3-year overall survival (Cooley et al., 2009 *Blood*, 113, 726-32). In a cohort of 1086 AML recipients of unrelated grafts, the same group later compared the contribution to HCT outcome of donor centromeric and telomeric group A and B *KIR* haplotypes. Donors homozygous for centromeric B gene content motifs (Cen B/B) most strongly associated with low risk of relapse and prolonged
15 survival (Cooley et al., 2010 *Blood*, 116, 2411-9). Among activating *KIR*, activating *KIR2DS2* is mapped on the centromeric region of several B group haplotypes, and may thus mediate the clinical benefit observed for Cen B/B donors through interaction with an unknown ligand expressed on leukemia cells (Cooley et al., 2010 *Blood*, 116, 2411-9).

20 [0033] Recent studies investigated the effect of telomeric activating *KIR3DS1* and *KIR2DS1* genes on transplantation outcome. Patients receiving unrelated grafts from *KIR3DS1* donors exhibited a lower risk for grade II-IV GvHD and mortality (Venstrom et al., 2010 *Blood*, 115, 3162-5; Venstrom et al., 2012 *N Engl J Med*, 367, 805-16). Activating *KIR2DS1* is found in approximately 1/3 Caucasians, and
25 commonly occurs in individuals positive for *HLA-C2* (*C1/C2*; *C2/C2*) (Cognet et al., 2010; Fauriat et al., 2010; Pende et al., 2009). *KIR2DS1* expression occurs in more than 20% NK cells (Pende et al., 2009), and 2DS1 single positive (*KIR2DS1*^{SP}) NK cells (i.e., lacking inhibitory *KIR* expression), may also be identified. In *HLA-C2* individuals, *KIR2DS1*^{SP} NK cells may potentially display auto-reactivity to normal
30 self-tissues. Compared with *HLA-C1* donors, *KIR2DS1*^{SP} NK cells from *HLA-C2* homozygous individuals are hyporesponsive to a *HLA-C2* positive target cell (Fauriat et al., 2010 *Blood*, 115, 1166-74). Similarly, mice studies described

hyporesponsiveness of activating receptor-positive NK cells resulting from *in vivo* chronic interaction with a viral ligand (Sun et al., 2008 *J Exp Med*, 205, 1819-28; Tripathy et al., 2008 *J Exp Med*, 205, 1829-41).

[0034] Therefore, using the example of HCT, it is apparent that HLA-KIR interactions play a crucial role in mediating clinically relevant NK alloreactivity phenomena. Thus, the genotypes of HLA and KIR of the donor individuals and the recipient individuals are important factors to consider to improve the efficacy and to reduce unwanted side-effects in NK cell therapy in HCT and other in other clinical applications using NK cells.

[0035] Early clinical studies explored the effects of low-dose IL-2 administration on NK cell expansion and cytotoxicity in patients with cancer. In advanced breast cancer and lymphoma, peripheral blood NK cells promptly expanded following IL-2 infusion, but did not show increased anti-tumor cytotoxicity (Miller et al., 1997, *Biol Blood Marrow Transplant* 3, 34-44). Adoptively transferred, *in vitro* activated autologous NK cells have proven safe in patients with relapsed lymphoma and metastatic breast cancer (Burns et al., 2003, *Bone Marrow Transplant* 32, 177-186); metastatic colorectal cancer and non-small cell lung cancer (Krause et al., 2004, *Clin Cancer Res* 10, 3699-3707); metastatic melanoma and metastatic renal cell carcinoma (Parkhurst et al., 2011, *Clin Cancer Res* 17, 6287-6297). These trials failed to show clinically relevant NK-mediated anti-tumor effects, mostly due to the inability of autologous NK cells to overcome self-tolerance. In one study, while the adoptively transferred NK cells were shown to persist *in vivo* for up to several months, they exhibited low NKG2D levels and weak anti-tumor cytotoxicity *in vitro* (Parkhurst et al., 2011, *Clin Cancer Res* 17, 6287-6297).

[0036] The clinical significance of NK-mediated graft-vs-leukemia effects in haplotype-mismatched HCT (Ruggeri et al., 2002, *Science* 295, 2097-2100; Ruggeri et al., 2007, *Blood* 110, 433-440), as well as evidence demonstrating the anti-tumor capabilities of IL-2 activated, haplotype mismatched donor lymphocytes (Slavin et al., 2010, *Cancer Immunol Immunother* 59, 1511-1519), prompted multiple clinical trials evaluating the safety and anti-tumor efficacy of haplotype-mismatched, allogeneic NK cells. In 2005, Miller and co-workers explored for the first time the

biological and clinical effects of haploidentical NK cells in patients with poor prognosis AML or various solid cancers, receiving concomitant systemic immunosuppression and IL-2 (Miller et al., 2005, *Blood* 105, 3051-3057). NK cell infusion induced complete hematologic remission in 5 of 19 (26%) patients with
5 AML (Miller et al., 2005, *Blood* 105, 3051-3057). Later on, infusion of mismatched NK cells with systemic immunosuppression proved safe and able to achieve transient engraftment in a cohort of pediatric patients with AML in first complete remission (Rubnitz et al., 2010, *J Clin Oncol* 28, 955-959). Finally, infusion of haploidentical NK cells after systemic immunosuppression was well tolerated by
10 elderly patients with high-risk AML, and induced complete remission in one patients (of 5 tested) with active AML (Curti et al., 2011).

[0037] The clinical benefits associated with infusion of haploidentical NK cells have prompted research in the field of large-scale expansion of clinical-grade NK cells. Current protocols for the expansion of NK cells from peripheral blood
15 mononuclear cells are generally based on the delivery of exogenous IL-2 and the use of different feeder cells, including EBV-transformed lymphoblastoid lines, genetically engineered K562 cells, or irradiated autologous cells (Berg et al., 2009, *Cytotherapy* 11, 341-355; Fujisaki et al., 2009, *Cancer Res* 69, 4010-4017; Gong et al., 2010, *Tissue Antigens* 76, 467-475; Siegler et al., 2010, *Cytotherapy* 12, 750-
20 763).

[0038] While several peripheral blood expansion procedures hold promise for NK-based immunotherapy, several caveats currently limit the clinical applicability of such protocols. NK expansion yields are typically inconsistent, and significant donor-to-donor variation is commonly observed. Moreover, contamination of NK
25 cells with other lymphocytes such as T cells is also common, which imposes additional limits on the therapeutic use of NK cells prepared by these methods, such as T cell-specific cytotoxicity and immune reactions. In addition, existing NK cell culturing protocols require added exogenous cytokines, i.e., culture media supplementation with exogenous cytokines, including notably IL-2 (Munz et al., J.
30 Exp. Med. 1997, 185: 385-91; Cella & Colonna, *Methods in Molecular Biology*, 2000, vol. 121; Hansasuta et al., *Eur. J. Immunol.* 2004, 34: 1673-1679; Morris et

al., Journal of Immunological Methods 307, 2005, 24– 33; Imai et al., Blood. 2005, 106:376-383; US Patent NO 8,026,097).

[0039] The presence of exogenous cytokines in cell culture media at concentrations much higher than their native concentrations in human blood may affect the biology of cultured NK cells in a variety of ways. For example, IL-2 promotes NK cell cytolytic activity and modulates other pathways in response to antigen (See Liao W. et al., Immunity. 2013, 24; 38(1):13-25). Thus, the ability to eliminate IL-2 from NK cell culture helps to preserve NK cell phenotype and native biological functions. Similar observations can be made for other added cytokines. Thus, it would be preferable to avoid addition of IL-2 or any other exogenous cytokine. Furthermore, all NK cells expansion techniques intended for clinical applications have been used to induce proliferation of crude, polyclonal NK cells, while the selective expansion of alloreactive, clonogenic NK cell subpopulations with a specific biological properties may be critical for the success of NK-based immunotherapy.

[0040] Currently available NK cell cloning protocols (Munz et al., J. Exp. Med. 1997, 185: 385-91; Cella & Colonna, Methods in Molecular Biology, 2000, vol. 121; Hansasuta et al., Eur. J. Immunol. 2004, 34: 1673–1679; Morris et al., Journal of Immunological Methods 307, 2005 24–33; Imai et al., Blood. 2005;106:376-383; US Patent NO 8,026,097) have not been designed for the expansion of NK clonal populations from individual NK cells with pre-determined phenotypic and/or functional characteristics.

SUMMARY OF THE DISCLOSURE

[0041] The present disclosure is based upon the observation that individual natural killer (NK) cells can be propagated and expanded *in vitro* in the presence of trans-presented IL-15, such as in co-culture with a feeder cell that presents on its surface an IL-15 that results, at least in part, from the exogenous expression of a nucleic acid encoding IL-15. It was further discovered that a clonal NK cell can be propagated and expanded *in vitro* without the addition of other factors, in particular without the addition of IL-2, to the culture medium. Thus, as disclosed herein, a

single NK cell clone can be propagated according to the present methods to a high cell density and/or number.

[0042] Moreover, the homogenous, clonogenic NK cell populations resulting from these methods maintain cell viability and the desired structural and functional characteristics of the initial NK cell clone. Thus, the present disclosure provides homogenous, clonogenic NK cell populations wherein each member of the cell population exhibits one or more phenotype and/or produces one or more protein such as, for example, one or more killer immunoglobulin-like receptors (KIR), one or more C-type lectin-like receptors (CLLR), one or more natural cytotoxicity receptors (NCR), and/or one or more chimeric antigen receptors (CAR), which provides to a clonogenic NK cell population one or more desired functionalities as described in further detail herein.

[0043] The advantageous properties exhibited by the presently disclosed clonogenic NK cell populations cannot be achieved with heterogeneous NK cell populations, such as heterogeneous NK cell populations that are isolated from human samples (*e.g.*, peripheral blood mononuclear cells (PBMC) or that result from the *in vitro* expansion of heterogenous, non-clonal NK cell populations. For example, by the present methods, clonogenic cell populations can be produced that match to particular recipient patient, and thus can be used in HCT and in cancer immunotherapy or other therapeutic methods that exploit a specific NK cell cytotoxicity and/or cytolytic activity, which cannot be provided in sufficient quantity or purity by a heterogeneous NK cell population.

[0044] Thus, within certain embodiments, the present disclosure provides clonogenic NK cell populations, including isolated and/or purified clonogenic NK cell populations. Within certain aspects of these embodiments, the clonogenic NK cell populations comprise at least about 10^5 NK cells that are derived from a single clone. The present disclosure describes isolated, purified clonogenic NK cell populations exhibiting a predetermined desirable phenotypic trait of interest. The number of cells in said NK cell population is at least of the order of 10^5 and said phenotype comprises expression of one or more cell surface receptors that modulate NK cell function and/or mediate NK cell cytotoxicity and/or cytolytic activity.

[0045] In some embodiments, the present disclosure describes certain clonogenic NK cell populations of at least 10^5 cells that express certain cell surface receptors that are important for the function of NK cells, including their cytotoxicity and cytolytic activity.

5 [0046] In some embodiments, the clonogenic NK cell populations express one or more cell surface receptors. The receptors can be a killer immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), or a chimeric antigen receptor (CAR). Additionally, the NK cell surface
10 receptors can be an inhibitory receptor, an activating receptor, or a combination of both. In some embodiments, the clonogenic NK cell population exhibits an expression pattern of KIR, and examples of KIR include, but are not limited to, KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2. In some
15 embodiments, the clonogenic NK cell population exhibits an expression pattern of NCR, including NKp46, NKp44, and NKp30. In some embodiments, the clonogenic NK cell population exhibits an expression pattern of CLLR, and examples of CLLR include, but are not limited to, NKG2D and NKG2D-DAP10-CD3 ζ . In some
20 embodiments, the clonogenic NK cell population exhibits an expression pattern of CAR, and examples of CAR include, but are not limited to, chimeric receptors comprising a CD19 peptide, a G(D2) peptide, a CS1 peptide, or a WT1 peptide. In other embodiments, the clonogenic NK cell population exhibits an expression
pattern of a combination of two or more of a KIR, a CLLR, an NCR, and a CAR (from the same or different categories among those disclosed herein).

[0047] In some embodiments, the present disclosure describes isolated, purified
25 NK cell populations that are isolated from donors with certain KIR and HLA class I genotypes that make them desirable for applications in HCT. In some embodiments, the present disclosure describes isolated, purified NK cell populations that express KIR2DS1 but without co-expression of inhibitory KIR with ligand specificity for HLA class I antigens. In some embodiments, the present disclosure describes
isolated, purified NK cell populations that are obtained from a HLA-C1:C1
30 homozygous or HLA-C1:C2 heterozygous, and KIR2DS1 positive donor. In some embodiments, the current disclosure describes isolated, purified NK cell populations

that are obtained from HLA-C1:C1 homozygous or HLA-C2:C2 heterozygous, and KIR2DS1 positive donor.

5 [0048] In some embodiments, the clonogenic NK cell population obtained from a single NK cell clone (i.e., a monoclonal NK cell population) has a cell number of at least 10^5 up to the number reached just before the cells stop dividing. In some embodiments, the clonogenic NK cell population obtained from a single NK cell clone has a cell number of the order of at least 10^6 and up to the order of 10^7 .

10 [0049] In some embodiments, the clonogenic NK cell population can be a polyclonal population containing a mixture of pooled selected monoclonal NK cell populations. The selection can be made based on a common phenotype of interest expressed by the populations, such as for example similarities in cell surface receptor expression pattern. In some embodiments, monoclonal NK cell populations with similar cell surface receptor expression patterns can be combined to produce a polyclonal NK cell population with a final cell number of at least 10^6 , 10^7 , 10^8 ,
15 or 10^9 . The polyclonal NK cell population can be used in cell therapy.

[0050] In addition to isolated, purified clonogenic NK cell populations, the present disclosure describes a biologic composition suitable for human administration. This biologic composition contains the isolated, purified clonogenic NK cell populations as described above. In some embodiments, the composition
20 comprising an aliquot of an isolated, purified clonogenic NK cell population expressing a phenotype of interest. In some embodiments, the composition contains monoclonal or polyclonal human NK cell populations of at least 10^5 to about 10^7 cells per clone or as high as the number of cells will reach before they stop proliferating.

25 [0051] Thus, within certain embodiments, the present disclosure provides methods for producing a population of clonogenic NK cells that have a desirable phenotypic trait of interest. After NK cells are isolated from a tissue sample, individual NK cell clones can be obtained. The phenotypic trait of interest can be the expression of certain cell surface receptors, including without limitation killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors (CLLR), natural
30 cytotoxicity receptors (NCR), and chimeric antigen receptors (CAR). Selection of

NK cell clones can be based on the expression of these receptors or any other phenotypic trait of interest.

[0052] Once NK cell clones are isolated and selected based on the predetermined phenotype, the present disclosure describes a novel method of expanding these single cell clones *in vitro*. This method comprises (a) culturing a human NK cell clone (i) in the presence of a feeder cell, wherein said feeder cell trans-presents human interleukin-15 (IL-15), and (ii) in a culture medium that is without added (exogenous) IL-2; and (b) maintaining said culture (i) under culturing conditions that support the proliferation of said human NK cell clone and (ii) for a period of time sufficient to achieve expansion of said human NK cell into said clonogenic NK cell population. In addition to NK cells that are isolated from a human tissue sample (i.e., native NK cells), this method can also be applied to expand genetically engineered NK cells (e.g., “designer” NK cells).

[0053] In some embodiments, the predetermined phenotypic trait of interest can comprise the expression of one or more cell surface receptors such as receptors that modulate NK cell cytotoxicity, cytokine production and/or other immune functions.

[0054] In some embodiments, the NK cell surface receptor can be a killer immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), or a chimeric antigen receptor (CAR). Additionally, the NK cell surface receptor can be an inhibitory receptor, an activating receptor, or a combination of both. In some embodiments, the NK cell clones can be selected based on the expression pattern of KIR, and examples of KIR include, but are not limited to, KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2. In some embodiments, the NK cell clones can be selected based on the expression pattern of NCR, and examples of NCR include, but are not limited to, NKp46, NKp44, and NKp30. In yet other embodiments, the NK cell clones can be selected based on the expression pattern of CLLR, and examples of CLLR include, but are not limited to, NKG2D and NKG2D-DAP10-CD3 ζ . In some other embodiments, the NK cell clones can be selected based on the expression pattern of CAR, and examples of CAR include, but are not limited to, chimeric receptors comprising a CD19 peptide, a G(D2) peptide, a CS1 peptide, or a WT1 peptide. In yet some other embodiments,

the NK cell clones can be selected based on the expression pattern of a combination of a KIR, a CLLR, an NCR, and a CAR.

5 [0055] The present disclosure describes a method of expanding NK cell clones using feeder cells that are genetically engineered to trans-present human IL-15. In some embodiments, the trans-presentation of IL-15 is achieved through co-expression of human IL-15 and human IL-15R α each of which may be induced.

10 [0056] In some embodiments, the feeder cells include cells that are HLA class I negative cells, including a pre-B-lymphocyte cell line, a bone marrow stromal cell line, an erythroleukemia cell line, a B lymphoblastoid cell line, a Burkitt lymphoma cell, and a Wilms tumor cell. In a preferred embodiment, the feeder cell is BaF/3. In another preferred embodiment, the BaF/3 cells are transfected with nucleic acids encoding for human IL-3. In other embodiments, the feeder cells include one or more of OP9, K562 and 721.221. In some embodiments, the feeder cells include one or more of Daudi cells, HFWT cells and HLA class I positive cells. In some
15 embodiments, the feeder cells are surface antigen mismatched relative to an inhibitory surface KIR receptor(s) on the NK cell clone within the same cell culture.

[0057] In addition to IL-15 trans-presenting feeder cells, the present disclosure describes additional feeder cells that would support the proliferation and maintenance of NK cells in culture. These additional feeder cells include without
20 limitation peripheral blood mononuclear cells (PBMC), EBV-B lymphoblastoid cells (EBV-BLCL), or RPMI8866 lymphoblastoid cells.

[0058] In some embodiments, all the feeder cells described in the present disclosure are rendered non-proliferative (e.g., by irradiation) before contacting NK cells in cell culture.

25 [0059] The present disclosure also describes a NK cell culture system that is without one or more cytokines, notably IL-2.

[0060] The method described by the present disclosure can allow for expansion of single cell NK clones to at least about 1×10^5 NK cells per clone. In some embodiments, single cell clones can be expanded to about 5×10^5 NK cells, to about

1.5 x 10⁶ NK cells, or to about 5 x 10⁶ NK cells. This represents an expansion rate of at least 10⁵ fold to as high as 5 x 10⁶ fold.

[0061] The method described by the present disclosure can produce a clonogenic NK cell population that retains the characteristics of the original single cell clone. In some embodiments, at least about 50% NK cells after the expansion still express the one or more cell surface receptors that have been used to select the clone.

[0062] The method described by the present disclosure can produce a clonogenic NK cell population that is highly viable. In some embodiments, at least 90% of said NK cell population is viable.

[0063] The method described by the present disclosure can produce a clonogenic NK cell population that exhibits normal NK cell functions, e.g., cytotoxicity and/or cytolytic activity.

[0064] The present disclosure also describes methods of combining monoclonal human NK cell populations exhibiting one or more phenotypic traits of interest to generate a larger NK cell populations exhibiting said traits.

[0065] In a more specific aspect, the present disclosure describes a method of producing a population of clonogenic NK cells expressing a phenotype of interest, and reacting to a specific HLA class I genotype of a recipient, said method comprising: (a) selecting at least one NK cell clone from a donor whose HLA class I genotype mismatches that of a recipient; (b) culturing said at least one NK cell clone in the presence of a feeder cell, wherein said feeder cell trans-presents human interleukin-15 (IL-15) and wherein said culturing is performed by addition of a culture medium that is without added IL-2; and (c) maintaining said culture (i) under conditions of temperature, humidity, and CO₂ that support the proliferation of said human NK cell clone and (ii) for a period of time sufficient to achieve expansion of said human NK cell into said clonogenic NK cell population; and wherein said phenotypic trait of interest of said NK clonogenic population comprises expression of at least one cell surface receptor having the property of detecting “missing self-HLA class I ligand” in said recipient.

[0066] In some embodiments, the genotype of KIR in the donor NK cell and the HLA class I genotype are the main criteria for selection. In some embodiments, the NK clones express at least one cell surface receptor selected from the group consisting of inhibitory KIR with ligand specificity for HLA class I and, optionally selected from the group consisting of activating KIR, c-type lectin-like receptors, natural cytotoxicity receptors, and NK-activating chimeric receptors. In some embodiments, the inhibitory KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1 and said at least one cell surface receptor is selected from the group consisting of KIR2DS1, KIR2DS2, NKG2D, NKp46, NKp44, and NKp30; NKG2D-DAP10-CD3 ζ , and a chimeric receptor comprising one or more peptide selected from the group consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.

[0067] The present disclosure also provides some examples that may be of particular interest for HCT and cancer immunotherapy. In some preferred embodiment, the donor NK cell clone and its preferred recipient genotype can be selected from a row in **Table VII**.

[0068] In another aspect, the present disclosure also describes IL-15 trans-presenting feeder cells that can promote human NK cell proliferation. In some embodiments, these feeder cells are immortalized cell lines that are of either human or murine origin.

[0069] In some embodiments, the present disclosure describes feeder cells that are genetically engineered to trans-present human IL-15. In some embodiments, the trans-presentation of IL-15 is achieved through co-expression (separately or together) of human IL-15 and human IL-15 receptor alpha (human IL-15R α).

[0070] In some embodiments, the present disclosure describes cells that are suitable as IL-15 trans-presenting feeder cells. In some embodiments, these cells are HLA class I negative cells, including a pre-B-lymphocyte cell line, a bone marrow stromal cell line, an erythroleukemia cell line, a B lymphoblastoid cell line, a Burkitt lymphoma cell, and a Wilms tumor cell. In more specific embodiments, the feeder cell is BaF/3. In another embodiment, the feeder cell is OP9. In another embodiment, the feeder cell is K562. In another embodiment, the feeder cell is

721.221. In another embodiment, the feeder cell is a Daudi cell. In another embodiment, the feeder cell is an HFWT cell. In some embodiments, the feeder cells can be HLA class I positive cells. In yet other embodiments, the feeder cells are surface antigen mismatched relative to said an inhibitory surface KIR receptor(s) on
5 the NK cell clone within the same cell culture.

[0071] Additionally, the present disclosure describes a method for treating a disease using the clonogenic NK cell populations that are described above. NK cells show promise in treating a variety of cancers and auto-immune diseases, including, but not limited to AML, ALL, melanoma, MDS, non-Hodgkin's lymphoma, neuroblastoma, multiple myeloma, transplant rejection, and GvHD. The method described by the present disclosure comprises the administration of isolated, purified clonogenic NK cell populations to a recipient individual, wherein said population comprises of the order of 10^5 to 10^7 cells per clone, and wherein said population expresses a phenotype of interest relevant for said disease in a recipient individual in
10 need thereof.

[0072] The clonogenic NK cell populations that the method of treatment comprises are selected and expanded according to a predetermined, desirable phenotypic trait of interest, e.g., expression of one or more cell surface receptors. In some embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more preferred cell surface receptors selected from
20 killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors (CLLR), natural cytotoxicity receptors (NCR), and chimeric antigen receptors (CAR). Additionally, in some embodiments, the method of treatment comprises clonogenic NK cell populations that express an NK inhibitory receptor, an NK activating receptor, or a combination of both. In some preferred embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more KIR, and examples of KIR include, but are not limited to, KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2. In some other embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more NCR, and examples of NCR include, but are not limited to, NKp46, NKp44, and NKp30.
25 30 In some embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more CLLR, and examples of CLLR include, but are

not limited to, NKG2D and NKG2D-DAP10-CD3 ζ . In some embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more CAR, and examples of CAR include, but are not limited to, chimeric receptors comprising a CD19 peptide, a G(D2) peptide, a CS1 peptide, or a WT1 peptide. In yet other embodiments, the method of treatment comprises clonogenic NK cell populations that express a combination of two or more of a KIR, a CLLR, an NCR, and a CAR (from the same or different categories).

[0073] The present disclosure also describes methods of treating certain diseases using a clonogenic NK cell population that is selected to increase the efficacy and/or to reduce the side effects of such treatment comprising NK cells. The expression of certain cell surface receptors in NK cells may be of particular importance to certain diseases. In some embodiments, the method of treatment comprises matching clonogenic NK cell populations with certain cell surface receptor expression pattern to a disease as selected from a row in Table VIII.

[0074] Details of one or more embodiments are set forth in the accompanying drawings and the description below. Other features, objects, and advantages featured in the invention will be apparent to those skilled in the art from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0075] **Fig. 1A** and **Fig. 1B** are a series of graphical representations of NK subsets and NK clones, respectively, according to FACS analysis with respect to specific receptor repertoires.

[0076] **Fig. 2A, Fig. 2B, Fig. 2C** and **Fig. 2D** are a series of plots showing the percentage of NK cell clone cytotoxicity with respect to effector and target cell HLA genotypes.

[0077] **Fig. 3** depicts a series of scatter plots of KIR mRNA copy number according to the number of NK clones. In the color version of Fig. 3 (Fig. 3—

Color) mRNA with protein is presented in green while mRNA without protein is presented in red. In the corresponding black and white version of Fig. 3 (Fig. 3—B&W), mRNA with protein is presented with the symbol “●” while mRNA without protein is presented with the symbol “X”.

- 5 [0078] **Fig. 4A** depicts a plot and a diagram of time-dependent changes in intracellular Ca^{2+} concentration; **Fig. 4B** and **Fig. 4C** are scatter plots of correlations between NK cell membrane receptor expression and cellular functions; and **Fig. 4D** and **Fig. 4E** are plots of cytotoxicity according to NK cell subsets.
- [0079] **Fig. 5** is a scatter plot of cytotoxicity according to NK cell subsets.
- 10 [0080] **Fig. 6** is a scatter plot of NK cell surface marker expression according to NK cell subsets.

DETAILED DESCRIPTION

- [0081] In some aspects, the present disclosure relates to novel methods for *in vitro* expansion of clonogenic natural killer (NK) cells. The methods utilize the
- 15 stimulatory effects of trans-presented IL-15 in cell culture using feeder cells to expand isolated NK cell clones with a predetermined phenotypic trait of interest. The expanded clonogenic NK cell populations are homogenous, in contrast to heterogenous, crude NK cells typically isolated and expanded from a human tissue sample. The present disclosure also relates to feeder cells that are genetically
- 20 engineered to trans-present IL-15 involving co-expressing human IL-15 and IL-15 receptor α subunit (IL-15R α) that are capable of stimulating the expansion of NK cell clones *in vitro*. The present disclosure further relates to viable, functional clonogenic NK cell populations with the predetermined phenotypic trait of interest obtained via the above *in vitro* expansion methods and their use in personalized
- 25 medicine to treat diseases, including leukemia, lymphoma and HCT, in drug screening and in basic and translational studies involving NK cells.

[0082] While various embodiments of the present disclosure are described in detail below, it should be appreciated that the present disclosure provides many applicable inventive concepts that can be embodied in a wide variety of specific

contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the products and methods discussed herein and do not limit the scope of the present disclosure.

5 [0083] It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition involved here, and vice versa. Furthermore, compositions of the present disclosure can be used to achieve methods discussed herein. It may be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the present disclosure. The principal features of the disclosed subject, 10 matters can be employed in various embodiments without departing from the scope of the present disclosure. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

15 [0084] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

20 [0085] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it may be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the 25 steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

30 [0086] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the technical field of this disclosure.

Definitions

[0087] The terms in quotes below are given the meanings ascribed to them in this section unless the context requires otherwise. Additional terms may be defined throughout the specification. The singular should be construed to include the plural.

5 [0088] The use of the word “**a**” or “**an**” when used in conjunction with the term “comprising” or the equivalent in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean
10 are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[0089] Unless otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “**about**” or
15 “**approximately**”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present disclosure. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each
20 numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches. Exemplary error margins are within 20%, typically, within 10%, and more typically, within 5% of a given value or range of values.

[0090] As used in this specification and claim(s), the terms “**comprising**” (and
25 any form of comprising, such as “**comprise**” and “**comprises**”), “**having**” (and any form of having, such as “**have**” and “**has**”), “**including**” (and any form of including, such as “**includes**” and “**include**”) or “**containing**” (and any form of containing, such as “**contains**” and “**contain**”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

30 [0091] The term “**combination**” or “**combination thereof**” as used herein refers to all permutations and combinations of the listed items preceding the term. For

example, “A, B, C, or combinations thereof is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or
5 term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0092] The term “**activating receptor**” includes immune cell receptors that bind
10 antigen, complexed antigen (e.g., in the context of antigen presentation by MHC or HLA molecules), or bind to antibodies. On the other hand, the term “**inhibitory receptor**” refers to a receptor capable of down-regulating a biological response mediated by another receptor, regardless of the mechanism by which the down-regulation occurs. To recognize and respond to inflamed or infected tissues, NK
15 cells express a variety of activating and inhibitory receptors and natural cytotoxicity receptors, as well as co-stimulatory receptors. These receptors recognize cellular stress ligands as well as major histocompatibility complex class I and related molecules, which can lead to NK cell responses. Examples of NK activating receptors include KIR2DS1, KIR3DS1, KIR2DS2, and examples of NK inhibitory
20 receptors include KIR2DL1, KIR2DL2, KIR2DL3, and KIR3DL1 (Pittari, G. et al., J Immunol, 2013: 190:4650-4660).

[0093] In the context of the present disclosure the terms “**activation**” and “**activated NK cells**” refer to NK cells that have received an activating signal. Activated NK cells are capable of killing cells with deficiencies in MHC class I
25 expression. Given their strong cytolytic activity and the potential for auto-reactivity, NK cell activity is tightly regulated. In order to kill cells with a missing or abnormal MHC class I expression the NK cells need to be activated.

[0094] The term “**agonist**” is used in the broadest sense and includes any molecule that mimics a biological activity of a native polypeptide or a
30 pharmaceutical agent. Conversely, the term “**antagonist**,” also used in the broadest sense, includes any molecule that partially or fully blocks, inhibits, or neutralizes a

biological activity of a native polypeptide disclosed herein. Suitable agonist or antagonist molecules specifically include agonist or antagonist antibodies or antibody fragments, fragments or amino acid sequence variants of native polypeptides, peptides, antisense oligonucleotides, small organic molecules, etc.

5 **[0095]** The term “**superagonist**” as used in the context of the present disclosure describes a property of some binding molecules, which by specifically binding to/interacting with particular epitopes of certain NK cell membrane receptors make it possible to elicit a stronger response in the NK cells than common agonists.

10 **[0096]** The term “**alloantigen**” refers to an antigen that is not recognized as self. Specifically, an alloantigen is defined by an MHC polymorphism between a host individual and a donor individual of the same species, or between any two individuals or between populations of cells. In the context of a tissue graft or transplant, alloantigens are the nonself MHC expressed by the cells of allografted tissue that can induce an intense immune response in the recipient host (e.g. host
15 versus graft) and which is aimed at eliminating the transplanted cells. The immune reaction is the result of the host immune cells recognizing the alloantigenic cells or tissue as originating from a nonself source. If an alloantigen is presented to a member of the same species that does not have the alloantigen, it will be recognized as foreign and induce an immune response.

20 **[0097]** The term “**allogeneic**” refers to two or more individuals, cells, tissues, or other biological materials that differ at the MHC. Host rejection of grafted tissues from unrelated donors usually results from T-cell responses to allogeneic MHC molecules expressed by the grafted tissues. As used herein, a B cell and a T cell are allogeneic when they differ at the MHC as a result of originating from different
25 individuals. In some contexts, these individuals are a transplant host and donor

30 **[0098]** The term “**allospecific**” refers to being reactive to, identifying, or binding cells or other biological components from genetically disparate individuals within the same species. Allospecific T cells can have effector or regulatory functions, and the relative proportions of the two populations activated following alloantigen presentation is one of the factors that determine the clinical outcome of a tissue graft or transplant, namely, graft rejection or persistence.

[0099] The term “**anergic**” with respect to an immune cell refers to a state of being nonresponsive to an antigen (“**anergy**”). T cells and B cells are said to be anergic when they are living but cannot respond to their specific antigen even under optimal conditions of stimulation

5 [00100] The term “**antigen**” refers to a compound, composition, or substance that can recognize and/or stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term “antigen” includes all
10 antigenic epitopes within the antigenic substance.

[00101] The term “**antibody**” is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), antibody fragments and engineered antibodies and minibodies so long as they exhibit the
15 desired biological activity. By “**specifically binds**” or “**immunoreacts with**” is meant that the antibody reacts with one or more antigenic determinants of the desired antigen and does not react (i.e., bind) with other polypeptides or binds at much lower affinity with other polypeptides. The term “antibody” also includes antibody fragments that comprise a portion of a full length antibody, generally the
20 antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab’, F(ab’)2, and Fv fragments; diabodies; linear antibodies; single-chain antibody (scFv) molecules; and chimeric molecules and multispecific antibodies formed from antibody fragments or from an antibody fragment and a heterofunctional molecule, such as adhesins. In certain embodiments of the
25 application, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In such a case, it may also be desirable to use an antibody fragment that has been modified by any means known in the art in order to increase its serum half-life.

[00102] The term “**binding affinity of an antibody**” means the strength of the
30 interaction between a single antigen-binding site on an antibody and its specific antigen epitope. The higher the affinity, the tighter the association between antigen

and antibody, and the more likely the antigen is to remain in the binding site. The affinity constant is the ratio between the rate constants for binding and dissociation of antibody and antigen. Typical affinities for IgG antibodies are 10^5 to 10^9 L/mole.

[00103] The term “**monoclonal antibody**” as used herein refers to an antibody
5 obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The monoclonal antibodies herein specifically include “chimeric” antibodies in which a portion of the heavy and/or light chain is identical with or homologous to
10 corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological
15 activity (U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855, 1984)).

[00104] “**Humanized**” forms of non-human antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in
20 which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and/or capacity. Methods for making humanized and other chimeric antibodies are known in the art.

[00105] The term “**autoimmune disease**” refers to a condition that results from an adaptive immune response directed at an individual's own cells and tissues expressing self-antigens. Autoimmunity can also be described as a loss of self-tolerance, the property of not mounting an immune response against self. The resulting immune response against self-tissues and cells can lead to various acute
25 and chronic disease states as a result of injury to vital organs and tissues. Examples of autoimmune diseases include, but are not limited to, Addison's disease, alopecia
30 of autoimmune diseases include, but are not limited to, Addison's disease, alopecia

areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, type I diabetes, dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves's disease, Guillain-Barre syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia
5 gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

[00106] The term “**autologous**” refers to any material derived from the same individual to whom it is later to be re-introduced.

10 [00107] The term “**B cell**,” or “**B lymphocyte**,” refers to one of the two major types of lymphocytes. Each B cell expresses a particular antigen receptor on its cell surface. On activation by an antigen, B cells differentiate into cells producing antibody molecules of the same antigen specificity as this receptor.

[00108] The terms “**biologically active**” or “**biologically active form of the protein**,” as used herein, are meant to include forms of the proteins or antigens that
15 are capable of effecting enhanced activated NK cell proliferation.

[00109] A “**form of the protein**” is intended to mean a protein that shares a significant homology with the IL-15 or the antigens and is capable of effecting stimulation and proliferation of NK cells.

20 [00110] The term “**cell culture**” refer to cells grown in suspension or grown adhered to a variety of surfaces or substrates in vessels such as roller bottles, tissue culture flasks, dishes, multi-well plates and the like. Large scale approaches, such as bioreactors, including adherent cells growing attached to microcarriers in stirred fermentors, are also encompassed by the term “cell culture.”

25 [00111] The terms “**cell culture medium**,” or “**culture medium**” refer to a chemical composition that supports the growth and/or differentiation of a cell, including a mammalian cell. Typical culture media include suitable nutrients (e.g. sugars, amino acids, proteins, and the like) to support the growth and/or differentiation of a cell. Media for the culture of mammalian cells are well known to
30 those of skill in the art and include, but are not limited to Medium 199, Eagle's Basal

Medium (BME), Eagle's Minimum Essential Medium (MEM), alpha modification MEM (MEM), Minimum Essential Medium with Non-Essential Amino Acids (MEM/NEAA), Dulbecco's Modification of Eagle's Medium (DMEM), McCoy's 5 A, Rosewell Park Memorial Institute (RPMI) 1640, modified McCoy's 5 A, Ham's
5 F10 and F 12, CMRL 1066 and CMRL 1969, Fisher's medium, Glasgow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), Leibovitz's L-15 Medium, McCoy's 5A medium, S-MEM, NCTC-109, NCTC-135, Waymouth's MB 752/1 medium, Williams' Medium E, and the like. Cell culture media are commercially available (e.g. from GibcoBRL, Gaithersburg, Md.) and
10 even custom-developed culture media are commercially available (see, e.g., Specialty Media, Cell and Molecular Technologies, Inc., Phillipsburg, N.J.). In some embodiments, the culture medium refers to GMP Serum-free Stem Cell Growth Medium (SCGM) by CellGenix.

[00112] The term “**conditioned medium**” refers to culture medium that has been
15 in contact with live cells and contains a range of cell-derived molecules (e.g. growth substances, etc.) that when placed in contact with a subsequent batch of cells may enhance the growth or differentiation of subsequent cells. The term “non-conditioned medium” refers to cell medium that has not been in contact with cells. In the absence of a description, media should be understood to mean non-
20 conditioned media.

[00113] The term “**cell product**” or “**cell products**” as used herein refers to any and all substances made by and secreted from a cell, including but not limited to, protein factors (i.e. growth factors, differentiation factors, engraftment factors, cytokines, morphogens, proteases (i.e. to promote endogenous cell delamination,
25 protease inhibitors), extracellular matrix components (i.e. fibronectin, etc.).

[00114] The term “**clone**” refers to a group of cells that share a common ancestry, meaning they are derived from the same cell. Thus there are terms like “polyclonal” - derived from many clones; “oligoclonal” - derived from a few clones; and, “monoclonal” - derived from one clone.

30 [00115] The term “**clonogenic**” population refers to a population of cells derived from the same precursor cell by continuous proliferation. A clonogenic population

may include precursor cells, activated cells and differentiated cells, or any combination thereof. A “monoclonal population” refers to cells derived from a single precursor cell. Thus a clonogenic collection of cells can be monoclonal or polyclonal but in either case it results from specific preselected clones.

5 [00116] The term “**cloning efficiency**” is defined as the percentage of cells which can form vital cell populations of preferably more than 50 cells after being deposited. If for example in a cell sorting operation 50 cells are distributed over 50 culture vessels and if 25 of these 50 individually deposited cells grow to form cultures, the cloning efficiency is 50% (25 out of 50).

10 [00117] The term “**cognate**” refers to two biomolecules that interact, such as a ligand and its receptor, an antibody and the antigen it is specific for, etc.

[00118] The term “**crude NK cells**” or “**crude NK cell populations**” refers to a heterogeneous NK cell preparation where total NK cells have been isolated from a tissue sample (e.g., human blood) using common NK markers (e.g. NK cells
15 obtained from PBMC using either a negative selection method, e.g. a cocktail of magnetically labeled mAbs specific for non-NK lineage antigens (Miltenyi Biotec), or using a positive selection method, e.g. a cocktail of magnetically labeled mAbs specific for NK lineage antigens (Miltenyi Biotec)) but where there has been no further clonogenic separation of the NK cells. Occasionally “**crude**” in the foregoing
20 term is replaced by “**bulk**” without a difference in meaning.

[00119] The term “**cytokines**” refers to peptide protein mediators that are produced by immune cells and that modulate immune cell functions. Examples of cytokines include, but are not limited to, IL-1b, IL-2, IL-6, IL-15, IFN-g, TGF-b, G-CSF, GM-CSF, and TNF α . In contrast, the term “in the absence of additional
25 exogenous cytokines” as used herein with respect to a cell culturing condition refers to culturing a cell *in vitro* without adding additional soluble exogenous cytokines (although cytokines may be trans-presented by feeder cells). In specific embodiments, it occurs in the absence of added IL-2 in excess of 1 IU/mL, which may also be referred to as “IL-2 free” culturing conditions. In further specific
30 embodiments, “in the absence of cytokines” refers to a culturing condition that

contains IL-15 trans-presentation using a soluble IL-15 agonist complex but no added exogenous cytokines.

[00120] The term “**cytotoxicity**” refers to the quality of being toxic to cells. Examples of toxic agents include chemical substances, or immune cells such as cytotoxic lymphocytes such as cytotoxic T cells, NK cells and NK like T-cells. NK cells and NK like T-cells stand out with their high cytotoxic capacity. A skilled person can determine the cytotoxicity using available methods. One way of determining if cells exhibit an increased cytotoxicity is to use the *in vitro* analysis of cell mediated cytotoxicity against BaF/3 or K562 cells using the standard ⁵¹Cr-release assay. Alternatively, the degranulation assay can be used. Both these methods are disclosed in the Examples.

[00121] The term “**differentiation**” refers to the process by which cells become more specialized to perform biological functions, and differentiation is a property that is totally or partially lost by cells that have undergone malignant transformation.

[00122] The term “**effective amount**” of an active ingredient of a composition refers to a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, e.g., an amount which results in the attenuation, elimination, or prevention, or delay of onset, or a decrease, in at least one clinical and/or subclinical parameter associated with a disease that is being treated. The amount administered to a subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight, and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

[00123] The term “**endogenous**,” when used in reference to a polypeptide, means that which is naturally produced (e.g., by an unmodified mammalian or human cell). As used herein, the terms “**endogenous**” and “**native**” are interchangeable.

[00124] The term “**expansion**” or “**expanding**” or “**expand**” refers to growing cells in culture to achieve a larger population of the cells.

[00125] The term “**expression**” as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a

- desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, and in situ hybridization to cellular RNA or by PCR, among other methods. Proteins encoded by a selected sequence can be quantified by various methods including, but not limited to, e.g., ELISA, Western blotting, radioimmunoassays, immunoprecipitation, assaying for the biological activity of the protein, or by immuno staining of the protein followed by FACS analysis.
- 10 [00126] The term “**feeder cell**” refers to a culture of cells that grows *in vitro* and provides support to the growth and/or maintenance of another cell of interest in culture. Feeder cells can secrete at least one factor into the culture medium to support the growth of the other cell, or can express at least one molecule on their surface that can aid the growth of the other cell. For example, a feeder cell can trans-
15 present IL-15 on its surface to promote the growth of NK cells in culture. A feeder cell can comprise a monolayer, where the feeder cells cover the surface of the culture dish with a complete layer, or can comprise cells in suspension. Desirably feeder cell proliferation is inhibited, by any suitable means, such as irradiation, to avoid contaminating the supported growth of cells.
- 20 [00127] The term “**feeder cell-free**” means culture media and also cultivation conditions which are characterized in that cells are grown in the absence of any feeder cells.
- [00128] The term “**nucleic acid construct**” refers to a DNA or RNA molecule that comprises a nucleotide sequence encoding a protein of interest. The coding
25 sequence includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the individual to whom the nucleic acid molecule is administered.
- [00129] The term “**graft versus host disease**” (GVHD) refers to a condition that
30 occurs when immune cells present in donor tissue mount an immune response

against the host, or recipient, of the allografted cells or tissue. GvHD thus amounts to a rejection of the host by immune cells of the graft.

[00130] The term “**HLA**” is an acronym for “**human leukocyte antigen**” and refers to the human major histocompatibility complex (MHC).

- 5 [00131] The term “**HLA haplotype**” refers to a linked set of genes associated with one haploid genome, which determines the HLA of cells from an individual.

[00132] The term “**host**” refers to an individual to whom transplanted cells, tissues, organs, or other biological material is transplanted. “Recipient” and “host” are used interchangeably.

- 10 [00133] The term “**HCT**” or “**HSCT**” refers to hematopoietic (stem) cell transplantation, the transplantation of multipotent hematopoietic stem cells, usually derived from bone marrow, peripheral blood, or umbilical cord blood. It is a medical procedure in the fields of hematology, most often performed for patients with certain cancers of the blood or bone marrow, such as multiple myeloma, lymphoma or
15 leukemia. In these cases, the recipient's immune system is usually destroyed with radiation or chemotherapy before the transplantation. Infection and graft-versus-host disease is a major complication of allogenic HSCT.

- [00134] The term “**IL-15**” refers to interleukin 15, a cytokine that stimulates NK cells (NM 172174) (Fehniger T A, Caligiuri MA. Blood 97(1): 14-32, 2001). The
20 term “**IL-15R α** ” refers to interleukin 15 receptor alpha protein (NM_002189).

- [00135] The term “**IL-15 trans-presentation**” refers to the chaperoning of IL- 15 to, and presenting on the cell surface of IL-15 expressing cells. For example, it has been reported that IL-15R α expressing cells can chaperone IL- 15 to the cell surface, where IL-15 is available to exert its function, e.g., activating NK cells, and
25 promoting NK cell proliferation, among other functions (Mortier, E., et al. 2008. J Exp Med 205:1213-1225; Ma A., et al. 2006. Annu Rev Immunol 24:657-679; Rubinstein, M.P., et al. 2006. Proc Natl Acad Sci USA 103:9166-9171). For example, to express membrane-bound IL-15 a construct consisting of human IL-15 mature peptide and another construct consisting of human IL-15 receptor alpha
30 peptide are co-transfected to a feeder cell.

[00136] The term “**immortalize**” encompasses the process of whereby a cell line can be passaged indefinitely in culture, while the cells in culture retain the functions and features of the primary cells in the culture the day the culture was begun.

[00137] The term “**immune response**” refers to the individual or concerted
5 targeted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by these cells or the liver (including antibodies, cytokines, and complement) that ultimately results in capture of or damage to, destruction of, or elimination from an individual's body of antigens or cells that originate from a source other than that the individual's body. In cases of
10 autoimmunity or pathological inflammation, the immune response is directed to normal cells or tissues of the same individual rather than to nonself cells.

[00138] As used herein the term “**isolated**” is meant to describe a polynucleotide, a polypeptide, or a cell that is in an environment different from that in which the polynucleotide, the polypeptide, or the cell naturally occurs. An isolated genetically
15 modified host cell may be present in a mixed population of genetically modified host cells. An isolated polypeptide will in some embodiments be synthetic. “Synthetic polypeptides” are assembled from amino acids, and are chemically synthesized *in vitro*, e.g., cell-free chemical synthesis, using procedures known to those skilled in the art.

[00139] The terms “**KIR**” or “**Killer cell immunoglobulin-like receptors**” refer to immune receptors expressed on cells of the innate immune system (NK cells and certain T-cells). KIR genes form a rapidly evolving and diverse gene family. KIRs recognize MHC molecules on cells of self and can inhibit natural killer cell activation. KIRs contribute to an important innate immune monitoring of steady
25 intracellular sampling and declaration of cell content on cell surfaces by MHC molecules. Some KIRs have an activating function on killer cells and probably evolved secondarily from inhibitory KIRs, possibly in response to pathogens that produce MHC-mimicking molecules. Humans have 15 different KIR genes encoding receptors specific for the polymorphic determinants of MHC class I
30 molecules (HLA-A, B and C).

[00140] The term “**MHC**”, as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism.

5 [00141] The term “**natural killer (NK) cells**” as used herein, refers to non-T, non-B lymphocytes that are defined by the ability to kill a target cell “naturally,” that is, in a spontaneous fashion that did not require any priming and was not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. Human NK cells are traditionally defined by their cell surface markers. The traditional cell surface phenotype defining human NK cells within the lymphocyte gate on a flow cytometric analyzer shows an absence of CD3 (thereby
10 excluding CD4 and CD8 T cells, T regulatory cells and NKT cells) and expression of CD56 (thereby excluding B cells, monocytes and dendritic cells). Additionally, NK cells may express additional cell surface markers such as NKp46, a member of the highly conserved natural cytotoxicity receptor (NCR) family of NK-activating
15 receptors.

[00142] The term “**phenotype of interest**” or “**phenotypic trait of interest**” refers to an observable physical or biochemical characteristic of a cell or a cell population, as determined by either genetic makeup or environmental influences. For example, the phenotypic trait could include observable expression of a particular
20 gene or a set of genes.

[00143] The term “**polypeptide**” is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, and to longer
25 chains, commonly referred to in the art as proteins. Polypeptides, as defined herein, may contain amino acids other than the 20 naturally occurring amino acids, and may include modified amino acids. The modification can be anywhere within the polypeptide molecule, such as, for example, at the terminal amino acids, and may be due to natural processes, such as processing and other post-translational
30 modifications, or may result from chemical and/or enzymatic modification techniques which are well known to the art.

[00144] The term “**positive selection**” refers to conditions which distinguish cells expressing the selective gene so that these cells can be easily isolated. Non-limiting examples of positive selection include Fluorescence Activated Cell Sorting (FACS) and magnetic bead sorting. Another example of positive selection is the CD3⁺CD56⁺ NKT Cell Isolation Kit from Miltenyi Biotech. On the other hand, the term “negative selection” refers to conditions which distinguish cells not expressing the selective gene so that these cells can be easily isolated. Non-limiting examples of negative include Fluorescence Activated Cell Sorting (FACS) and magnetic bead sorting. An example of negative selection is the MACSxpress NK Cell Isolation Kit from Miltenyi Biotech.

[00145] The term “**preferential expansion**” refers to conditions that favor the growth or proliferation of one cell type versus another in a mixed population of cells. In one example, a preferential expansion of NK cells refers to conditions where the number of NK cells in a culture increase (on a percentage basis) to a greater extent than non-NK cells in the culture. For example, a preferential expansion of NK cells may be an increase in the cell number that is at least 5%, at least 10%, at least 25%, at least 50%, at least 75%, or at least 100% greater than the increase in the number of non-NK cells. In one embodiment, only NK cells proliferate (and non-NK cells do not proliferate) in response to the culture conditions.

[00146] The term “**primary cells**” encompasses cells derived from the original tissue as obtained and manipulated to generate primary cultures.

[00147] The term “**purified**” with respect to a compound or a cell of interest means that the same has been separated from components that accompany it in nature. “Purified” can also be used to refer to a compound or a cell of interest separated from components that can accompany it during manufacture (e.g., in chemical synthesis). In some embodiments, a compound is substantially pure when it is at least 50% to 60%, by weight, free from organic molecules with which it is naturally associated or with which it is associated during manufacture. In some embodiments, the preparation is at least 50%, at least 75%, at least 90%, at least 95%, or at least 99%, by weight of the compound, or by number of the cells of

interest. Purity can be measured by any appropriate method, e.g., immune-chromatography, FACS, cell sorting, mass spectroscopy, high performance liquid chromatography analysis, etc.

5 [00148] The term “**receptor**” is broadly defined to include membrane-bound or membrane associated macromolecules that may be targeted by a pharmaceutical agent, or that are the target of a physiological ligand. The term “receptor” also includes macromolecules that are covalently or non-covalently associated with the outside surface of the plasma membrane, and not necessarily inserted into the phospholipid bilayer of the plasma membrane. The term “cell surface receptor”
10 refers to cell membrane-bound or membrane-associated macromolecules.

[00149] The term “**rejection**” is used in the present disclosure in a context of cell/tissue/organ transplant, and is related to the process by which a transplanted cell, tissue and/or organ is rejected by the immune system of the recipient, which destroys the transplanted cell, tissue and/or organ.

15 [00150] The term “**sample**” or “**specimen**” is any mixture of macromolecules obtained from a person or other subjects. This includes, but is not limited to, blood, plasma, urine, semen, saliva, lymph fluid, meningeal fluid, amniotic fluid, glandular fluid, and cerebrospinal fluid. This also includes experimentally separated fractions of all of the preceding. Each of these terms also includes solutions or mixtures
20 containing solid material, such as feces, cells, tissues, and biopsy samples.

[00151] The term “**self-antigen**” refers to an antigen that is expressed by a host cell or tissue.

[00152] The term “**side effects**” encompasses unwanted and adverse effects of a therapy. Unwanted effects are not necessarily adverse. An adverse effect from a
25 therapy might be harmful or uncomfortable or risky. Examples of side effects include, but are not limited to, graft versus host diseases, host versus graft diseases, rhinitis, diarrhea, cough, gastroenteritis, wheezing, nausea, vomiting, anorexia, abdominal cramping, fever, pain, loss of body weight, dehydration, alopecia, dyspnea, insomnia, dizziness, mucositis, nerve and muscle effects, fatigue, dry
30 mouth, and loss of appetite, rashes or swellings at the site of administration, flu-like symptoms such as fever, chills and fatigue, digestive tract problems and allergic

reactions. Additional undesired effects experienced by patients are numerous and known in the art. Many are described in the Physician's Desk Reference (58th ed., 2004).

5 [00153] The term “**single positive**” refers to a cell that contains only one cell surface target that is bound by a polypeptide agent, as described herein. The term “double positive” refers to a cell that contains two different cell surface targets (different target species) that are bound by a polypeptide agent described herein. The polypeptide agents described herein bind double positive cells with high avidity.

10 [00154] The term “**solid support**” means any surface capable of having an agent attached thereto and includes, without limitation, metals, glass, plastics, polymers, particles, microparticles, co-polymers, colloids, lipids, lipid bilayers, cell surfaces and the like. Essentially any surface that is capable of retaining an agent bound or attached thereto. A prototypical example of a solid support used herein, is a particle such as a bead.

15 [00155] The term “**substantially free of**” with respect to a population of cells, e.g. NK cells, refers to a population that is at least 50% free of non-NK cells, or in certain embodiments at least about 60, 70, 80, 85, 90, 95 or 99% free of non-NK cells.

20 [00156] The term “substantially pure” with respect to a population of cells, e.g. NK cells, refers to a population that is at least 50% NK cells, or in certain embodiments at least 60, 70, 80, 85, 90, 95 or 99% of NK cells.

[00157] The term “**syngeneic**” means genetically identical members of the same species.

25 [00158] The term “**tolerance**” or “**tolerant**”, as used herein, refers to the inhibition of a transplant recipient's ability to mount an immune response, e.g., to a donor antigen, which would otherwise occur, e.g., in response to the introduction of a non self MHC antigen into the recipient. Tolerance can involve humoral, cellular, or both humoral and cellular responses. The concept of tolerance includes both complete and partial tolerance. In other words, as used herein, tolerance include any

degree of inhibition of a graft recipient's ability to mount an immune response, e.g., to a donor antigen.

[00159] The term “**treatment**” or “**treating**” means any administration of a therapeutic agent which can be for example cells or a compound and include (1) inhibiting (slowing down or arresting) the progression of disease in an animal or a human that is experiencing or displaying at least one abnormal value or at least one symptom in a relevant parameter of the disease (i.e., arresting or slowing down further development of at least one abnormal value in a relevant parameter or at least one symptom), or (2) ameliorating the disease in an animal or a human that is experiencing or displaying at least one abnormal value or at least one symptom in a relevant parameter the disease (i.e., reversing at least one abnormal value or at least one symptom in a relevant parameter).

[00160] The term “**viability**” with respect to a cell refers to relative amounts of living and dead cells, present with a population of cells at any given time. Cell viability may be determined by measuring the relative numbers of living and dead cells in any given sample of the population. Cell viability may also be estimated by measuring the rate of cell proliferation of the entire population which represents the overall balance of the rates of cell growth and cell death. Rates of cell growth may also be directly measured, by counting the number of cells, and by using any number of commercially available cell proliferation assays which directly scores the rate of cell growth.

[00161] The term cell “**yield**” shall refer to the number of viable cells after *in vitro* expansion divided by the number of viable cells before the process. It will be appreciated that the number of viable cells can be ascertained in various ways known to those of skill in the art and as described herein.

Methods for the Production of Clonogenic Natural Killer (NK) Cell Populations

[00162] In some aspects, the present disclosure provides methods for *in vitro* expansion of clonogenic natural killer (NK) cells. The methods utilize the stimulatory effects of trans-presented IL-15 in cell culture using feeder cells to expand isolated NK cell clones with a predetermined phenotypic trait of interest. The expanded clonogenic NK cell populations are homogenous, in contrast to

heterogenous, crude NK cells typically isolated and expanded from a human tissue sample, and such populations constitute another aspect described in the present disclosure.

[00163] The present disclosure also relates to feeder cells that are genetically engineered to trans-present IL-15 involving co-expressing human IL-15 and IL-15 receptor α subunit (IL-15R α) that are capable of stimulating the expansion of NK cell clones *in vitro*. The present disclosure further relates to viable, functional clonogenic NK cell populations with the predetermined phenotypic trait of interest obtained via the above *in vitro* expansion methods and their use in personalized medicine to treat diseases, including leukemia, lymphoma and HCT, in drug screening and in basic and translational studies involving NK cells.

1. Methods for Isolating, Selecting, and Expanding Human NK Cell Clones
In vitro

[00164] The present disclosure provides methods of isolating primary NK cell clones from mammalian tissues, and methods for expanding these clones in the presence of IL-15 trans-presentation. These methods are able to expand NK clones to more than 0.1 million, and sometimes more than 1 million from a single cell, thus achieving a population of defined, homogenous NK cells that can be used in clinical applications such as cell therapy and for basic and translational research. In addition, the present disclosure provides methods of culturing, expanding, and cloning NK cells to produce a population of NK cells that are substantially free of contaminating cell types, e.g., CD8⁺ T cells (e.g., killer T cells), which are common problems found with NK cells prepared by existing protocols. As these contaminating cells are biologically active themselves with functions different from NK cells, their contamination would be likely to cause side effects in NK cell therapy or bias the NK cells and thereby reduce their efficacy. Thus, the current disclosure provides methods for producing NK cells that are suitable for clinical and research use with defined populations and significantly higher clinical efficacy and significantly less side effects. Accordingly, the present disclosure provides highly efficient methods of generating a population of clonogenic NK cells from single cell NK clones. These methods are particularly useful in generating therapeutic amounts of clonogenic NK cells with desirable characteristics and functions.

[00165] The present disclosure is based on the concept that clonogenic expansion of human NK cells *in vitro* could generate purified populations of NK cells in large numbers that possess a desirable phenotypic trait of interest. And this purified clonogenic NK cell population has advantages over crude NK cells isolated from human samples, e.g., PBMC, or *in vitro* expanded NK cells from such crude NK cells. For example, NK cells are heterogeneous with regard to the expression pattern of cell surface receptors, including killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors (CLLR), natural cytotoxicity receptors (NCR), and chimeric antigen receptors (CAR). Expansion of NK clones that occur naturally with low frequency and that possess a desirable phenotypic trait of interest, such as a certain expression pattern of cell surface receptors, can result in isolated purified clonogenic NK cell populations that express a specific phenotype and comprise a large number of cells. In some embodiments, these populations can match to particular recipient patients in HCT and in cancer immunotherapy, or are substantially more efficient with regard to exerting NK cell cytotoxicity and cytolytic activity compared to crude NK cells isolated from a tissue sample.

a). Isolation and Selection of NK Cell Clones

[00166] The present disclosure describes methods for producing a population of clonogenic NK cells that have a predetermined, desirable phenotypic trait of interest. After NK cells are isolated from a tissue sample, individual NK cell clones can be obtained by a variety of methods described below, preferentially through targeted cell sorting. In some embodiments, the NK cells are isolated from a tissue sample, such as a blood sample. NK cells can be isolated from PBMC by methods that recognize NK lineage markers, such as, e.g., CD56.

[00167] In particular embodiments, preferred clonogenic populations for further expansion are identified based upon their having one or more desirable characteristics, such as, e.g., cytotoxic activities; cytolytic activities; or expression of one or more cell markers with a predetermined functionality. In certain embodiment, clonogenic populations are screened for a desired characteristic using high throughput methods. Screening may be performed using a variety of techniques available (See J.S. Bonifacino et al., Current Protocols in Cell Biology, ISBN: 9780471143031). For example, screening for cytoplasmic or nucleic markers may

be performed by immunocytochemistry-based assays or polymerase chain reaction (PCR)-based assays, such as reverse transcriptase-PCR (RT-PCR), using antibodies or oligonucleotides that bind to a polypeptide or gene more highly expressed in cells having the desired phenotype as compared to other cells.

5 **[00168]** In some embodiments, the present disclosure provides a method of isolating NK clones from a tissue sample, comprising isolating crude NK cells according to a phenotype of interest, e.g., by their cell surface markers of NK lineage, and further separating the obtained NK cells according to the phenotype of interest. The tissue sample can be obtained from a healthy donor or a diseased
10 patient, with the provenance depending on the ailment aimed to be treated. For example, if the disease is HIV-1 infection, the NK cells are usually obtained from the patient; if the disease is AML being treated with HCT, the NK cells are obtained from a healthy donor (HLA matched or HLA mismatched with the patient). In a particular embodiment, the tissue sample is PBMC from peripheral blood. PBMC is
15 a primary source of NK cells, and PBMC can be obtained either from blood supplies at hospitals or blood banks or from a particular donor or patient. PBMC can be isolated from blood via a variety of methods by those skilled in the art. For example, PBMC can be isolated by centrifugation of peripheral blood using a Ficoll density gradient medium. PBMC contains a mixture of different cell types, including NK
20 cells, T cells, B cells, macrophages, etc.

[00169] A tissue sample may be isolated from a patient or donor by any means available in the art. In one embodiment, the tissue sample is a primary tissue explant. In certain embodiments, tissue is isolated by surgical removal or withdrawal using a needle biopsy. A variety of additional procedures are described in U.S. Pat. Nos.
25 6,020,196 and 5,744,360. Furthermore, tissue may be isolated from any suitable location on an animal, depending upon the type of tissue being isolated. In some embodiments, this tissue sample can be a blood sample, either obtained directly from a donor, or from a blood bank. In some other embodiments, this tissue sample can be a biopsy sample that contains NK cells, e.g., a spleen sample, a lymphatic
30 fluid sample.

[00170] NK cells are purified from other tissue components after or concurrent with the processing of a tissue sample. In one embodiment, NK cells are purified from other cells and tissue components after the tissue sample has been treated. NK cells may be obtained by routine methods, such as removing and centrifuging the media to pellet cells therein, and washing the cells remaining in the culture dish with a solution such as phosphate-buffered saline (PBS) or D-Hanks to remove those cells loosely attached to the adherent cell layer. This wash solution may then also be centrifuged to obtain cells.

[00171] In order to obtain purified NK cells, the cells purified from the tissue sample are sorted using one or more reagents that bind to cell surface (or internal) markers indicative of NK cells. For example, the present disclosure contemplates any suitable method of employing monoclonal antibodies to separate NK cells from other cells recovered from the tissue sample. These methods include, e.g., contacting a cell suspension comprising the cells purified from the tissue sample with one or a combination of monoclonal antibodies that recognize an epitope on NK cells; and separating and recovering from the cell suspension the cells bound by the monoclonal antibodies.

[00172] In one embodiment, cells are selected using antibodies bound to magnetic beads and a magnetic cell sorter device. In one embodiment, cells are selected by fluorescence activated cell sorting (FACS) using fluorescently labeled antibodies. In other embodiments, the monoclonal antibodies may be linked to a solid-phase and utilized to capture NK cells from tissue samples. The bound cells may then be separated from the solid phase by known methods depending on the nature of the antibody and solid phase. Examples of monoclonal antibody-based systems appropriate for preparing the desired cell population include magnetic cell sorting, FACS, magnetic bead/paramagnetic particle column utilizing antibodies for either positive or negative selection; separation based on biotin or streptavidin affinity; and high speed flow cytometric sorting of immunofluorescent-stained stem cells mixed in a suspension of other cells. Exemplary cell surface or internal markers of NK cell lineage include, but are not limited to, CD56, CD16, etc. Monoclonal antibodies that specifically bind to NK cells are known and commercially available, and many of these are specific for NK cells of certain subpopulations.

[00173] In another particular embodiment, monoclonal antibody-labeled magnetic beads are used to isolate NK cells. In some embodiments, NK cells are isolated by monoclonal antibody-labeled magnetic beads using positive selection. In another particular embodiment, NK cells are isolated by monoclonal antibody-labeled magnetic beads using negative selection, (e.g., depletion of other cells types and producing untouched NK cells). In another particular embodiment, a cocktail of magnetically labeled mAbs specific for non-NK lineage antigens from Miltenyi Biotec is used to produce NK cells from PBMC.

[00174] In one embodiment, the present disclosure provides a method of isolating and selecting NK cell clones with desirable characteristics. NK cells isolated from PBMC by lineage markers are heterogeneous populations of NK cells with different cell surface marker makeups. Clones of NK cells with defined characteristics can be obtained via a variety of methods by those skilled in the art (Brooks CG. Methods Mol Biol. 2000;121:13-24; Pittari et al., J. Immunol. 2013 190:4650-4660). Selection of NK cell clones can be done by a variety of methods.

[00175] In some preferred embodiments, NK cell clones are selected based on their cell surface receptor expression patterns, which can be done via a variety of methods, including FACS or immuno-conjugated magnetic beads. For example, fluorescent probe-labeled antibodies against NK cell surface receptors can be used to in FACS to separate NK cells isolated from a human tissue sample into subsets expressing and not-expressing the cell surface receptors that the antibodies recognize. Sometimes a combination of different antibodies can be used in such protocols to isolate NK cells with a particular phenotypic trait of interest. Antibodies to NK cell surface receptors are commercially available.

[00176] This phenotypic trait of interest can be the expression of certain cell surface receptors. These receptors include inhibitory NK receptors and activating NK receptors. Non-limiting examples of NK cell surface receptors that can be used in the selection of NK clones include killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors (CLLR), natural cytotoxicity receptors (NCR), and chimeric antigen receptors (CAR) (Pittari et al., J. Immunol. 2013 190:4650-4660). Non-limiting examples of KIR include KIR2DL1, KIR2DL2/3, KIR3DL1,

KIR2DS1, and KIR2DS2. Non-limiting examples of NCR include NKp46, NKp44, and NKp30. Non-limiting examples of CLLR include NKG2D or NKG2D-DAP10-CD3 ζ . Non-limiting examples of CAR include chimeric receptors comprising a CD19 peptide, a G(D2) peptide, a CS1 peptide, or a WT1 peptide.

5 [00177] Thus, once a desirable NK cell clone regarding to the expression or the non-expression of certain cell surface receptors is determined, such NK cell clones can be selected from a tissue sample, from a crude NK cell isolation, or from a subset of NK cells. Methods of selecting desirable NK clones with a predetermined trait include FACS, immuno-conjugated beads, RT-PCR, among others. In some
10 preferred embodiments, FACS is used to isolate desirable NK cell clones using fluorescent probe-conjugated mAbs (Pittari et al., J. Immunol. 2013 190:4650-4660). Non-limiting examples of fluorophore-labeled mAbs include: CD3-PE/Texas Red (S4.1, Invitrogen), CD56-PE/Cyanine7 (MEM-188, BioLegend), KIR2DL1/S1-PerCP/Cyanine5.5 (HP-MA4, eBioscience), KIR2DL1-Allophycocyanin (143211,
15 R&D Systems), KIR2DL2-3/S2-FITC (CH-L, Miltenyi Biotec), KIR3DL1-Alexa Fluor700 (DX9, BioLegend), KIR3DL1/S1-PE (Z27, Beckman Coulter), NKG2A-PE (131411, R&D Systems), CD85j/ILT2 (LILRB1)-PE (HP-F1, Beckman Coulter).

[00178] Both positive selection and negative selection methods can be used to isolate NK cell clones with a predetermined phenotypic trait. In both cases, the
20 selection of NK cell clones for further *in vitro* expansion can be based upon prior knowledge on the function of NK cell surface receptors. Positive selection entails the selection of NK cells that express the predetermined receptor or receptors. For example, KIR2DS1 has been shown to play an important role in HCT (Pittari et al., J. Immunol. 2013 190:4650-4660), thus it would be desirable to isolate NK cell
25 subsets that express KIR2DS1. On the other hand, target selection of NK cell clones can use a negative selection method, which encompasses the use of antibodies against NK cell surface molecules that an investigator wishes to exclude. For example, if NK clones without expression of KIR2DS1 cells are desired, an mAb against KIR2DS1 can be used to bind and eliminate KIR2DS1-expression NK cells.
30 Therefore, both positive and negative selection methods can be used to select NK cell clones of interest.

[00179] In some embodiments, the selection of NK clones with a desirable trait of interest depends on the application of the NK clones, when expanded, to a recipient. For example, in HCT, certain NK clones can be selected, expanded *in vitro*, and transplanted into a recipient undergoing cancer immunotherapy. The selection of
5 NK clones from a donor demands such clones to have increased efficiency, and reduced side-effects. Thus, the present disclosure describes the selection of NK cell clones based on the genotype of HLA class I molecules from the donor and the recipient. In some embodiments, at least one NK cell clone is selected from a donor whose HLA class I genotype mismatches that of the recipient, i.e. the NK cell clones
10 from the donor have the property of detecting “missing self-HLA class I ligand” in said recipient.

[00180] The present disclosure provides exemplary criteria for selecting certain NK cell clones with regard to the genotype of a recipient. In some embodiments, the genotype of KIR in the donor NK cell and the HLA class I genotype are the main
15 criteria for selection. In some embodiments, the NK clones express at least one cell surface receptor selected from the group consisting of inhibitory KIR with ligand specificity for HLA class I and, optionally selected from the group consisting of activating KIR, c-type lectin-like receptors, natural cytotoxicity receptors, and NK-activating chimeric receptors. In some embodiments, the inhibitory KIR is selected
20 from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1 and said at least one cell surface receptor is selected from the group consisting of KIR2DS1, KIR2DS2, NKG2D, NKp46, NKp44, and NKp30; NKG2D-DAP10-CD3ζ, and a chimeric receptor comprising one or more peptide selected from the group consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.

25 [00181] The present disclosure also provides some examples that may be of particular interest for HCT and cancer immunotherapy. In some embodiments, the donor NK cell clone and its preferred recipient genotype can be selected from a row in Table VII.

[00182] In addition to targeted NK cell selection, NK cell clone can be obtained
30 by limited dilution or single cell sorting, using established protocols known to those skilled in the art. Alternatively, NK cells can be selected according to other chemical

or physical characteristics using common techniques, such as the size of the cell (e.g., size exclusion chromatograph, HPLC), the density of the cell (e.g., density gradient centrifugation), the morphology of the cell (e.g., microscope-aided cell capture and isolation), among others.

5 **[00183]** In some embodiments, single NK cell clones with a desirable phenotypic trait of interest can be isolated and deposited into a cell culture vessel as preferentially single cells, or multiple cells alternatively. These cells can be then maintained in cell culture with suitable culture media and maintained under conditions with suitable temperature, humidity and CO₂ concentration for cell
10 maintenance. Alternatively, these clones can be stored for later use under conditions suitable for such purposes, e.g., liquid N₂. In a preferred embodiment, as demonstrated in the Examples, NK cell clones isolated from human PBMC are selected based upon their cell surface receptor expression by FACS and are deposited into U-shaped polystyrene 96-well plates as single cell conditions, and are
15 cultured in a CellGro SCGM medium supplemented with heat-inactivated AB human, penicillin, streptomycin and L-glutamine. These clones are then ready for *in vitro* expansion.

[00184] The NK cells that can be cultured and expanded using methods described by the present disclosure are not limited to native NK cells that can be obtained from
20 a tissue sample. In some embodiments, the NK cells can be *in vitro* differentiated NK cells from precursor cells, such as NK progenitor cells or from stem cells. The stem cells can be HSC, ESC or iPSC. These precursor cells can be differentiated into NK cells using methods known by those skilled in the art (Luevano M., et al., Cell Mol Immunol. 2012, Jul;9(4):310-20; Vandekerckhove B. et al., Front Biosci
25 (Landmark Ed). 2011, 16:1488-504).

[00185] The NK cells that can be cultured and expanded using methods described by the present disclosure are also not limited to NK cells isolated from a tissue sample or differentiated by a precursor cell. These NK cells could undergo further modifications before being cultured using methods described by the present
30 disclosure. For example, these NK cells can be genetically modified, e.g., certain genes can be inserted, deleted or modified, before being cultured by the present

methods. Genes that could potentiate NK cell cytotoxicity could be introduced to these designer NK cells to increase their effects for cell therapy. For example, pro-apoptotic serine protease granzyme B (GrB) plays an important role in NK cell killing activity, and human NK cells transduced with pre-pro-GrB showed
5 augmented tumor cell killing activity (Oberoi P. et al., PLoS One. 2013: 8(4):e61267).

b). *In vitro* Expansion of NK Cell Clones

[00186] The present disclosure provides a method of expanding NK clones *in vitro*. For cell therapy, NK cells need to be expanded to certain numbers for clinical
10 use. Additionally, other applications of NK cells, such as for basic and translational studies, drug screening, etc., may also require a large quantity of NK cells. Furthermore, some subsets of NK cells with a desirable phenotypic trait of interest may be rare *in vivo* in humans, and such low frequency NK cell subsets need to be expanded *in vitro* to achieve the sufficient numbers for clinical, research and drug
15 screening purposes.

[00187] Thus, an efficient protocol that is capable of expanding NK cell clones with desired traits is very important for clinical applications of NK cells in therapy and for studies involving NK cells. Previous methods of expanding NK cells can achieve at most an expansion ratio (i.e., the ratio of final cell numbers to the initial
20 cell numbers) in the order of 10^3 (See US Patent NO 8,026,097; see also patent application WO 2013/094988). In contrast, methods described by the present disclosure can routinely expand single cell NK clones to more than 10^5 , and often more than 10^6 (i.e., expansion ratio of 10^5 or 10^6), which is more than 100 to 1000-times more efficient compared to previous methods. Previous methods of expanding
25 NK cells also suffer from low cloning efficiency, e.g., 1%~5% (i.e., out of 100 NK cell clones, about 1%~5% could expand to more than 10^4 cells) (N.M. Valiante et al., Immunity, Vol. 7, 739-751, 1997). In contrast, methods described by the present disclosure can routinely expand more than 30% of clones to a final cell number of more than 10^5 cells per clone.

30 [00188] Another advantage of the present disclosure over previous methods of human NK cell culturing is that previous methods rely on high concentrations of

cytokines. The addition of cytokines to cell culture media may have undesirable effects on NK cells. Some cytokines can activate NK cells, and promote phenotypic changes in NK cell surface receptor expression. Some cytokines can induce NK cell differentiation, also changing the characteristics of the original NK cell clone. In order to preserve the characteristic of the original NK cell clone, the addition of cytokines to cell culture media should be reduced or eliminated. In particular, virtually all existing NK cell culture protocol involves the addition of IL-2 at various concentrations from 10 IU/mL to more than 200 IU/mL. An advantage of the present disclosure is that it eliminates the requirement of IL-2 from the cell culture media. In some embodiments, the cell culture medium is without any added cytokine, including IL-2 and IL-15.

[00189] Once a NK cell clone is selected or obtained, the present disclosure describes a novel method of expanding these single cell clones *in vitro*. This method comprises (a) culturing a human NK cell clone (i) in the presence of a feeder cell, wherein said feeder cell trans-presents human interleukin-15 (IL-15), and (ii) in a culture medium that is without added IL-2; and (b) maintaining said culture (i) under conditions of temperature, humidity, and CO₂ that support the proliferation of said human NK cell clone and (ii) for a period of time sufficient to achieve expansion of said human NK cell into said clonogenic NK cell population.

[00190] In addition to NK cells that are isolated from a human tissue sample (i.e., native NK cells), this method can also be applied to expand genetically engineered NK cells (e.g., “designer” NK cells). In addition to human NK cells, the method of NK cell expansion described by the present disclosure may also be applied to NK cells isolated from animals. For example, the method may be applied to expand murine NK cells to aid in studies involving NK cells in *in vivo* murine models.

[00191] An important feature of the present disclosure is that it utilizes IL-15 trans-presentation to stimulate the growth and maintenance of NK cell cultures. Another important feature of the current disclosure is that it reduces the need for the addition of exogenous cytokines, especially IL-2, for the growth and maintenance of NK cell cultures.

c). IL-15 Trans-presentation

[00192] It has been found that IL-15 trans-presentation plays a vital role in stimulating NK cell proliferation in cell culture. In some embodiments, the present disclosure describes a method of expanding NK cell clones using feeder cells that are genetically engineered to trans-present human IL-15. Here, the definition of feeder cells is broad, and include not only cells that secrete growth factors into the cell culture media, but also cells that express certain molecules on their cell surface, which molecules support the proliferation and maintenance of another cell of interest in the cell culture. IL-15 trans-presentation plays a key role in activating NK cells and promoting NK cell proliferation. And feeder cells can provide a stable platform for IL-15 trans-presentation.

[00193] Multiple cell types can be used as the feeder cell. The selection criteria for such a feeder cell include, but are not limited to, its feasibility for IL-15 trans-presentation, its native expression and/or secretion of cytokines and/or growth factors that may interfere with NK cell culture, its expression of HLA molecules that would interact with certain NK cells, in addition to general considerations for feeder cells (e.g., easy to maintain, high proliferation rate, easy to transfect, etc.). Non-limiting examples of such feeder cells include pre-B-lymphocyte BaF/3 cell line, bone marrow stromal OP9 cell line, erythroleukemia K562 cell line, 721.221 B lymphoblastoid cell line, Burkitt lymphoma Daudi cell line, and Wilms tumor HFWT cell line. In some embodiments, the feeder cells include cells that are HLA class I negative cells, including a pre-B-lymphocyte cell line, a bone marrow stromal cell line, an erythroleukemia cell line, a B lymphoblastoid cell line, a Burkitt lymphoma cell, and a Wilms tumor cell. In a specific embodiment, the feeder cell is BaF/3. In another specific embodiment, the BaF/3 cells are additionally transfected with nucleic acids encoding for human IL-3. Human IL3 mRNA sequence is published (NM_000588) and its cDNA can be obtained commercially (e.g., RC210109 from OriGene). In other embodiments, the feeder cells include one or more of BaF/3, OP9, K562 and 721.221. Daudi cells, HFWT cells and HLA class I positive cells. In some embodiments, the feeder cells are surface antigen mismatched relative to an inhibitory surface KIR receptor(s) on the NK cell clone within the same cell culture.

[00194] These feeder cells normally do not express IL-15 at a high level (e.g., K562), or do not express human IL-15 (e.g., BaF/3), and IL-15 trans-presentation is thus achieved by modifying the feeder cells prior to use. To facilitate IL-15's expression on cell surface, IL-15R α is also expressed in the same feeder cell. In
5 some embodiments, the trans-presentation of IL-15 is achieved through co-expression of human IL-15 and human IL-15R α . The co-expression of IL-15 and IL-15R α can be achieved through delivery of nucleic acids encoding these two genes via a variety of methods.

[00195] Delivery of nucleic acids encoding IL-15 and IL-15R α to a cell can be
10 achieved through a variety of methods by one skilled in the art. Common techniques include viral transfections, and transfections using chemical and physical means (See M. Kriegler, Transfer and Expression: A Laboratory Manual. pp.96-107. ISBN 0716770040). Briefly, viral transfection can be achieved retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, lentivirus vectors. Chemical-
15 based transfections can use calcium phosphate, liposomes, polymers, cyclodextrin, or nanoparticles. Physical methods of transfection include electroporation, sonoporation, and optical transfection. Other methods of transfection include gene gun, magnetofection, and methods involving microprojectiles carrying the nucleic acids.

[00196] The introduction of IL-15 and IL-15R α can be achieved separately, i.e., nucleic acids encoding the two polypeptides being introduced separately. For example, nucleic acids encoding IL-15 and IL-15R α can be packed into 2 separate vectors. Or alternatively the introduction of IL-15 and IL-15R α can be achieved simultaneously, e.g., nucleic acids encoding the two polypeptides can be packed into
25 the same vector. Furthermore, the trans-presentation of IL-15 in these feeder cells can be inducible or constitutive depending on the requirement by using different vector design and transfection methods. The density of feeder cells can be optimized to support NK cell growth, and in some embodiments, the feeder cell density can range from about 10^4 /mL to about 10^6 /mL.

[00197] In one embodiment, the present disclosure provides a method of achieving IL-15 trans-presentation in feeder cells through transfection of IL-15. IL-

15 nucleic acid construct can contain a native IL-15 or a genetically modified IL-15, such as an IL-15 fusion protein (Imai et al., Blood. 2005;106:376-383). A chaperone protein, e.g. IL-15R α , can also be transfected at the same time with IL-15 to facilitate IL-15 trans-presentation. In a particular embodiment, a nucleic acid
5 construct containing native human IL-15 and another nucleic acid construct containing native human IL-15R α are transfected to feeder cells simultaneously. In a particular embodiment, the sequence of IL-15 and IL-15R α nucleic acid constructs are provided in SEQ ID NO:1 and SEQ ID NO:2, respectively. In a particular embodiment, the disclosure further encompasses nucleic acid molecules that are
10 substantially identical to the nucleic acid products described herein (e.g., SEQ ID NO:1 and SEQ ID NO:2), such that they are at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5% or greater.

15 **[00198]** In one embodiment, the present disclosure provides a method of clonogenic NK cell expansion involving additional NK stimulating factors in addition to IL-15 trans-presentation. Non-limiting examples of such stimulating factors include 4-1BBL. For example, 4-1BBL has been shown to enhance NK cell proliferation in *in vitro* cultures (Imai et al., Blood. 2005;106:376-383). In a
20 particular embodiment, the feeder cells are further transfected with a nucleic acid construct that contains human 4-1BBL. In some embodiments, one feeder cell expresses both 4-1BBL and trans-presents IL-15. In some preferred embodiments, the feeder cell is transfected with IL-15 and IL-15R α , and 4-1BBL. In some embodiments, 4-1BBL expression and IL-15 trans-presentation occur on different
25 feeder cells.

[00199] In one embodiment, the present disclosure provides a method of clonogenic NK cell expansion involving additional feeder cell types other than the feeder cells with IL-15 trans-presentation. Non-limiting examples of such additional feeder cells include a peripheral blood mononuclear cell (PBMC), an EBV-B
30 lymphoblastoid cell (EBV-BLCL), and an RPMI8866 lymphoblastoid cell. For example, allogenic PBMC have been used frequently in NK cell cultures (Munz et al., J. Exp. Med. 1997 185: 385-91; Cella & Colonna, Methods in Molecular

Biology, 2000: vol. 121). Allogeneic, human EBV-B lymphoblastoid (BLCL) cells have also been used (Pittari et al., J. Immunol. 2013 190:4650-4660). In a particular embodiment, these additional feeder cells include allogenic PBMC. In another particular embodiment, these additional feeder cells further include allogenic EBV-
5 BLCL. In a particular embodiment, the feeder cells undergo treatment to inhibit proliferation, e.g., irradiation. In a particular embodiment, the feeder cell density is between the order of 10^3 /mL and the order of 10^6 /mL.

[00200] Within certain aspects of the present disclosure, it is contemplated that feeder cells may be replaced with a solid support that mimics the trans-activation
10 achieved by a feeder cell. Assay systems may be developed to test the conditions for achieving optical cell proliferation, which assay systems are well known and readily available to those of skill in the art. Such supports will have attached on its surface at least one molecule capable of binding to NK cells and inducing a primary activation event and/or a proliferative response or capable of binding a molecule
15 having such an affect thereby acting as a scaffold. The support may have attached to its surface the IL-15 protein or an IL-15 receptor antibody. Preferably, the support will also have IL-15 receptor bound on its surface.

[00201] The invention is intended to include the use of fragments, mutants, or variants (e.g., modified forms) of the IL-15 or antigens that retain the ability to
20 induce stimulation and proliferation of NK cells. A “form of the protein” is intended to mean a protein that shares a significant homology with the IL-15 or the antigens and is capable of effecting stimulation and proliferation of NK cells. The terms “biologically active” or “biologically active form of the protein,” as used herein, are meant to include forms of the proteins or antigens that are capable of effecting
25 enhanced activated NK cell proliferation. One skilled in the art can select such forms based on their ability to enhance NK cell activation and proliferation upon introduction of a nucleic acid encoding said proteins into a cell line. The ability of a specific form of the IL-15 or antigens to enhance NK cell proliferation can be readily determined, for example, by measuring cell proliferation or effector function
30 by any known assay or method, e.g. a MTS/MTT assay (Pittari et al., J. Immunol. 2013, 190:4650-4660).

[00202] In one embodiment, the present disclosure provides a method of clonogenic NK cell expansion without feeder cells. A feeder cell-free culture system provides a more definite composition in cell culture media and may be suitable for certain applications. Although feeder cells could provide consistent, stable IL-15 trans-presentation, soluble IL-15 complexes have been reported to achieve IL-15 trans-presentation. These soluble IL-15 complexes, or so called IL-15 superagonists, can be used to replace IL-15 trans-presented feeder cells to stimulate NK cell proliferation. Non-limiting examples of soluble IL-15 complexes include IL-15/IL-15R α and ALT-803. For example, it has been reported that murine IL-15/IL-15R α soluble complex can expand NK cells *in vitro* (Dubois et al., The Journal of Immunology, 2008, 180: 2099–2106). In a particular embodiment, the soluble IL-15 complex is a human IL-15/IL-15R α complex in a concentration between 0.1 nM and 10nM. In a particular embodiment, the soluble IL-15 complex is ALT-803 in a concentration between 0.1 nM and 10nM.

15 d). Exogenous Cytokines for NK Expansion

[00203] In some embodiments, the present disclosure provides a method of clonogenic NK cell expansion without added exogenous IL-2 in the culture medium and in some embodiments without any other added exogenous cytokines in the culture medium. The addition of cytokines to cell culture media may have undesirable effects on NK cells. Some cytokines can activate NK cells, and promote phenotypic changes in NK cell surface receptor expression. Some cytokines can induce NK cell differentiation, also changing the characteristics of the original NK cell clone. In order to preserve the characteristic of the original NK cell clone, the addition of cytokines to cell culture media should be reduced or eliminated. In particular, virtually all existing NK cell culture protocols involve the addition of IL-2 at various concentrations from 10 IU/mL to more than 200 IU/mL. IL-2 has been commonly added in addition to IL-15 trans-presentation for NK cultures (Imai et al., Blood. 2005;106:376-383; Cho et al., Clin Cancer Res; 2010 16(15); 3901–9; US Patent 8,026,097). However, the IL-2 exerts multiple effects on NK cells and could affect NK cell biology. For example, IL-2 promotes NK cell cytolytic activity and modulates other pathways in response to antigen (See Liao W. et al., Immunity. 2013 Jan 24;38(1):13-25). Thus, the ability to reduce or eliminate IL-2 from NK cell

culture helps to preserve NK cell nature phenotype and biological functions. An advantage of the present disclosure is that it eliminates the requirement of IL-2 from the cell culture media. In some embodiments, the cell culture medium contains no IL-2 or IL-15.

5 e). Basal Media

[00204] In one embodiment, the present disclosure provides a method of clonogenic NK cell expansion using a medium that can sustain NK proliferation. A variety of cell culture media have been used in the art to culture NK cells. Non-limiting examples of media suitable for NK culturing include RPMI (Munz et al., J. Exp. Med. 1997, 185: 385-91; Hansasuta et al., Eur. J. Immunol. 2004, 34: 1673–1679), GBGM (US Patent Application 2012/0148553), DMEM (US Patent Application 2012/0148553), and SCGM (Pittari et al., J. Immunol. 2013, 190:4650-4660). In a particular embodiment, SCGM is used as the basal medium to expand NK clones *in vitro*.

15 f). General Culture Conditions

[00205] To produce clonogenic populations of NK cells, purified cells may be plated at a density of one cell clone per well ratio. In certain embodiments, cells are plated in plates, e.g., multiwell plates, precoated with basement membrane or extracellular matrix components, such as the solubilized basement membrane preparation, BD MatrigelTM (BD Biosciences). After seeding the culture medium, the culture medium is maintained under conventional conditions for growth of mammalian cells. In some embodiments, the culture medium contains from about 0% to about 20% human serum. The cell culture is maintained at conditions that are suitable for NK cell proliferation. In some embodiments, the temperature, humidity and CO₂ contents are controlled, with temperature from about 35°C and about 39°C and CO₂ is from about 3% to about 7%. In some preferred embodiments, the temperature is maintained at about 37°C and the CO₂ about 5%.

[00206] Additionally, fresh media may be conveniently replaced, in part, by removing a portion of the media and replacing it with fresh media. Various commercially available systems which have been developed for the growth of mammalian cells to provide for removal of adverse metabolic products,

replenishment of nutrients, and maintenance of oxygen. For example, these include automated or semi-automated systems such as Rotary Cell Culture Systems (Synthecon, Inc.). These and other systems, as well as cell culture protocols have been summarized excellently in Joanna Picot, Editor, Human Cell Culture Protocols
5 (Methods in Molecular Medicine), 2005, ISBN-10: 1588292222. By employing these systems, the medium may be maintained as a continuous medium, so that the concentrations of the various ingredients are maintained relatively constant or within a predetermined range. Such systems can provide for enhanced maintenance and growth of the subject cells using the designated media and additives.

10 g.) Maintenance, Harvesting, and Storage of Clonogenic NK Cells

[00207] In some embodiments, the present disclosure provides methods of clonogenic NK cell expansion using a combination of feeder cell or lack thereof, a desired NK cell clone and an appropriate medium, for a duration sufficient to produce a high population of clonogenic NK cells.

15 [00208] After NK cell clones are separated and deposited into a culture vessel, cell culture media and supplements are added to the vessel to sustain cell growth. Periodically the culture media is replenished or replaced to ensure continuing NK cell growth. In some embodiments, NK cell culture can be maintained from about 10 days to about 35 days or from about 15 days to about 30 days or from about 20 days
20 to about 25 days. In some embodiments, the culture time is from about 20 days to about 25 days, which will produce NK cells with high cell density and/or number while maintaining NK cell function. The method described by the present disclosure can expand single cell NK clones to at least about 1×10^5 NK cells per clone. In some embodiments, single cell clones can be expanded to about 5×10^5 NK cells, to
25 about 1.5×10^6 NK cells, to about 5×10^6 NK cells. This represents an expansion rate of at least 10^5 fold to as high as 5×10^6 fold, which is feasible in NK cell culturing.

[00209] Periodically, cell culture media are replenished with fresh media and supplements, and feeder cells, if required, to ensure continued cell growth. NK cell
30 growth could be monitored by a variety of assays. Non-limiting examples include counting of cells under the microscope using dye exclusion method (e.g., trypan

blue), cytometric methods, and metabolic activity growth (e.g., MTT, resazurin) assays. For example, colorimetric change (purple to yellow) of the microculture supernatant can be detected.

5 [00210] If the initial cell culture vessel proves to be limiting for cell growth, proliferating NK clones are collected, transferred to multiple culturing vessels or to a larger culturing vessel and supplemented with additional medium as described above. When cell number or density reaches predetermined thresholds or culturing time reaches predetermined duration, NK clones are harvested, functionally characterized and cryopreserved for subsequent molecular studies. In some
10 embodiments, the total time required between initial cell deposition to harvesting can range between about 5 and 50 days, preferably between about 20 and 35 days. In some other embodiments, NK cells are screened for clonality and receptor expression by immunostaining or cytometry. Certain steps of the methods of the present disclosure, including obtaining tissue samples and culturing cells, may be
15 performed using procedures and reagents known and available in the art.

[00211] The method described by the present disclosure can expand a high percentage of single cell NK clones to above at least 1×10^5 NK cells per clone, i.e. high cloning efficiency and yield. The cloning efficiency for the clonogenic NK cell populations is typically at least about 15% (i.e., out of 100 NK single cell clones, at
20 least 15 can be expanded to a cell number of at least 1×10^5) and can be as high as over 50%.

[00212] As described above, the methods of the present disclosure may be used to prepare a cell population enriched in clonogenic NK cell populations. Thus, in various embodiments, the purified cell population comprises at least 75%, at least
25 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% clonogenic NK cell populations, as indicated by the presence of one or more NK cell lineage markers, such as CD56 or CD16, and/or the presence (instead or in addition) of one or more other cell surface molecules, such as inhibitory killer immunoglobulin-like receptors, activating killer immunoglobulin-
30 like receptors, c-type lectin-like receptors, and natural cytotoxicity receptors.

[00213] In some embodiments, the present disclosure provides a method of clonogenic NK cell expansion with an overall cloning efficiency of at least 10%, at least 20%, at least 30%, at least 40%, or at least 50%.

[00214] The method described by the present disclosure can produce a clonogenic NK cell population that is highly viable. In some embodiments, at least 90% of said NK cell population is viable. Viability of NK cells can be monitored by a variety of methods. Non-limiting examples of viability assays include ATP test, Calcein AM assay, clonogenic assay, ethidium homodimer assay, evans blue staining, fluorescein diacetate hydrolysis/Propidium iodide staining (FDA/PI staining), flow cytometry, formazan-based assays (MTT/XTT), lactate dehydrogenase (LDH) assay, propidium iodide-based DNA staining, trypan Blue staining (dye only crosses cell membranes of dead cells), and TUNEL assay.

[00215] The method described by the present disclosure can produce a clonogenic NK cell population that is functional as NK cells. These *in vitro* expanded NK cells can exhibit common NK cell functions such as, but not limited to, cytotoxicity and cytolytic activity. Several assays can be used to determine such activities, including the common ⁵¹Cr-labeled target cell killing assay ((Moretta et al., 1990; Valiante et al., 1997).

[00216] The methods of the present disclosure, therefore, provide a variety of advantages over the prior art. Use of IL-2 or one or more added exogenous cytokines can be avoided and preferably no such cytokines should be added to the culture medium. Thus the culture's dependence on cytokines is preferably confined to those needed to promote growth of NK cells and feeder cells. When clonogenic NK cells are expanded *in vitro*, they are only exposed to IL-15 trans-presentation, without the need for added IL-2 in the culture medium. Second, the methods of the present disclosure, in certain embodiments, include the cloning of individual NK cells, which allows the selection of clonogenic populations having desired attributes, such as, e.g., expression of specific cell markers, including surface markers present on desired subpopulations of NK cells and robust cell growth, and cytoplasmic markers such as myosin, nucleic makers and transcription factors. Selection of clones having a desired attribute may be performed by high throughput methods, which allows the

rapid screening of a large number of clones. Fourth, the final sorting and purification of the clonogenic NK cells based upon expression of a NK cell lineage marker (and optionally a NK cell functional marker, e.g., KIR, NCR, CLLR, CAR, etc.) may be adapted to purify subpopulations of clonogenic NK cells having a desired phenotype or expressing a marker that indicates it possesses desired functionality.

[00217] The expanded mammalian clonogenic NK cell populations of the present disclosure have a variety of uses, including both autologous and allogeneic therapeutic uses. Accordingly, tissue samples may be obtained from patients to be treated with the clonogenic NK cell populations or donors. Tissue samples may be obtained from any animal, including, e.g., humans, primates, and domesticated animals and livestock. In embodiments, tissue samples are obtained from mammals. Tissues may include any tissue comprising NK cells and/or their precursors, including, e.g., peripheral blood, spleen. In related embodiments, tissue may be ectodermal, mesodermal, or endodermal in origin.

[00218] Cells prepared according to the methods of the disclosure may be used immediately or stored prior to use. The cells may be used without any further culturing, or they may be cultured and/or differentiated prior to use. The cells may be stored temporarily under cool conditions, e.g., under refrigeration, or at approximately 2-10°C., or the cells may be frozen under liquid nitrogen for long-term storage. A variety of methods of freezing cells for long term storage and recovery are known in the art and may be used in combination with the present methods, including freezing cells in a medium comprising fetal bovine serum and dimethylsulfoxide (DMSO) (Julca I et al., *Biotechnol Adv.* 2012, 30(6):1641-54; Hubel A. *Transfus Med Rev.* 1997, 11(3):224-33).

25 2. Clonogenic NK Cell Populations and Methods of Use Thereof

[00219] The present disclosure describes isolated, purified clonogenic NK cell populations. Although NK clones have been expanded *in vitro* before, none of the previous protocols could consistently expand a substantial percentage of single cell NK clones to a high number, e.g., 1×10^5 per clone. The low cell density and/or number of clonogenic NK cell population and the low efficiency of successfully expanding various NK cell clones to a high cell density and/or number by previous

methods thus severely limit the potential clinical application of purified clonogenic NK cell populations. In contrast, the present disclosure describes isolated, purified clonogenic NK cell populations with a predetermined desirable phenotypic trait of interest, wherein the number of cells in said NK cell population is at least of the order of 10^5 and said phenotype comprises expression of one or more cell surface receptors that modulate NK cell function and/or mediate NK cell cytotoxicity and/or cytolytic activity.

[00220] Clonogenic NK cell populations produced by the present disclosure possess several advantages over NK cells expanded using other methods. One advantage of current disclosure, compared to the state of the art, is the capability of producing clonogenic NK cell populations with defined characteristics, in contrast to previous methods that can only expand crude NK cells. Another advantage of the current disclosure is the ability to expand single NK clones to more than 10^5 , and sometimes to more than 10^6 cells. This ability, coupled with the defined nature of these clonogenic populations, enables the application of these clonogenic NK cells for therapeutic and research uses. The present disclosure also provides populations of clonogenic NK cells, which are substantially free of or free of contaminating T cells and other cells. These populations are advantageous over previously described populations of purified NK cells, including those prepared by using common NK lineage markers, since they possess defined cell surface molecules involved in biological activity. In some embodiments, these cell populations do not include T cells, which can lead to undesired side effects when used in cell therapy. In addition, contaminating cells, such as fibroblasts, can proliferate more rapidly than NK cells and compete with NK cells in repopulating a tissue site when administered therapeutically.

[00221] Accordingly, the cell populations of the present disclosure include three desirable features not previously present in populations of NK cells prepared from a mammalian tissue sample: (1) defined and definite functional activity characterized by exact cell surface molecule makeup; (2) defined clonogenic cell populations, and freedom from contaminating T cells and other cell types; and (3) large numbers of individual clones that are suitable for therapeutic use. Thus, in various embodiments, a purified cell population of the present disclosure comprises at least 75%, at least

80%, at 10 least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% clonogenic NK cell populations, as indicated by the presence of one or more NK lineage markers, such as CD56 and CD16 and the presence of one or more NK cell surface molecules, such as KIRs.

5 [00222] Clonogenic NK cell populations have many potential clinical applications. In some embodiments, clonogenic NK cells produced by the present invention have broad applications in a variety of disease including, but not limited to, lung cancer, melanoma, breast cancer, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, neuroblastoma, rhabdomyosarcoma, leukemia,
10 lymphoma, multiple myeloma, transplant rejection, and GvHD. In addition, clonogenic NK cell populations can be used in drug screening in search for compounds that modulate diseases processes involving NK cells. Furthermore, clonogenic NK cell populations can be used in basic and translation studies involving NK cells. For example, these cells can be used for either *in vitro* or *in vivo*
15 studies of NK cell biology.

[00223] In some embodiments, NK cell clones can be expanded *in vitro* for use in adoptive cellular immunotherapy in which infusions of such cells have been shown to have anti-tumor reactivity in a tumor-bearing host. The compositions and methods of this invention can be used to generate a population of NK cells that deliver both
20 primary and co-stimulatory signals for use in immunotherapy in the treatment of cancer, in particular the treatment of leukemia, lymphoma, multiple myeloma, transplant rejection, and GvHD. For example, NK cells have been used in combination with rituximab, aldesleukin, and chemotherapy in treating patients with relapsed Non-Hodgkin Lymphoma and chronic lymphocytic leukemia (See
25 ClinicalTrials.gov Identifier: NCT00625729). Similarly, NK cells have been used with epratuzumab to treat relapsed Acute Lymphoblastic Leukemia (ALL) (See ClinicalTrials.gov Identifier: NCT00941928). The compositions and methods described in the present invention may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so
30 forth. Likewise, the presently disclosed compositions and methods may be used in conjunction with other types of therapy for GvHD such as a steroid and/or immunosuppressant.

[00224] In some embodiments, the clonogenic NK cell populations produced of the present disclosure possess at least two characteristics. The first characteristic is said NK cells possess at least one NK lineage marker to ensure the NK lineage of the cells. Non-limiting examples of NK lineage marker include, e.g., CD56 and
5 CD16. This first characteristic distinguishes NK cells produced by the present disclosure from other cells, such as cytolytic T cells, that may be functionally similar to NK cells. The second characteristic is said NK cells possess a phenotypic trait of interest that is predetermined. This predetermined phenotypic trait of interest distinguishes certain clonogenic NK cell populations from other clonogenic NK cell
10 populations.

[00225] In some embodiments, clonogenic NK cell populations are selected based on the characteristics of the donor of such NK cells and the recipient of the expanded NK cells. The criteria for selecting clonogenic NK cell populations for a given recipient are based on maximizing NK cell potency (e.g., the cell's
15 cytotoxicity and cytolytic activities), and/or minimizing NK cell-associated graft-versus-host effects. For example, in the case of cancer immunotherapy involving NK cells, it is desirable to select clonogenic NK cell populations that have increased graft-versus-tumor effects. In addition, clonogenic NK cell populations can be selected based on the HLA genotypes of the donor from which NK cells are isolated
20 and the genotype of the recipient of the NK cell therapy.

[00226] In some embodiments, said phenotypic trait of NK cells can be expression of at least one cell marker that exerts effects on NK cell activation, NK cell interaction with other NK cells and with other cells not NK cells, NK cell proliferation, NK cell apoptosis, NK cell secretion of cytokines, NK cell
25 tissue/organ distribution, among others.

[00227] In some embodiments, the cell marker can be a NK-cell specific marker (i.e., primarily expressed by only NK cells, but not other cells, e.g., KIR2DS1, KIR3DS1). In other embodiments, the cell marker can be any cell marker expressed by a NK cell, including cytokines, chemokines, cell surface receptors (e.g., TGF- β ,
30 IL-1 β). Non-limiting examples of said cell markers include inhibitory killer immunoglobulin-like receptors, activating killer immunoglobulin-like receptors, c-

type lectin-like receptors, and natural cytotoxicity receptors. In some embodiments, said cell populations are expanded from NK cell clones from donors with certain HLA genotypes. Non-limiting examples of HLA genotype include HLA-C1/C1, HLA-C1/C2, HLA-C2/C2, Bw4, Bw6, and any of their combinations.

5 **[00228]** In some embodiments, the phenotypic trait of interest of NK cells is the expression of at least one cell marker that is important for NK cell activities as described above. In some embodiments, the phenotypic trait of interest is the lack of expression of at least one cell marker that is important for NK cell activities as describe. In yet some other preferred embodiments, the phenotypic trait of interest is
10 a combination of the expression of at least one cell marker and the lack of expression of at least another cell marker.

[00229] In some embodiments, the clonogenic NK cell populations by this disclosure can be applied alone, or as part of a biologic composition, to a recipient patient with certain HLA genotypes. In some embodiments, the phenotypic trait of
15 interest of the selected and expanded NK clonogenic population comprises expression of at least one cell surface receptor having the property of detecting “missing self-HLA class I ligand” in said recipient. Non-limiting examples of such selection process is listed in Table VII.

[00230] In some embodiments, a cell population of the present disclosure
20 comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a predetermined pattern of inhibitory killer immunoglobulin-like receptor (KIR) (e.g., some inhibitory KIRs are expressed while some others are not expressed), wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at
25 least 99%, or 100% of the cells in the cell population have such an expression pattern. Non-limiting examples of inhibitory KIR include KIR2DL1, KIR2DL2/3, and KIR3DL1.

[00231] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage
30 marker, such as, e.g., CD56 and CD16, and expressing a predetermined pattern of activating killer immunoglobulin-like receptor (KIR) (e.g., some activating KIRs are

expressed while some others are not expressed), wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR. Non-limiting examples of activating KIR include
5 KIR2DS1, KIR2DS2, and KIR3DS1.

[00232] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a predetermined pattern of c-type lectin-like receptor (e.g., some c-type lectin-like receptors are expressed while
10 some others are not expressed), wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and c-type lectin-like receptor(s). Non-limiting examples of c-type lectin-like receptors include NKG2D.

[00233] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a predetermined pattern of natural cytotoxicity receptor (e.g., some NCRs are expressed while some others are not expressed), wherein at least 75%, at least 80%, at least 85%, at least 90%, at
20 least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and natural cytotoxicity receptor(s). Non-limiting examples of natural cytotoxicity receptors include NKp46, NKp44, and NKp30.

[00234] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 or CD16 or both, and expressing a predetermined pattern of chimeric receptors (e.g., some chimeric receptors are expressed while
25 some others are not expressed), wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or
30 100% of the cells in the cell population express both said NK lineage marker(s) and chimeric receptors. Non-limiting examples of chimeric receptors comprising a

peptide for CD19, G(D2), CS1, or WT1. Non-limiting applications of clonogenic NK cell populations expressing such chimeric receptors include treatment for CD19+ leukemia and lymphoma and neuroblastoma (Shimasaki N. et al., 2013, Methods Mol Biol. 969:203-20; Altvater B. et al., 2009, Clin Cancer Res. 15(15):4857-66).

[00235] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a specific combination of inhibitory KIR: KIR2DL1^{pos}/KIR2DL2-3^{neg}/KIR3DL1^{neg}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR phenotype. In a specific embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of HLA-C2/C2 or C1/C2. In another preferred embodiment, said cell population can be applied as part of a biologic composition to a recipient patient with a HLA genotype of HLA-C1/C1.

[00236] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a specific combination of inhibitory KIR: KIR2DL1^{neg}/KIR2DL2-3^{pos}/KIR3DL1^{neg}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR phenotype. In a specific embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of HLA-C1/C1 or C1/C2. In another embodiment, said cell population can be applied as part of a biologic composition to a recipient patient with a HLA genotype of HLA-C2/C2.

[00237] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a specific combination of inhibitory KIR: KIR2DL1^{neg}/KIR2DL2-3^{neg}/KIR3DL1^{pos}, wherein at least 75%, at

least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR phenotype. In an embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of Bw4. In another embodiment, said cell population can be applied as part of a biologic composition to a recipient patient with a HLA genotype of Bw6.

[00238] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing a specific combination of inhibitory KIR: KIR2DL1^{pos}/KIR2DL2-3^{neg}/KIR3DL1^{pos}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR phenotype. In a preferred embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of *C2:C2;Bw4* or *C1:C2;Bw4*. In another preferred embodiment, said cell population can be applied as part of a biologic composition to a recipient patient with a HLA genotype of *C1:C1;Bw6*

[00239] In some preferred embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 or CD16 or both, and expressing a specific combination of inhibitory KIR: KIR2DL1^{neg}/KIR2DL2-3^{pos}/KIR3DL1^{pos}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and inhibitory KIR phenotype. In a more specific embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of *C1:C1;Bw4* or *C1:C2;Bw4*. In another preferred embodiment, said cell population can be applied as part of a biologic composition to a recipient patient with a HLA genotype of *C2:C2;Bw6*.

[00240] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing an activating KIR phenotype:

KIR2DS1^{SP}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and activating KIR. In a more specific embodiment, said cell population is expanded from a NK cell clone
5 isolated from a donor with a HLA genotype of *C1:C1* or *C1:C2*.

[00241] In some embodiments, a cell population of the present disclosure comprises clonogenic NK cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and expressing an activating KIR phenotype: KIR3DS1^{SP}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least
10 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and activating KIR. In a preferred embodiment, said cell population is expanded from a NK cell clone isolated from a donor with a HLA genotype of *C1:C1* or *C1:C2*. In some embodiments, a cell population of the present disclosure comprises clonogenic NK
15 cell populations expressing at least one NK lineage marker, such as, e.g., CD56 and CD16, and the phenotype: NKG2D-DAP10-CD3 ζ ^{pos}, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the cells in the cell population express both said NK lineage marker(s) and c-type lectin-like receptors.

[00242] In certain embodiments, the purified cell populations of the present disclosure are present within a composition, e.g., a biologic composition, adapted for and suitable for delivery to a patient, i.e., physiologically compatible. Accordingly, the present disclosure includes compositions comprising at least one clonogenic NK cell population of the present disclosure and one or more of buffers (e.g., neutral
25 buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDT A or glutathione, adjuvants (e.g., aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents,
30 thickening agents and/or preservatives.

[00243] In related embodiments, the present disclosure provides a biologic composition that comprises the purified cell populations provided herein and a biological compatible carrier or excipient, such as 5-azacytidine, cardiogenol C, or ascorbic acid.

5 [00244] In related embodiments, the purified cell populations are present within a composition adapted for or suitable for freezing or storage. For example, the composition may further comprise fetal bovine serum and/or dimethylsulfoxide (DMSO).

[00245] The present disclosure further provides methods of treating or preventing
10 injuries and diseases or other conditions, comprising providing a cell population of the present disclosure, i.e., clonogenic NK cell populations, to a patient suffering from said injury, disease or condition. In particular embodiments, the cell population was generated using a tissue sample obtained from the patient being treated (i.e., autologous treatment). In other embodiments, the cell population was obtained from
15 a donor, who may be related or unrelated to the patient (i.e., allogeneic treatment). The donor is usually of the same species as the patient, although it is possible that a donor is a different species (i.e., xenogeneic treatment).

[00246] In various embodiments, the clonogenic NK cell populations by this disclosure and related compositions are used to treat a variety of cancers and auto-
20 immune diseases, including, but not limited to AML, ALL, melanoma, MDS, non-Hodgkin's lymphoma, neuroblastoma, multiple myeloma, transplant rejection, and GvHD. The method described by the present disclosure comprises the administration of an effective amount of an isolated, purified clonogenic NK cell population to a recipient individual, wherein said population comprises of the order
25 of 10^5 to 10^7 cells per clone, and wherein said population expresses a phenotype of interest relevant for said disease in a recipient individual in need thereof.

[00247] The clonogenic NK cell populations that the method of treatment comprises are selected and expanded according to a predetermined, desirable phenotypic trait of interest, e.g., expression of one or more cell surface receptors. In
30 some embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more cell surface receptors selected from one or

more of the following classes: killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors (CLLR), natural cytotoxicity receptors (NCR), and chimeric antigen receptors (CAR). Additionally, in some embodiments, the method of treatment comprises clonogenic NK cell populations that express an NK inhibitory
5 receptor, an NK activating receptor, or a combination of both. In some embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more KIR, and examples of KIR include, but are not limited to, KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2. In other
10 embodiments, the method of treatment comprises clonogenic NK cell populations that express one or more NCR, and examples of NCR include, but are not limited to, NKp46, NKp44, and NKp30. In some embodiments, the method of treatment comprises infusion in a patient of effective amounts of clonogenic NK cell populations that express one or more CLLR, and examples of CLLR include, but are not limited to, NKG2D and NKG2D-DAP10-CD3ζ. In some other embodiments, the
15 method of treatment comprises clonogenic NK cell populations that express one or more CAR, and examples of CAR include, but are not limited to, chimeric receptors comprising a CD19 peptide, a G(D2) peptide, a CS1 peptide, or a WT1 peptide. In yet other embodiments, the method of treatment comprises clonogenic NK cell populations that express a combination of one or more receptors in the following
20 classes: KIR, CLLR, NCR, and CAR.

[00248] In some preferred embodiments, the clonogenic NK cell populations by this disclosure can be selected according to the disease that the cells are intended to treat. Certain clonogenic NK cell populations may be stronger effectors for the disease compared to other clonogenic NK cell populations. The criteria for selecting
25 clonogenic NK cell populations for certain disease are based on the intended effects of NK cell transplants and the characteristics of the disease. For example, in the case of cancer immunotherapy involving NK cells, it is desirable to select clonogenic NK cell populations that have increased graft-versus-tumor effects. In addition, when NK cells are used in patients undergoing HCT, clonogenic NK cell populations can
30 be selected based on the HLA genotypes of the donor from which NK cells are isolated and the genotype of the recipient of the NK cell therapy. In addition, clonogenic NK cells with the phenotype KIR2DS1^{pos} and inhibitory KIR^{neg} may

have stronger effects for AML patients undergoing HCT (Venstrom, J. M., et al. 2012. N. Engl. J. Med. 367:805-816). Non-limiting examples of selecting clonogenic NK cell populations according to target disease are listed in Table VIII.

[00249] In some preferred embodiments, the present disclosure provides methods for treating or preventing AML, including but not limited to AML patients who undergo HCT. These methods comprise providing at least one clonogenic NK cell population of the present disclosure, wherein said cell population expresses KIR2DS1, to a patient diagnosed with an AML disease or injury. In a preferred embodiment, said cell population is expanded from a NK cell clone isolated from a donor with HLA genotype HLA-C1/C1 or C1/C2.

[00250] In specific embodiments, the present disclosure provides methods for treating AML, including but not limited to AML patients who undergo HCT. These methods comprise providing at least one clonogenic NK cell population of the present disclosure, wherein said cell population expresses KIR2DS2, to a patient diagnosed, suspected of having, or being at risk of an AML disease or injury.

[00251] In some preferred specific embodiments, the present disclosure provides methods for treating or preventing HCT-related complications. These methods comprise providing at least one clonogenic NK cell population of the present disclosure, wherein said cell population expresses KIR3DS1, to a patient diagnosed, suspected of having, or being at risk of a HCT-related complication.

[00252] In specific embodiments, the present disclosure provides methods for treating or preventing HIV-1 infection and complications. These methods comprise providing at least one clonogenic NK cell population of the present disclosure, wherein said cell population expresses KIR3DS1, to a patient diagnosed, suspected of having, or being at risk of a HIV-1 infection and complication. In a preferred embodiment, said patient has the genotype KIR3DS1^{neg} and Bw4-80I.

[00253] In specific embodiments, the present disclosure provides methods for treating or preventing post-transplant cytomegalovirus (CMV) reactivation. These methods comprise providing at least one clonogenic NK cell population of the present disclosure, wherein said cell population expresses at least one activating

KIR, to a patient diagnosed, suspected of having, or being at risk of a post-transplant CMV reactivation. Non-limiting examples of activating KIR include KIR2DS1, KIR2DS2, and KIR3DS1.

[00254] In specific embodiments, the present disclosure provides methods for
5 treating or preventing a MICA/MICB^{pos} or ULBP^{pos} cancer. These methods
comprise providing at least one clonogenic NK cell population of the present
disclosure, wherein said cell population expresses NKG2D, to a patient diagnosed,
suspected of having, or being at risk of a MICA/MICB^{pos} or ULBP^{pos} cancer. In a
preferred embodiment, said cell population is expanded from a NK cell clone
10 isolated from a donor who is negative for inhibitory KIRs.

[00255] In specific embodiments, the present disclosure provides methods for
treating or preventing a NCR ligand-positive cancer. These methods comprise
providing at least one clonogenic NK cell population of the present disclosure,
wherein said cell population expresses at least one NCR, to a patient diagnosed,
15 suspected of having, or being at risk of a NCR ligand-positive cancer. In a preferred
embodiment, said cell population is expanded from a NK cell clone isolated from a
donor who is negative for inhibitory KIRs. Non-limiting examples of NCR include
NKp30, NKp44 and NKp46.

[00256] The methods of the present disclosure can be used to isolate and culture
20 large number of clonogenic NK cell populations with defined and definite
characteristics and free of contaminating cells such as T cells, which is important for
clinical applications. The NK cell populations prepared by methods described in the
present disclosure can be monoclonal or polyclonal. In addition, two or more such
clonogenic NK cell populations can be combined to increase the cell number or to
25 obtain NK cells with more than one desirable phenotypic traits of interest. These
defined clonogenic NK cell populations have advantages over crude NK isolations
from a tissue samples, and NK cells expanded from such crude NK cell isolations.
For example, US Patent 8,026,097 recently reported a method to culture NK cells *in*
vitro isolated from human PBMC. However, that method only cultures crude, total
30 NK cells instead of clonogenic NK cell populations. NK cells isolated from PBMC
are very heterogeneous in nature and can be distinguished at least by their cell

surface receptor expression patterns, such as inhibitory killer immunoglobulin-like receptors, activating killer immunoglobulin-like receptors, c-type lectin-like receptors, and natural cytotoxicity receptors, chimeric antigen receptors, among others. Some NK cell clones have opposite functions and some NK cell clones have demonstrated advantages for certain therapeutic applications, as we have demonstrated (Pittari et al., J. Immunol. 2013 190:4650-4660). For example, NK cell clones from a donor with a HLA genotype that mismatches that of a recipient exhibit higher cytotoxicity and cytolytic activity, and thus are more suitable for therapeutic use (Pittari et al., J. Immunol. 2013 190:4650-4660). In addition, the current disclosure could expand NK cell clones at a very high cloning efficiency and is also able to expand single NK cell clones to sometimes more than 5 million cells. Thus, the current disclosure overcomes several key limitations of previous methodologies of NK cell culture and expansion *in vitro* and can produce clonogenic NK cell populations with homogenous populations and with desirable characteristics.

[00257] Cell populations and related compositions of the present disclosure may be provided to a patient by a variety of different means. In certain embodiments, they are provided locally, e.g., to a site of actual or potential injury or disease. In one embodiment, they are provided using a syringe to inject the compositions at a site of possible or actual injury or disease. In other embodiments, they are provided systemically. In one embodiment, they are administered to the bloodstream intravenously or intra-arterially. The particular route of administration will depend, in large part, upon the location and nature of the disease or injury being treated or prevented. Accordingly, the disclosure includes providing a cell population or composition of the disclosure via any known and available method or route, including but not limited to oral, parenteral, intravenous, intra-arterial, intranasal, and intramuscular administration.

[00258] The development of suitable dosing and treatment regimens for using the cell populations and compositions described herein in a variety of treatment regimens, including e.g., oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, will again be driven in large part by the disease or injury being treated or prevented and the route of administration. The determination

of suitable dosages and treatment regimens may be readily accomplished based upon information generally known in the art and obtained by a physician. Crude NK cells that are isolated from a tissue sample or expanded from such cells have been used in cell therapy at dosages between 0.8×10^6 to 100×10^6 /kg body weight (Cheng M. et al., 2013, Cellular & Molecular Immunology 10, 230–252). Compared to crude NK cells, clonogenic NK cells have the potential to exhibit higher efficacy and higher potency. Thus the dosages of clonogenic human NK cells to be used in cell therapy may be lower compared to those of crude NK cells. Of course, exact dosages and regimens are best determined in Phase I trials and will depend on the individual situation. In some embodiments, clonogenic NK cells can be introduced to a patient at dosages in the order of 10^5 , 10^6 , 10^7 , 10^8 , 10^9 /kg body weight. In some preferred embodiment, clonogenic NK cells can be introduced to a patient at dosages in the order of 10^5 , 10^6 , 10^7 /kg body weight. In some embodiments, these clonogenic NK cells are monoclonal NK cells. In some other embodiments, these clonogenic NK cells are polyclonal NK cells.

[00259] Treatment may comprise a single treatment or multiple treatments. In particular, for preventative purposes, it is contemplated in certain embodiments that purified cell populations of the disclosure are administered during or immediately following a stress that might potentially cause injury, such as, e.g., AML or ALL.

[00260] The present disclosure also provides cell culture systems useful in the preparation and/or use of the purified cell populations of the present disclosure. For example, in one embodiment, a feeder cell useful in the expansion of clonogenic NK cell populations is provided that comprises cells that are capable of IL-15 trans-presentation. In order to illustrate the methods of expanding human NK cell clones, the clonogenic NK cell populations and the methods of treatments utilizing these cells as described in the present disclosure, the following examples are given. It is worth noting that the following examples are illustrative, not limiting in nature, and the scope of the present invention is not limited to the following examples.

EXAMPLES

[00261] The present disclosure is next described by means of the following examples. The use of these and other examples anywhere in the specification is

illustrative only, and in no way limits the scope and meaning of the disclosure or of any exemplified form. Likewise, the disclosure is not limited to any particular preferred embodiments described herein. Indeed, modifications and variations of the disclosure may be apparent to those skilled in the art upon reading this specification, and can be made without departing from its spirit and scope. The disclosure is therefore to be limited only by the terms of the claims, along with the full scope of equivalents to which the claims are entitled.

Example 1

IL-15 trans-presentation and in vitro clonogenic expansion of NK cells

[00262] This example describes novel materials and methods to expand human clonogenic NK cells *in vitro* using IL-15 trans-presentation. This example especially describes a novel feeder cell system that co-expresses IL-15 and IL-15R α and that has proven efficacious in promoting the proliferation of clonogenic NK cells *in vitro*. Using the protocol as described below, a monoclonal NK cell population of more than 1×10^6 can be achieved. This example also characterizes some clonogenic NK cell populations obtained by these methods.

NK cell donors

[00263] NK cells were obtained from 7 individuals (5 healthy donors and 2 transplant recipients). HLA class I genotyping was performed on genomic DNA by a combination of PCR amplification with sequence-specific primers or sequence-specific oligonucleotide probes (Hsu et al., 2005). KIR genotyping was performed by KIR sequence-specific primers (KIR genotyping SSP Kit, Invitrogen) and KIR haplotypes and genotypes were assigned (Khakoo et al., 2006) (**Table I**). NK cells from healthy donors were negatively selected from freshly isolated PBMC obtained from 30 ml peripheral blood, using a cocktail of magnetically labeled mAbs specific for non-NK lineage antigens (Miltenyi Biotec) (Chewning et al., 2007). For all experiments, post-isolation NK cell purity was >90%. NK cells from transplant recipients were directly FACS-sorted from bulk PBMC (see *NK cloning*).

Institutional Review Board Approval

[00264] Informed written consent was obtained from all donors according to Memorial Sloan-Kettering Cancer Center Institutional Review Board Protocol (IRB# 95-054A for healthy donors and IRB# 09-141 for transplant recipients).

5 *IL-15 Trans-presented Feeder Cells -- BaF/3 IL-15R α /IL-15 Transfectants*

[00265] pSFG retroviral vectors containing full length cDNA of human IL-15R α or IL-15 (kindly provided by Dr. Thomas A. Waldmann, Metabolism Branch, National Cancer Institute) were transfected into Phoenix E packaging cell line, to
 10 produce retroviral supernatants. BaF/3 cells were incubated with retroviral supernatants, for 6-8 h, in fibronectin-coated plates (Takara Biomedicals). Clones of IL-15R α /IL-15 double-transfected pre-B-lymphocyte BaF/3 cells (BaF/3 IL-15R α /IL-15) were obtained by limiting dilution, and stable expression of IL-15 and IL-15R α was confirmed by monthly mAb staining. The cell line was maintained in
 15 RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine (all provided by the Core Media Preparation Facility, Memorial Sloan-Kettering Cancer Center).

NK Cloning and Generation of Clonogenic NK Cell Populations

[00266] NK clones were developed following single cell deposition (**Fig. 1**) and
 20 propagated by IL-15 trans-presentation. Fig.1A and Fig.1B show the generation of NK clones from single NK cells with specific receptor repertoires. *A*, Flow cytometric representation of NK subsets identified by HP-MA4 and 143211 mAbs. HP-MA4 recognizes NK cells expressing 2DS1, 2DL1 or both (subset 1+2). Combined use of HP-MA4 and 143211 mAbs allows discrimination between
 25 2DS1^{pos}/2DL1^{neg} (subset 1) and 2DS1^{pos}/2DL1^{pos} or 2DS1^{neg}/2DL1^{pos} (subset 2) NK cells. Resting NK cells obtained from a healthy donor are depicted. *B*, P75 and P81: 2DS1^{pos}/2DL1^{neg} (HP-MA4^{pos}/143211^{neg}) NK clones. P74, P107, P89 and P73: 2DL1^{pos} (HP-MA4^{pos}/143211^{pos}) NK clones. In 2DL1^{pos} NK clones, 2DS1 expression is verified by real time RT-qPCR.

30 [00267] NK cell subpopulations displaying specific combinations of KIR/NKG2A expression were identified by the following mAbs: CD3-PE/TexasRed

(S4.1, Invitrogen), CD56-PE/Cyanine7 (MEM-188, BioLegend), 2DL1/S1-PerCP/Cyanine5.5 (HP-MA4, BioLegend), 2DL1-Allophycocyanin (143211, R&D Systems), 2DL2-3/S2-FITC (CH-L, Miltenyi Biotec), 3DL1-Alexa Fluor700 (DX9, BioLegend), 3DL1/S1-PE (Z27, Beckman Coulter), NKG2A-PE (131411, R&D Systems), CD85j/ILT2 (LILRB1)-PE (HP-F1, Beckman Coulter). Single NK cells from selected subpopulations were FACS-sorted (Aria III, BD Biosciences) and deposited into U-shaped polystyrene 96-well plates (one cell/well) containing 100 μ l CellGro SCGM medium (CellGenix GmbH) supplemented with 10% heat-inactivated AB human serum (Gemini Bioproducts), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine.

[00268] The following feeders were added to the medium: 10^4 allogeneic EBV-B lymphoblastoid cell line (EBV-BLCL) (JY); 4×10^4 PBMC obtained from 3 different donors, and 3×10^3 BaF/3 IL-15R α /IL-15 cells. Feeders were gamma irradiated (EBV-BLCL and PBMC: 5.2 Gray; BaF/3 IL-15R α /IL-15: 13.9 Gray). After sorting, plates were centrifuged at 500 rpm for 1 min and incubated in a 37 °C, 5% CO₂ humidified atmosphere. On d 5, 100 μ l fresh medium and irradiated EBV-BLCL (10^4), PBMC (4×10^4) and BaF/3 IL-15R α /IL-15 (3×10^4) were added to each well. On d 10, 80 μ l supernatant were removed and substituted with medium and irradiated feeders, as on d 5. On d 15, NK cell growth could be detected by a colorimetric change (purple to yellow) of the microculture supernatant. Proliferating NK clones were collected, transferred in 48-well plates and supplemented with 800 μ l medium and irradiated BaF/3 IL-15R α /IL-15 (5×10^5). On d 22, 300 μ l supernatant were removed from all wells, and replaced with 500 μ l medium and irradiated BaF/3 IL-15R α /IL-15 (5×10^5). Between d 28 and d 32, NK cells were screened by flow cytometry to determine viability, clonality and receptor expression. NK clones were harvested, functionally characterized and cryopreserved for subsequent molecular studies.

IL-15 trans-presentation supports generation of 2DS1^{pos} NK clones

[00269] 2DS1^{pos} clones have previously been obtained from donors lacking cognate HLA-C2 ligand (i.e., donors homozygous for the HLA-C1 ligand). In contrast, very few 2DS1^{pos} clones were obtained from donors expressing HLA-C2

(Chewning et al., 2007). Since IL-15 trans-presentation is the major growth and survival signal for NK cells (Dubois, S., et al. 2002. Immunity 17:537-547; Burkett, P. R., et al. 2004. J. Exp. Med. 200:825-834; Koka, R., et al. 2004. J. Immunol. 173:3594-3598; Huntington, N. D., et al. 2007. Nat. Immunol. 8:856-863), we
5 investigated, if human NK cloning efficiency and clone survival could be enhanced by IL-15 trans-presentation *in vitro*.

[00270] Trans-presentation was achieved by co-culture of FACS-sorted NK cells with murine BaF/3 cells transfected with human IL-15R α and human IL-15. This procedure supported clone development from all donors, irrespective of their *HLA-C*
10 genotype.

[00271] Each clone reached 0.25-4 x 10⁶ cells, and the overall cloning efficiency was 35%-40%. Clones were developed from seven donors, representing the three *HLA-C* genotypes: *C1:C1*; *C1:C2* and *C2:C2*. *HLA-KIR* ligand groups and *KIR* genes for each NK donor are listed in Table I. The inventors analyzed 386 clones,
15 which included the 2DS1^{SP} phenotype and other 2DS1^{POS} phenotypes (Table II). For example, 2DS1^{SP} clones were obtained from donors with each *HLA-C* genotype (Table II, *Columns A* and *B*). Similarly, twenty-four 2DS1^{SP}, which also expressed the inhibitory receptor CD94/NKG2A were obtained (Table II, *Column F*). Accordingly, clones with a broad KIR repertoire, including 2DS1, can be obtained
20 from donors with any *HLA-C* genotype, when IL-15 trans-presentation is the NK growth factor.

[00272] Therefore, this example describes novel materials and a novel method to expand human clonogenic NK cells *in vitro* using IL-15 trans-presentation. Compared to the state of the art, the present invention is able to expand clonogenic
25 NK cell populations at a very high cloning efficiency and is able to obtain clonogenic NK cell populations for more than 0.25 x 10⁶ cells per clone, and is able to obtain clonogenic NK cell populations from all donors tested, irrespective of their *HLA-C* genotype.

Table I. Donor HLA class I and KIR

Sample ID	HLA-KIR		KIR Genes		KIR Haplotype ^a
	Ligand Group		Activating	Inhibitory	
	C	B			
Healthy Volunteers					
UDN ^b 001	C1:C1	Bw4	2DS1 / — ^c / 3DS1	2DL1 / 2DL3 / 3DL1	A, B [A1, B3]
UDN 002	C1:C1	Bw4	2DS1 / 2DS2 / 3DS1	2DL1 / 2DL2-3 / 3DL1	A, B [A1, B15]
UDN 003	C2:C2	Bw4	2DS1 / — / 3DS1	2DL1 / 2DL3 / 3DL1	A, B [A1, B3]
UDN 004	C1:C2	Bw4	2DS1 / 2DS2 / 3DS1	2DL1 / 2DL3 / 3DL1	B, B [B4, B17]
UDN 005	C1:C2	Bw4	2DS1 / 2DS2 / 3DS1	2DL1 / 2DL2-3 / 3DL1	B, B [B7, B24]
HCT Donors					
UDN 006	C1:C2	Bw6	2DS1 / 2DS2 / —	2DL1 / 2DL2-3 / 3DL1	B, B [B31, B34]
UDN 007	C1:C2	Bw4	2DS1 / — / 3DS1	2DL1 / 2DL3 / 3DL1	A, B [A1, B3]

^a KIR haplotype numbers from Khakoo and Carrington (18).^b Unique donor number.^c Absence of *KIR* gen

Table II. NK cloning strategy

Number of NK clones per FACS sorting gate ^a								
	Donor							Total NK clones
	<i>HLA-C</i>	A	B	C	D	E	F	by <i>HLA-C</i> genotype
Total NK clones by sorting gate	<i>C1:C1</i>	55	33	18	37	3	0	146
	<i>C1:C2</i>	43	23	36	19	12	0	133
	<i>C2:C2</i>	30	25	15	9	4	24	107
		128	81	69	65	19	24	386
Possible		2DS1 ^b <i>or</i>		2DL1 ^b <i>or</i>		2DS1 <i>and</i>		
KIR phenotypic		2DL1 ^b <i>or</i>						
combinations		2DS1/2DL1	2DS1	2DS1/2DL1	2DL2/2DL3/	2DS1/3DL1	2DS1/NKG2A	
		_{<i>b</i>}		_{<i>b</i>}	2DS2 ^c			

^a Sorting of single NK cells using the following mAb: anti-2DL1/2DS1 (HP-MA4), anti-2DL1 (143211), anti-2DL2/2DL3/2DS2 (CH-L), anti-3DL1 (DX9), anti-NKG2A (131411).

FACS sorting gates:

A: HP-MA4^{pos}B: HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{neg}C: HP-MA4^{pos}, 143211^{pos}D: HP-MA4^{pos}, 143211^{neg}, CH-L^{pos}, DX9^{neg}E: HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{pos}F: HP-MA4^{pos}, 143211^{neg}, CH-L^{neg}, DX9^{neg}, 131411^{pos}^b Possible expression of additional KIR with ligand specificity for HLA-class I.^c Expression of at least one inhibitory (2DL2/2DL3) or activating (2DS2) receptor.

Example 2

Clonogenic NK Cell Populations Possess Clone-specific Biology and Functions and Their Roles in Recipient-specific Cytotoxicity

[00273] This example describes clone-specific properties of clonogenic NK cell populations obtained using the novel materials and methods outlined in Example 1. This example emphasizes the heterogeneous nature of NK cells isolated from human donors, and highlights that clonogenic NK cell populations may possess properties that are not available in unseparated crude NK cells (e.g., NK cells isolated from PBMC using common commercial immune-beads). This is important because NK cells have been used in cell therapy in the treatment of cancers and other diseases, and current trials use crude NK cells that are heterogeneous and undefined in nature. Thus, this example provides evidence that clonogenic NK cell populations expanded by this invention are, in contrast, homogenous and defined in nature, and may have higher efficacy and less side effects compared to crude NK cells. This example focuses on the study of the cytotoxicity of different clonogenic NK cell populations according to their cell surface receptors. In addition, these clonogenic NK cell populations can also be used in basic and translational research of NK cell biology, especially to study the recipient-specific cytotoxicity of clone-specific NK cells. This example demonstrates that the novel method of clonogenic expansion of human NK cell clones as described in the present disclosure, when combined to other cellular and molecular biology techniques (e.g., RT-qPCR), may help attain a profound understanding of NK cell biology that cannot be achieved by studying crude NK cell isolations from a tissue sample (e.g., total NK cells isolated from PBMC).

25 *Characterization of NK clones*

[00274] *KIR/NKG2A receptor expression.* KIR and NKG2A expression was tested by flow cytometry. mRNA copy numbers for individual KIR (see *Quantitative PCR*) were used for estimation of KIR surface expression when KIR receptors could not be individually recognized by monospecific mAbs. Normalized mRNA copy numbers for 2DL1, 2DS1, 2DL2-3 and 3DS1 were used to determine the lowest number associated with surface expression. Cell surface expression of 2DL1, 2DS1, 2DL2-3 and 3DS1 was assigned to one of three groups: KIR

expression present; KIR expression absent; and KIR surface expression not tested. Because the z27 mAb used to detect 3DS1 receptor also recognizes 3DL1 (Trundley et al., 2007), the analysis for determination of the relationship between 3DS1 cell surface expression and mRNA copy numbers was exclusively based on clones
 5 lacking 3DL1 expression (i.e., DX9^{neg}). The lowest KIR transcript number associated with detectable receptor surface expression was 40 copies for 2DL1, 13 copies for 2DS1, 23 copies for 2DL2-3, and 98 copies for 3DS1. These values were set as minimal copy number of transcripts, necessary for surface expression of each KIR. This procedure for identification of NK clones with KIR surface expression
 10 was applied to evaluate the effects of inhibitory KIR for self-HLA class I on anti-HLA-C2 reactivity by 2DS1^{pos} clones. In addition, it was used in the analysis of a possible effect of 3DS1 on anti-HLA-C2 cytotoxicity mediated by 2DS1.

[00275] Mean fluorescence intensity (MFI) values were used to determine expression levels of 2DS1 receptor.

15 *Cytotoxicity*

[00276] Cytotoxicity against EBV-BLCL was measured in standard ⁵¹Cr release assays performed in triplicate (or in duplicate for clones with limited cell number) for 4h, 37 °C, at effector to target cell ratio (E:T) 10:1 (3 x 10³ target cells/well). Where indicated, effectors were tested in the presence of 10 µg/ml anti-human
 20 NKG2A, 4E (anti-HLA-B/C), or control anti-mouse F(ab')₂ fragment. EBV-BLCL target cells were obtained from the International Histocompatibility Working Group (IHWG, <http://www.ihwg.org/reference/index.html> Consanguineous_Reference_Panel) or generated in our laboratory. EBV-BLCL possessed the following HLA class I genotypes: GK, A*02:01/*03:01, B*40:01/*15:01, Cw*03:04/*03:04 (B group: Bw6; C group: C1); KA, A*03:01/*68:01, B*15:01/*51:01, Cw*04:01/*07:04 (B group: Bw4; C group: C1:C2); 9036, A*02:01/*02:01; B*44:02/*44:02, Cw*05:01/*05:01 (B group: Bw4; C group: C2); DD, A*02:01/03:01, B*35:02/41:01, Cw*04:01/17:01 (B group: Bw6; C group: C2). Targets were maintained in RPMI 1640 supplemented with 10% FCS,
 30 100 U/ml penicillin, 0.1 mg/ml streptomycin up to 3 mo before being discarded. All EBV-BLCL were tested for expression of HLA-E, using PE-conjugated anti-HLA-E mAb (3D12, BioLegend).

[00277] The inventors determined the values for nonspecific ^{51}Cr release in 2DS1^{pos} clones by performing 101 cytotoxicity assays where 2DS1-mediated activation could not be involved in target lysis. In 60 assays, 2DL1^{pos}/2DS1^{neg} clones were tested against a *HLA-C2* group positive EBV-BLCL and in 41 assays, 2DS1^{SP} clones were tested against a *HLA-C2* group-negative EBV-BLCL. No cytotoxicity from such combinations could be accounted for by “missing self-HLA class I recognition”. The uppermost % lysis observed in these assays was 13.1%. A threshold at 13.1% lysis was therefore set to distinguish between nonspecific ^{51}Cr release and specific 2DS1-mediated NK response.

10 *Supported Lipid Bilayers and Live Imaging*

[00278] Bilayers were generated as described from small unilamellar vesicles containing a 10:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine and biotinyl cap phosphoethanolamine (Avanti Polar Lipids) (Abeyweera et al., 2011). After formation, bilayers were incubated with streptavidin, washed with PBS, and then incubated with 2 mg/ml biotinylated EB6 mAb (anti-2DL1/S1), 1 mg/ml biotinylated ICAM-I, and 1 mg/ml biotinylated HLA-E. For comparative studies in which one or more constituents were left out of the bilayer, the nonstimulatory biotinylated mouse MHC molecule H2-D^b was added to keep the total protein concentration for that experiment constant. After protein loading, bilayers were stored at room temperature for up to 4 h prior to use.

[00279] Prior to imaging, NK cell clones were loaded with 5 mg/ml Fura 2-AM and then transferred into RPMI supplemented with 5% FCS and lacking phenol red. 2×10^5 NK cells were added to chambered coverglass bearing stimulatory supported lipid bilayers and then imaged using an inverted fluorescence video microscope (IX-81, Olympus) fitted with a 20X objective lens (0.75 NA, Olympus) and attached to an EM-CCD camera (Hamamatsu Photonics). Ratiometric Fura 2 images were collected using a DG-4 Xenon lamp (Sutter Instrument Company) with 340 and 380 nanometers bandpass filters in place. Differential interference contrast and Fura 2 fluorescence images were acquired every 30 s for 20-30 min after addition of cells to the stimulatory bilayer. All experiments were performed at 37 °C. Data were collected using Slidebook Software (Intelligent Imaging Innovations) and analyzed

using Slidebook and Excel by computing the average Fura 2 ratio for all cells in the imaging field for each time point.

Quantitative PCR

[00280] *Primers and Probes.* Sequences for 2DL1 primers, and 2DS1, 2DL2-
 5 3/S2, 3DS1 and NKG2A primers and probes were previously reported (Uhrberg et
 al., 1997). 2DL1 probe and GAPDH primers were designed in our laboratory.
 Nanomolar (nM) oligonucleotide concentrations for real-time RT-qPCR reactions
 (indicated in parentheses) were established by optimization matrix for both primers
 (range: 100 to 900 nM) and probes (range: 50-250 nM). Shorter probes with
 10 conjugated minor groove binder groups were preferred over standard DNA probes to
 increase reaction specificity. 2DS1: Fwd: 5'-TCTCCATCAGTCGCATGAR-3'
 (500); Rev: 5'-AGGGCCCAGAGGAAAGTT-3' (500); Probe: 5'-6FAM-
 AGGTCTATATGAGAAACCT-MGB-3' (150). 2DL1: Fwd: 5'-
 GCAGCACCATGTCGCTCT-3' (300); Rev: 5'-GTCACTGGGAGCTGACAC-3
 15 (100); Probe: 5'-6FAM-CACATGAGGGAGTCCAC-MGB-3' (100). 2DS2: Fwd:
 5'-TGCACAGAGAGGGGAAGTA-3' (300); Rev: 5'-
 CACGCTCTCTCCTGCCAA-3' (300); Probe: 5'-6FAM-
 GTCATCACAGGTCTATATGA-MGB-3' (100). 2DL2: Fwd: 5'-
 GGAGGGGGAGGCCCATGAAT-3' (300); Rev: 5'-
 20 GTCGGGGGTTACCGGTTTTTA-3' (500); Probe: 5'-6FAM-
 CCAAGGTCAACGGAACA-MGB-3' (150). 2DL3: Fwd: 5'-
 CCACTGAACCAAGCTCCG-3' (500); Rev: 5'-CAGGAGACAACCTTTGGATCA-
 3' (500); Probe: 5'-6FAM-CTGGTGCTGCAACAA-MGB-3' (150). 3DS1: Fwd:
 5'-GCACCCAGCAACCCCA-3' (300); Rev: 5'-TAGGTCCCTGCAAGGGCAC-
 25 3' (500); Probe: 5'-6FAM-AATTTCTCCATCGGTTCCATGA-MGB-3' (150).
 NKG2A: Fwd: 5'-CTCCAGAGAAGCTCATTGTTGG-3' (500); Rev: 5'-
 CACCAATCCATGAGGATGGTG-3' (500); Probe: 5'-6FAM-
 CGATAGTTGTTATTCCCTCTACA-MGB-3' (150). GAPDH: Fwd: 5'-
 TTCGCTCTCTGCTCCTCCTG-3'; Rev: 5'-CTTCCCGTTCTCAGCCTTGA-3'.
 30 *Recombinant plasmids.* KIR, NKG2A and GAPDH cDNA was PCR amplified from
 2 donors using the above listed primers (500 nM). Thermal cycling conditions were:
 stage 1: 2 min, 94 °C; stage 2: 30 cycles of [30 s, 94 °C; 30 s, 60 °C; 30 s, 72 °C];

stage 3: 7 min, 72 °C. PCR products were ligated (pGEM-T Easy Vector, Promega) and transformed into MAX Efficiency DH5 α TM Competent Cells (Invitrogen). Recombinant plasmid DNA was extracted, the insert sequenced and the concentration determined at 260 nanometers (NanoDrop 1000, Thermo Scientific).

5 [00281] *cDNA synthesis from NK clones.* cDNA for real-time RT-qPCR was extracted from cryopreserved NK clones using the MACS® One-Step cDNA technology (Miltenyi Biotec). Briefly, poly(A)⁺ tails of mRNA in cell lysates were hybridized with oligo(dT) microbeads. Magnetically labeled mRNA retained in micro columns was used as template for cDNA synthesis (1h, 42 °C). Prior to
10 reverse transcription, RNase-free DNase I (Applied Biosystems) was added to mRNA (10 U, 2 min, room temperature), to completely remove traces of genomic DNA. RNase H from E. coli (New England Biolabs) was added for in-column digestion of mRNA-bound cDNA (2 U, 30 min, 37 °C). cDNA was stored at -20 °C.

[00282] *PCR amplification of cDNA.* PCR amplifications used 2 μ l NK clone
15 cDNA in buffer solution in a 50 μ l reaction mix containing FastStart Universal Probe Master (Roche Applied Science) and the primer/probe oligonucleotides described above. Quantification of housekeeping GAPDH was performed by TaqMan Gene Expression Assay for GAPDH (HS99999905_m1, Applied Biosystems). Reactions were performed in duplicate using ABI 7300 PCR System
20 (Applied Biosystems), under the following thermal cycling conditions: stage1: 2 min, 50 °C; stage 2: 10 min, 95 °C; stage 3: 40 cycles of [15 s, 95 °C]; stage 4: 1 min, 60 °C. Non-template controls were set up in triplicate for each reaction.

[00283] *Absolute quantification of NK clone transcripts.* Samples containing 10-fold serial dilutions (KIR and NKG2A: 3×10^5 to 30; GAPDH: 3×10^6 to 3×10^2) of
25 known gene copy numbers in recombinant plasmids were amplified in triplicate along with each real-time RT-qPCR run. 5-point standard curves were generated to quantify each KIR and NKG2A transcript. Standard curves with a linearity (r^2) ≥ 0.985 and an efficiency ranging from 85% to 110% were considered acceptable. Threshold cycle values >36 were considered non-specific and discarded.
30 Normalization of copy numbers for KIR and NKG2A: [KIR or NKG2A copy number/GAPDH copy number] $\times 10^3$.

[00284] Fig. 3 shows mRNA copy numbers for KIR receptors with ligand specificity for HLA-C antigens. Quantitative determination of mRNA expression for 2DL1, 2DS1, 2DL2-3 and 3DS1 was performed by real time RT-qPCR. Dots represent individual NK clones: mRNA expression with protein surface expression; mRNA expression without surface expression, and mRNA expression with untested surface expression. Dotted lines in each plot identify the minimal mRNA copy number values associated with KIR surface expression. 2DL1: 166 clones, minimal value: 40 copies; 2DS1: 285 clones, minimal value: 13 copies. 2DL2-3: 229 clones, minimal value: 23 copies. 3DS1: 30 clones, minimal value: 98 copies. For each sample, mRNA copy number determination is performed in duplicate.

[00285] In the color version of Fig. 3 (Fig. 3—Color) mRNA with protein is presented in green while mRNA without protein is presented in red. In the corresponding black and white version of Fig. 3 (Fig. 3—B&W), mRNA with protein is presented with the symbol “•” while mRNA without protein is presented with the symbol X.

[00286] *Statistical analysis.* Frequencies of cytotoxic 2DS1^{pos} clones possessing different receptors or different *HLA-C* genotypes were compared using the two-sided chi-square test. Specific cytotoxicity values or receptor expression levels observed in different clone groups were compared by non-parametric two-tailed Mann-Whitney or Wilcoxon signed rank tests for independent or paired observations, respectively. Correlation between NKG2A gene expression and NKG2A MFI or % cytotoxicity was determined by Spearman's rank correlation coefficient. All statistical tests were performed using Prism 5 for Mac OS X (GraphPad). *p* values ≤ 0.05 were considered significant.

[00287] Frequency of 2DS1^{SP} NK clones with anti-HLA-C2 cytotoxicity is only decreased in donors homozygous for HLA-C2.

[00288] A total of 91 2DS1^{SP} clones were isolated of which 56 had anti-HLA-C2 cytotoxicity (Table III). Clones with anti-HLA-C2 cytotoxicity were obtained from any donor, regardless of the *HLA-C* genotype. Anti-HLA-C2 cytotoxicity was detected in 29 of 42 clones with the *C1:C1* genotype (69%) and in 19 of 22 clones with the *C1:C2* genotype (86%). In contrast, anti-HLA-C2 cytotoxicity was only

observed in 8 of 27 clones with the *C2:C2* genotype (30%) (*C1:C1* vs *C2:C2*, $p=0.001$ and *C1:C2* vs *C2:C2*, $p<0.0001$) (Fig. 2A and Table IIIA).

[00289] Fig. 2A, Fig. 2B, Fig. 2C and Fig. 2D are a series of plots showing the percentage of NK cell clone cytotoxicity with respect to effector and target cell HLA genotypes. Fig. 2A, *C1:C1* (n=42), *C1:C2* (n=22), and *C2:C2* (n=27) 2DS1^{SP} clones were tested for cytotoxicity against *C2:C2* EBV-BLCL (IHWG 9036) targets cells. Fig. 2B, *C1:C2* 2DS1^{SP} clones (n=22) were tested for cytotoxicity against *C1:C2* EBV-BLCL (KA) target cells. The same *C1:C2* clones were tested for cytotoxicity against *C2:C2* target cells, shown in Fig. 2A. Statistical analysis compares the frequency of anti-HLA-C2 cytotoxic *C1:C2* clones detected using *C2:C2* or *C1:C2* target cells. Fig. 2C, *C2:C2* 2DS1^{pos}/2DL3^{pos} clones (n=13) were tested for cytotoxicity against *C2:C2* EBV-BLCL (IHW 9036) targets cells. Statistical analysis compares the frequency of anti-HLA-C2 cytotoxic 2DS1^{pos}/2DL3^{pos} *C2:C2* clones, shown here, and of 2DS1^{SP} *C2:C2* clones, shown in Fig. 2A, to the same *C2:C2* target cells. Fig. 2D, Donor-derived clones obtained from HCT recipients were tested against *C2:C2* EBV-BLCL (IHW 9036) target cells. Case 1: HCT #1. Cytotoxicity of 2DL1^{pos} (n=6) and 2DS1^{SP} (n=10) *C1:C2* clones, obtained from a *C2:C2* HCT recipient 100 d post-transplantation. Case 2: HCT #2. Cytotoxicity of 2DL1^{pos} (n=16) and 2DS1^{SP} (n=4) *C1:C2* clones, obtained from a *C1:C2* HCT recipient 200 d post-transplantation.

[00290] These figures show 2DS1^{SP} NK clones with anti-HLA-C2 cytotoxicity. A, B and C, Clones from healthy donors; D, Donor-derived clones obtained from hematopoietic stem cell transplantation recipients. Collected data are from fourteen independent experiments. Assays were performed in duplicate or triplicate, E:T ratio 10:1. Dotted line: 13.1% cut-off between clone cytotoxicity and non-cytotoxicity. Closed circles: 2DS1^{pos} clones with anti-HLA-C2 cytotoxicity; open circles: 2DS1^{pos} clones without anti-HLA-C2 cytotoxicity; open squares: 2DL1^{pos} clones. 2DS1^{SP} denotes clones expressing the activating 2DS1 receptor but no inhibitory KIR with ligand specificity for HLA class I (2DL1, 2DL2, 2DL3 and 3DL1). ** $p=0.001$; *** $p<0.0001$.

[00291] The frequency of anti-HLA-C2 cytotoxicity among 2DS1^{SP}, *C1:C2* clones was similar to the frequency observed among *C1:C1* clones (**Fig. 2A**, *Left* and *Center*). This demonstrates that clonal deletion or clonal anergy is not characteristic for 2DS1^{SP} clones from donors heterozygous for *HLA-C2*. The 22 clones from *C1:C2* heterozygous donors were tested on *HLA-C2* heterozygous and homozygous target cells. The *C1:C2* clones were significantly less frequently cytotoxic against target cells with the autologous *C1:C2* genotype ($p<0.0001$) (Fig. 2A, *Center* and Fig. 2B; Table IIIB). Thus, 2DS1^{SP} clones with anti-HLA-C2 reactivity derived from donors with the *C1:C2* genotype are rarely cytotoxic to autologous targets. This decrease in frequency of anti-HLA-C2 cytotoxicity cannot be ascribed to the effect of inhibitory KIR expressed by the clones, since they all are 2DS1^{SP}.

[00292] The inventors finally determined the effect of inhibitory KIRs with ligand specificity for non-self-HLA class I on the function of 2DS1^{pos} clones. Thirteen 2DS1^{pos}, *C2:C2* clones, which also expressed the inhibitory receptor 2DL3 with ligand specificity for HLA-C1, were obtained. Six of 13 (46%) clones had anti-HLA-C2 reactivity, which is not significantly different from the results obtained with 2DS1^{SP}, *C2:C2* clones (**Fig. 2A**, *Right* and Fig. 2C; **Table IIIC**). Therefore, 2DS1^{pos}, *HLA-C2* homozygous clones with non-self inhibitory KIR display comparable anti-HLA-C2 reactivity as 2DS1^{SP} clones from the same donor.

3DS1 Does Not Contribute to Anti-HLA-C2 Reactivity of 2DS1^{SP} Clones

[00293] Because the genes encoding the two activating receptors 2DS1 and 3DS1 are in strong positive genetic linkage disequilibrium (Hsu et al., 2002; Venstrom et al., 2012), they frequently occur together. It is therefore potentially difficult to distinguish between a 3DS1 and a 2DS1-mediated effect. The minimal 3DS1 mRNA copy numbers needed for expression of 3DS1 receptor was 98 (**Fig. 3**). This value was used as reference for determination of 3DS1 receptor expression. Ninety 2DS1^{SP} clones from donors with different *HLA-C* genotypes were tested for 3DS1 expression. Sixty-five clones (72%), had the 2DS1^{SP}/3DS1^{pos} phenotype with 3DS1 mRNA copy numbers consistent with 3DS1 cell surface expression (median: 544; range: 102-2340). The remaining twenty-five clones had the 2DS1^{SP}/3DS1^{neg} phenotype. Their 3DS1 mRNA copies were either undetectable, or present in

numbers below the calculated minimal value, needed for expression (median: 0; range: 0-79). The frequency of anti-HLA-C2 cytotoxicity was compared between 2DS1^{SP}/3DS1^{pos} and 2DS1^{SP}/3DS1^{neg} clones. The analysis was done separately for different *HLA-C* groups of donors, because the *HLA-C* genotype affects the frequency of anti-HLA-C2 reactive 2DS1^{SP} clones (**Fig. 2**). It is demonstrated in **Table VI**, that presence or absence of 3DS1 does not affect anti-HLA-C2 reactivity in 2DS1^{SP} clones. This was observed in clones from *HLA-C1* positive (i.e., *C1:C1* and *C1:C2*), and clones from *HLA-C2* homozygous donors. Therefore, 3DS1 does not contribute to anti-HLA-C2 cytotoxicity by 2DS1^{SP} clones.

[00294] It is surprising that 2DS1^{pos} clones from donors heterozygous for *HLA-C2* do not display any evidence of reduced *in vitro* responses to HLA-C2. Determination of HLA-C2 antigen binding to 2DS1 *in vitro* demonstrates very weak binding affinity (Biassoni, R., et al. Eur. J. Immunol. 27:3095-3099; Stewart, C. A., et al. 2005. Proc. Natl. Acad. Sci. U S A 102:13224-13229). Because HLA antigens are co-dominantly expressed, the gene products from both *HLA-C* alleles will be displayed. *HLA-C2* homozygous individuals will therefore express twice the amount of HLA-C2 as *HLA-C2* heterozygous donors. Our study indicates that the amount of HLA-C2 ligand expressed by *HLA-C2* homozygous host cells is sufficient to induce tolerance in 2DS1^{pos} NK cells, while the amount expressed by *HLA-C2* heterozygous donors is insufficient for activation of 2DS1. We also demonstrate lack of ligand induced receptor down-modulation of 2DS1 on NK cells from *HLA-C2* homozygous donors. In contrast, the Ly49H receptor is down-regulated in mice expressing the m157 viral ligand (Sun, J. C., and L. L. Lanier. 2008. J. Exp. Med. 205:1819-1828; Tripathy, S. K., et al. 2008. J. Exp. Med. 205:1829-1841). Collectively, these results support that 2DS1 interactions with HLA-C2 is a weak interaction, which agrees with results from binding affinity studies (Biassoni, R., et al. Eur. J. Immunol. 27:3095-3099; Stewart, C. A., et al. 2005. Proc. Natl. Acad. Sci. U S A 102:13224-13229).

[00295] The present disclosure addresses the functional effects of interactions between the activating receptor, 2DS1, and its ligand, HLA-C2. But 2DS1 and the gene for another activating receptor, 3DS1, frequently exist together due to strong positive genetic linkage disequilibrium (Hsu, K. C., et al. 2002. J. Immunol.

169:5118-5129). Clinical genetic association studies of hematopoietic transplantation in AML have demonstrated different functional associations for the two genes in transplantation outcome. Specifically, *2DS1*, but not *3DS1*, was found to be associated with protection against post-transplantation leukemia relapse, while
5 *3DS1* was associated with improved survival (Venstrom, J. M., et al. 2012. N. Engl. J. Med. 367:805-816). In agreement with these clinical findings, we here demonstrate that the anti-HLA-C2 reactivity of *2DS1*^{SP} clones is independent of presence or absence of *3DS1* expression. It is currently not known how *3DS1* affects NK function, but studies of AIDS patients with HIV-1 indicate, that the *3DS1*
10 receptor might bind HLA-B (Bw4-80I) in HIV-I infected cells and target NK cells towards infected cells (Alter, G., et al. 2007. J. Exp. Med. 204:3027-3036). Collectively, these and the findings disclosed herein suggest that important NK effector functions are mediated by activating receptors with ligand specificity for HLA class I.

15 [00296] The inventors demonstrate that *2DS1*^{pos} clones are readily obtained from normal donors irrespective of their *HLA-C* genotype. Presence of both the activating *2DS1* receptor and its cognate ligand does not result in extensive deletion of such NK cells. Furthermore, *2DS1*^{pos} clones from *HLA-C2* heterozygous donors display anti-HLA-C2 reactivity *in vitro* similar to *HLA-C1* homozygous donors, who do not
20 carry the cognate ligand. Therefore, donors heterozygous for *HLA-C2* do not express sufficient ligand to induce *2DS1* tolerance. In contrast, *2DS1*^{pos} clones from *HLA-C2* homozygous donors have significantly reduced frequency of anti-HLA-C2 reactive clones. These results demonstrate that NK cells with an activating KIR specific for a self major-histocompatibility antigen are not all deleted from the repertoire, but are
25 rendered tolerant when sufficient density of the ligand is expressed. NK cell tolerance has been reported in mouse models of NK cells that express activating receptors for self-antigens (Ogasawara, K., et al. 2005. Nat. Immunol. 6:938-945; Oppenheim, D. E., et al. 2005 Nat. Immunol. 6:928-937; Wiemann, K., et al. 2005. J. Immunol. 175:720-729; Sun, J. C., and L. L. Lanier. 2008. J. Exp. Med.
30 205:1819-1828; Tripathy, S. K., et al. 2008. J. Exp. Med. 205:1829-1841), but the differential effect of tolerance induction by homozygous versus heterozygous expression of the activating ligand has not previously been reported. NK tolerance

was also observed in mice with mixed allogeneic bone marrow chimerism. NK cells in these mice expressed the activating Ly49D receptor and one strain also expressed the putative MHC-class I ligand, H2-D^d (Zhao, Y., et al. 2003. J. Immunol. 170:5398-5405; Hanke, T., et al. 1999. Immunity 11:67-77; George, T. C., et al. 5 1999. J. Immunol. 163:1859-1867). These reports as well as the present study demonstrate self-tolerance of activating MHC class I specific receptors in absence of known inhibitory receptors to self-MHC-class I.

[00297] In conclusion, this example describes clone-specific properties of clonogenic NK cell populations. These observations emphasize the heterogeneous 10 nature of NK cells isolated from human donors, and highlight that clonogenic NK cell populations may possess properties that are not available in unseparated crude NK cells (e.g., NK cells isolated from PBMC using common commercial immune-beads). Thus, the current invention, which enables the ability to obtain clonogenic NK cell populations of desirable and defined characteristics, can provide specific 15 NK cell populations that are superior to crude NK cells.

Table III. Cytotoxicity of 2DS1^{SP} NK clones

Donor <i>HLA-C</i> Genotype	KIR Phenotype	Target <i>HLA-C</i> Genotype	<i>n</i>	Cytolytic		% Lysis Median (range)	<i>p</i> ^{<i>a</i>}	<i>p</i> ^{<i>b</i>}
				Yes, <i>n</i> (%)	No, <i>n</i> (%)			
<i>A</i>								
<i>C1:C1</i>	2DS1 ^{SP}	<i>C2:C2</i>	42	29 (69)	13 (31)	34.3 (13.3-67.5)	1	1
<i>C1:C2</i>	2DS1 ^{SP}	<i>C2:C2</i>	22	19 (86)	3 (14)	39.6 (13.3-62.6)	NS	NS
<i>C2:C2</i>	2DS1 ^{SP}	<i>C2:C2</i>	27	8 (30)	19 (70)	24.9 (13.5-43.4)	0.001	NS
<i>B</i>								
<i>C1:C2</i>	2DS1 ^{SP}	<i>C2:C2</i>	22	19 (86)	3 (14)	39.6 (13.3-62.6)	1	1
<i>C1:C2</i>	2DS1 ^{SP}	<i>C1:C2</i>	22	3 (14)	19 (86)	24.3 (22.9-29.7)	<0.0001	NS
<i>C</i>								
<i>C2:C2</i>	2DS1 ^{SP}	<i>C2:C2</i>	27	8 (30)	19 (70)	24.9 (13.5-43.4)	1	1
<i>C2:C2</i>	2DS1 ^{pos} /L3 ^{pos}	<i>C2:C2</i>	13	6 (46)	7 (54)	17.7 (13.2-27.7)	NS	NS

^a Frequency of anti-*HLA-C2* cytolytic clones in each group is compared.

^b Magnitude of anti-*HLA-C2* cytotoxic responses for each clone group is compared.

5

Example 3

Clonogenic NK Cell Populations in Allogeneic Hematopoietic Stem Cell

Transplantation

[00298] This example describes the role of clonogenic NK cell populations in clinical settings. The clonogenic activities of NK cells were examined in donors and recipients of HSCT. *In vitro* expansion of clonogenic NK cell populations according to their KIR/HLA genotypes showed that these different cell populations have differential functions both *in vitro* and *in vivo*, as evidenced by the cytotoxicity experiments. Thus, this example provides evidence that expansion of clonogenic NK cell populations may be used to produce specific NK populations that match a donor's HLA genotype and exhibit better therapeutic effects than crude NK cell populations.

[00299] NK alloreactivity is known to affect hematopoietic stem cell engraftment. Rejection of murine parental bone marrow grafts by F1 hybrid NK cells is regulated by missing self-MHC-class I recognition, in combination with signals from activating receptor-ligand interactions. In some mouse strains the activating NKG2D and its ligands are dominating, while in other strains the activating Ly49D receptor in presence of H2-D^d mediates graft rejection (Beilke et al., 2010; George et al.,

1999). Allogeneic NK cells also participate in protecting HCT recipients against leukemia relapse and this effect is primarily observed in patients with acute myeloid leukemia (AML). The initial clinical studies involved HLA haploidentical transplants where the recipients lacked HLA class I ligands for inhibitory KIRs present in the donor (Ruggeri et al., 2002). Donor-derived NK alloactivation was interpreted as being caused by “missing self-HLA class I ligand” in the recipient. Another mechanism for development of alloreactive NK cells is HLA-C2 mediated activation of 2DS1^{pos} NK cells from *HLA-C1* homozygous individuals (Chewning, J. H., et al. 2007. J. Immunol. 179:854-868; Sivori, S., et al. 2011. Blood 117:4284-4292). We have recently demonstrated protection from relapse of AML following HCT from 2DS1 donors with the *HLA-C* genotypes *C1:C1* and *C1:C2*, while this benefit is absent if the HCT donor has the *C2:C2* genotype.

[00300] Allogeneic, myeloablative hematopoietic stem cell transplantation (HCT) provides a possibility for evaluating *de novo* development of donor-derived 2DS1^{pos} NK cells in the presence of cognate HLA-C2 ligand. The inventors observed that 2DS1^{SP} NK clones with anti-HLA-C2 reactivity are present in recipients of 2DS1^{pos} allogeneic hematopoietic stem cell transplantation. The inventors investigated two cases, where the graft was obtained from 2DS1^{pos} *C1:C2* donors. 2DS1^{SP} NK cells were identified post-HCT in both recipients (**Fig. 2D**). In the first case, ten 2DS1^{SP} clones were obtained 100 day post-HCT from a *HLA-C2* homozygous recipient. Four of the 2DS1^{SP} clones displayed anti-HLA-C2 cytotoxicity (**Fig. 2D, Case 1**). In the second case, four 2DS1^{SP} clones were obtained from a *HLA-C1:C2* heterozygous recipient, 200 day post-HCT. Two clones displayed anti-HLA-C2 cytotoxicity (**Fig. 2D, Case 2**). Therefore, donor-derived 2DS1^{SP} NK cells with ability to mediate anti-HLA-C2 cytotoxicity can be identified in an allogeneic host that expresses the cognate HLA-C2 ligand.

[00301] Nearly all 2DS1^{pos} clones with anti HLA-C2 reactivity are 2DS1^{SP} or 2DS1^{pos} with irrelevant, non-self-inhibitory KIR. These clones would be expected to display hypo-responsiveness due to lack of NK licensing (Anfossi et al., 2006 Immunity, 25, 331-42; Kim et al., 2005 Nature 436: 709-713. It is therefore surprising that 2DS1^{SP} clones from *HLA-C1* homozygous and heterozygous donors display potent anti-HLA-C2 responses, since they lack inhibitory receptors for self

MHC-class I. These findings are, however, consistent with the recently reported phenomenon termed “functional NK plasticity” described in mouse models. Here, mature NK cells were transferred between wild type mice and MHC-class I-deficient hosts. These mature NK cells displayed functional plasticity and adapted to the MHC environment of the host (Elliott et al., 2010 J. Exp. Med. 207: 2073–2079; Joncker et al., 2010 J. Exp. Med. 207: 2065–2072). Such studies confirm and extend these findings by demonstrating NK plasticity of developing NK cells in both the syngeneic and allogeneic *HLA-C2* positive host.

[00302] The present study of 2DS1^{pos} clones with anti-*HLA-C2* reactivity derived from donors with different *HLA-C* genotypes provides a mechanistic interpretation of previous clinical observations: while *2DS1* donors with the *C1:C1* and *C1:C2* genotypes have similar ability to generate a large number of anti-*HLA-C2* clones, *HLA-C2* homozygous donors have significantly reduced frequency of such clones. These results support a model where rejection of developing leukemic cells in many cases may be mediated by NKG2D activation of NK cells by NKG2D ligands (Boissel et al., 2006; Druker et al., 1996). Such NKG2D responses frequently require additional stimulatory signals. Such amplifying, stimulatory signals might be provided by “missing self-*HLA* class I ligand”, as observed in *HLA*-haploidentical HCT (Ruggeri et al., 2002) or by donor-derived 2DS1^{pos} NK cells activated by *HLA-C2* antigens in the recipient (Venstrom et al., 2012).

[00303] In conclusion, 2DS1^{pos} NK clones were developed by the inventors from donors with all three *HLA-C* genotypes *C1:C1*; *C1:C2*; and *C2:C2* for the purpose of determining the effect of the natural ligand, *HLA-C2*, on their frequency, phenotype, and tolerance to the self-ligand. The inventors report that 2DS1^{pos} NK clones with anti-*HLA-C2* reactivity, can be obtained from individuals with any *HLA-C* genotype. The frequency of 2DS1^{SP} clones with anti-*HLA-C2* reactivity is equally high for donors with the *HLA-C* genotypes *C1:C1* and *C1:C2*. In contrast, 2DS1^{pos} clones from donors homozygous for *HLA-C2* have significantly decreased frequency of anti-*HLA-C2* reactivity, consistent with tolerance of 2DS1 to *HLA-C2*. They also find that the inhibiting receptor CD94/NKG2A is not a critical regulator of tolerance to *HLA-C2* in *HLA-C2* homozygous NK cells. Finally, they observe

that 2DS1-mediated anti-HLA-C2 cytotoxicity in all donors almost exclusively is restricted to 2DS1^{SP} clones.

Example 4

Clonogenic NK Cell Populations According to Cell Surface Receptors and Their Heterogeneous Functions: Interplay Between NK Activating and Inhibitory Cell Surface Receptors among Clonogenic NK Cells

[00304] This example provides further characterization of the NK cell heterogeneity by clonogenic expansion of NK cells according to additional NK cell surface receptors. This example also describes mechanistic studies between NK cell heterogeneity and NK cell function. This example thus complements Example 2 and provides additional evidence on the importance of separating NK cells according to the make-up of their cell surface receptors.

15 *2DS1^{pos} NK clones expressing at least one inhibitory KIR for self-HLA class I are tolerant*

[00305] Among the inhibitory KIR with HLA class I ligand specificity, only 2DL1 and 3DL1 can be individually recognized by mAbs. The remaining inhibitory KIR 2DL2, 2DL3 and the activating receptor 2DS2 cannot be distinguished by monospecific Abs. Similarly, 2DS1 is not distinguishable from 2DL1, when both

20 receptors are present on the same cell. KIR phenotyping was therefore supplemented with determination of mRNA copy numbers for each of the KIRs with ambiguous phenotypes. Absolute RT-qPCR quantification assays were performed for 2DL1 (166 clones); 2DS1 (285 clones) and 2DL2-3 (229 clones) KIR transcripts. The mRNA copy numbers for such KIRs were determined and the minimal copy number

25 associated with KIR surface expression was identified, as described in Example 2 and in **Figure 3**. These minimal copy number values were used as reference to assign a KIR receptor phenotype to a total of 29 clones, whose surface expression of one or more KIR could not be unambiguously determined by flow cytometric analysis alone. This method allowed distinction of 2DL3 expression from 2DS2

30 expression in 16 clones, and detection of 2DS1 expression in 12 2DL1^{pos} clones. Moreover, we could identify 2DL1 and 2DS1 expression in 3 clones that were characterized by a mAb with specificity for both 2DL 1 and 2DS1.

[00306] Forty-six 2DS1^{pos} clones expressing at least one inhibitory KIR for self-HLA class I were obtained. Thirty-two clones were heterozygous for *HLA-C2* and 14 clones were homozygous for *HLA-C2*. Only one clone displayed anti-*HLA-C2* cytotoxicity (2%) (**Table IV**). Therefore, 2DS1^{pos} NK cells expressing at least one
 5 inhibitory KIR for self-HLA class I very rarely mediate anti-*HLA-C2* cytotoxicity, when the donor has *HLA-C2*. In contrast, 2DS1^{pos}, *HLA-C1* homozygous clones co-expressing inhibitory KIR for self-HLA class I frequently display anti-*HLA-C2* cytotoxicity (**Table V**, and Chewning, J. H., et al. 2007. J. Immunol. 179:854-868).

*Anti-*HLA-C2* cytotoxicity of 2DS1^{SP} clones expressing the inhibitory
 10 receptor LILRB1*

[00307] The leukocyte Ig-like receptor (LILR) B1 is expressed on NK cell subsets and other cells belonging to the myeloid and lymphoid lineage (Colonna, M., et al. 1997. J. Exp. Med. 186:1809-1818; Vitale, M., et al. 1999. Int. Immunol. 11:29-35), and delivers inhibitory signals upon interaction with a wide range of
 15 HLA class I antigens (Borges, L., et al. 1997. J. Immunol. 159:5192-5196; Colonna, M., et al. 1998. J. Immunol. 160:3096-3100). Twenty-two 2DS1^{SP} clones expressed LILRB1. Eight clones were obtained from *HLA-C1* homozygous donors and 14 clones from *HLA-C2* homozygous donors. All clones from C1 donors displayed anti-*HLA-C2* cytotoxicity. In contrast, anti-*HLA-C2* cytotoxicity was only observed
 20 in 5 of 14 clones from *C2* donors (*C1:C1* vs *C2:C2*, $p=0.003$). Therefore, LILRB1 may in some instances contribute to inhibition and maintaining tolerance of 2DS1 signals in *HLA-C2* homozygous donors.

[00308] The reduced frequency of anti-*HLA-C2* reactivity observed in *HLA-C2* homozygous, 2DS1^{SP} clones is consistent with NK tolerance observed in mice transgenic for activating receptor ligands (Ogasawara, K., et al. 2005. Nat. Immunol. 6:938-945;
 25 Oppenheim, D. E., et al. 2005 Nat. Immunol. 6:928-937; Wiemann, K., et al. 2005. J. Immunol. 175:720-729; Sun, J. C., and L. L. Lanier. 2008. J. Exp. Med. 205:1819-1828; Tripathy, S. K., et al. 2008. J. Exp. Med. 205:1829-1841). In humans,
 30 the inhibitory receptor CD94/NKG2A could potentially counteract 2DS1 activation by the *HLA-C2* ligand. We first determined if the 2DS1 receptor is signaling-competent in 2DS1^{SP}, *HLA-C2* homozygous clones expressing CD94/NKG2A. EB6

mAb cross-linking of the 2DS1 receptor in the presence of ICAM-I induces Ca^{++} flux. This activation signal is inhibited when HLA-E, the ligand for CD94/NKG2A (Braud, V. M., et al. 1998. Nature 391:795-799), is added (**Fig. 4A**).

[00309] **Fig. 4A** depicts a plot and a diagram of time-dependent changes in intracellular Ca^{2+} concentration; **Fig. 4B** and **Fig. 4C** are scatter plots of correlations; and **Fig. 4D** and **Fig. 4E** are plots of cytotoxicity according to NK cell subsets. These figures together show that the inhibitory receptor CD94/NKG2A attenuates but does not block 2DS1-mediated activation signals. Fig. 4A, Determination of intracellular Ca^{++} concentration of a representative C2:C2 2DS1^{SP}/NKG2A^{pos} clone triggered by EB6 mAb cross-linking of the 2DS1 receptor in the presence of ICAM-I, with or without HLA-E ligand for NKG2A inhibitory receptor. Activation was measured during exposure to mAb- and/or ligand-coated lipid bilayers (0-29.5 min). Shifts of intracellular Ca^{++} concentrations were determined by assessing changes of Fura 2-AM 340/380 fluorescence ratio. Results are representative of four independent experiments. EB6: anti-KIR2DL1/S1; HLA-E: hNKG2A ligand; ICAM-I: Inter Cellular Adhesion Molecule-I. Second from top line: ICAM-I; top line: EB6+ICAM-I; bottom linear-shape line: EB6+ICAM-I+HLA-E; bottom bell-shape line: EB6+HLA-E. Fig. 4B, Correlation between NKG2A mRNA copy numbers and NKG2A receptor MFI in 2DS1^{SP}/NKG2A^{pos} clones. This analysis was performed on C1:C1 (n=12) and C2:C2 (n=18) clones. Fig. 4C, Correlation between NKG2A mRNA copy numbers and anti-HLA-C2 cytotoxicity in 2DS1^{SP}/NKG2A^{pos} clones. This analysis was performed on C2:C2 (n=26) clones. Fig. 4D and Fig. 4E, Non-cytolytic (n=10) (Fig. 4D) and cytolytic (n=14) (Fig. 4E) 2DS1^{SP}/NKG2A^{pos} C2:C2 clones were tested against C2:C2 EBV-BLCL (IHWG 9036), with or without anti-NKG2A F(ab')₂-mediated NKG2A inhibition. Data were generated in three independent experiments. Each test was performed in duplicate, E:T ratio 10:1. F(ab')₂ concentration was 10 µg/ml. 4E F(ab')₂: anti-HLA-B/C F(ab')₂; NKG2A F(ab')₂: anti-hNKG2A F(ab')₂. The dotted line represents the 13.1% cut-off between cytotoxicity and non-cytotoxicity.

30 **p* < 0.05; ****p* ≤ 0.0001.

[00310] Similar results were obtained with 3 additional clones, demonstrating that the 2DS1 receptor is signaling-competent. Next, we determined the correlation

between NKG2A expression levels, and anti-HLA-C2 reactivity of 2DS1^{SP}/NKG2A^{pos} clones. NKG2A mRNA transcript copy numbers correlated well with NKG2A receptor MFI in 2DS1^{SP}/NKG2A^{pos} clones from *C1:C1* and *C2:C2* donors ($p=0.002$) (**Fig. 4B**). This data validated the inclusion of NKG2A mRNA
5 copies for our correlation studies. We expected that high cytotoxicity would be observed in clones with low NKG2A mRNA copies. However, NKG2A mRNA copy numbers were not found to correlate with anti-HLA-C2 cytotoxicity in 2DS1^{SP}, *C2:C2* clones (**Fig. 4C**). Therefore, expression of CD94/NKG2A inhibitory receptor does not predict if 2DS1^{SP}, *HLA-C2* homozygous clones mediate anti-HLA-C2
10 reactivity, or if they are tolerant to HLA-C2.

[00311] In order to directly test the inhibitory function of CD94/NKG2A on 2DS1^{SP}/NKG2A^{pos}, *HLA-C2* homozygous clones, the inventors determined the effect of anti-NKG2A F(ab')₂ fragment on cytotoxicity of 10 non-cytolytic (**Fig. 4D**) and 14 cytolytic (**Fig. 4E**) clones. Anti-HLA-C2 cytotoxicity was determined
15 using HLA-A*02:01 homozygous target (BLCL 9036), which expresses several HLA class I alleles with HLA-E binding leader peptides (Lee et al., 1998). HLA-E expression on this target was confirmed by mAb staining. Only one non-cytolytic clone changed from non-cytolytic to cytolytic, when the CD94/NKG2A receptor was blocked with anti-NKG2A F(ab')₂ (**Fig. 4D**). Furthermore, all 14 2DS1^{SP} clones
20 with anti-HLA-C2 reactivity displayed enhanced cytotoxicity following blocking with anti-NKG2A F(ab')₂ (**Fig. 4E**). Therefore, CD94/NKG2A only provides a modulatory, attenuating effect on 2DS1-mediated anti-HLA-C2 cytotoxicity, and is not a major factor controlling 2DS1 tolerance to HLA-C2 in *HLA-C2* homozygous donors. These results also agree with a recent report of a single 2DS1^{pos}/NKG2A^{pos}
25 clone, where 2DS1-mediated cytotoxicity was unaffected by presence of CD94/NKG2A (Foley et al., 2008, Int. Immunol. 20:555-563).

Table IV. 2DS1^{pos} NK clones with one or more inhibitory KIR for self-HLA-class I

Donor <i>HLA-C</i> Genotype	Effector Cell KIR Phenotype	Target Cell <i>HLA-KIR</i> Ligands	<i>n</i>	Cytolytic	
				Yes, <i>n</i> (%)	No, <i>n</i> (%)
<i>C1:C2</i>		<i>C1:C2;Bw4</i>	32	1 (3)	31 (97)
<i>C1:C2</i>	2DS1/2DL1	<i>C1:C2;Bw4</i>	5	0	5
<i>C1:C2</i>	2DS1/2DL1/2DL3	<i>C1:C2;Bw4</i>	1	0	1
<i>C1:C2</i>	2DS1/2DL1/2DL3/3DL1	<i>C1:C2;Bw4</i>	1	0	1
<i>C1:C2</i>	2DS1/2DL3	<i>C1:C2;Bw4</i>	14	0	14
<i>C1:C2</i>	2DS1/3DL1	<i>C1:C2;Bw4</i>	11	1	10
<i>C2:C2</i>		<i>C2:C2;Bw4</i>	14	0 (0)	14 (100)
<i>C2:C2</i>	2DS1/2DL1	<i>C2:C2;Bw4</i>	5	0	5
<i>C2:C2</i>	2DS1/2DL1/3DL1	<i>C2:C2;Bw4</i>	3	0	3
<i>C2:C2</i>	2DS1/3DL1	<i>C2:C2;Bw4</i>	6	0	6
Total			46	1 (2)	45 (98)

Example 5

5 2DS1 Activation, Missing Self-HLA Class I Recognition and KIR Inhibition

[00312] To illustrate the importance of purified clonogenic NK cell populations with defined characteristics, this example demonstrates that the novel method of clonogenic expansion of human NK cell clones as described in the present disclosure, when combined to other cellular and molecular biology techniques (e.g., RT-qPCR), may help attain a profound understanding of NK cell biology that cannot be achieved by studying crude NK cell isolations from a tissue sample (e.g., total NK cells isolated from PBMC).

[00313] Another aim of the present disclosure was to illustrate the genetic basis for 2DS1 activation and ligand induced tolerance. NK cells are regulated by inhibiting and activating cell surface receptors. Most inhibitory receptors recognize MHC-class I antigens, and protect healthy cells from NK cell-mediated auto-aggression. However, certain activating receptors, including the human killer cell Ig-like receptor (KIR) 2DS1, also recognize MHC-class I. This raises the question of how NK cells expressing such activating receptors are tolerized to host tissues. The inventors investigated whether the presence of HLA-C2, the cognate ligand for 2DS1, induces tolerance in 2DS1-expressing NK cells. Anti-HLA-C2 activity could be detected *in vitro* in some 2DS1 positive NK clones irrespective of presence or

absence of HLA-C2 ligand in the donor. The frequency of anti-HLA-C2 reactivity was high in donors homozygous for *HLA-C1*. Surprisingly, there was no significant difference in frequency of anti-HLA-C2 cytotoxicity in donors heterozygous for *HLA-C2* and donors without *HLA-C2* ligand. However, donors homozygous for *HLA-C2* had significantly reduced frequency of anti-HLA-C2 reactive clones as compared to all other donors. 2DS1 positive clones that express inhibitory KIR for self-HLA class I were commonly non-cytotoxic, and anti-HLA-C2 cytotoxicity was nearly exclusively restricted to 2DS1 single positive clones lacking inhibitory KIR. 2DS1 single positive NK clones with anti-HLA-C2 reactivity were also present post-transplantation in *HLA-C2* positive recipients of hematopoietic stem cell transplants from 2DS1 positive donors. These results demonstrate that many NK cells with anti-HLA-C2 reactivity are present in *HLA-C1* homozygous and heterozygous donors with 2DS1. In contrast, 2DS1 positive clones from *HLA-C2* homozygous donors are frequently tolerant to HLA-C2.

[00314] The inventors explored some possible mechanisms for tolerance to self in 2DS1^{pos} NK cells in donors homozygous for *HLA-C2*. Inhibition of 2DS1 activation by CD94/NKG2A only occurs very rarely. In contrast, inhibitory KIR with ligand specificity for self-HLA class I almost invariably suppressed 2DS1 activity. The effect of LILRB1 on *HLA-C2* homozygous 2DS1^{SP} clones was also tested, as shown in the previous example. Such clones frequently did not display anti-HLA-C2 reactivity. But the inventors also demonstrate that the *HLA-C* genotype influences the frequency of anti-HLA-C2 reactivity, which is high in *HLA-C1* homozygous, and low in *HLA-C2* homozygous donors. Therefore, their results only suggest a possible contribution of LILRB1 receptor to tolerance development and maintenance.

[00315] The inventors investigated the relationship between 2DS1 activation by HLA-C2 ligand, NK activation by missing self-HLA class I and inhibition of NK cell activation by inhibitory KIR to self-HLA class I. Eight 2DS1^{pos} clones that also expressed the inhibitory receptor 3DL1 were obtained from a donor heterozygous for *C1:C2* and homozygous for *HLA-Bw4*. The clones were tested against a panel of target cells homozygous for the 2DS1 activating ligand (i.e., *C2:C2* homozygous) or lacking the activating ligand (i.e., *C1:C1* homozygous). Presence of inhibitory

ligand for 3DL1 (i.e., *Bw4* homozygous) or absence of 3DL1 ligand (i.e., *Bw6* homozygous) was similarly tested in different combinations of target cells (**Fig. 5**). **Fig. 5** shows 3DL1 interaction with cognate HLA-Bw4 can override 2DS1-activation. 2DS1^{pos}/3DL1^{pos} clones (n=8) were obtained from a 2DS1 healthy donor with the *HLA-C1:C2;Bw4* genotype, and tested for cytotoxicity against EBV-BLCL target cells with different *HLA-C* and *-B* genotype. Left: cytotoxicity against *C2:C2;Bw4* EBV-BLCL (IHWG 9036) target cells. Center: cytotoxicity against *C2:C2;Bw6* EBV-BLCL (DD) target cells. Right: cytotoxicity against *C1:C1;Bw6* EBV-BLCL (GK) target cells. Data were generated in two independent experiments. Assays were performed in duplicate, E:T ratio 10:1. Dotted line: 13.1% cut-off between clone cytotoxicity and non-cytotoxicity. Degree of cytotoxic response between different groups is compared. ***p*<0.01.

[00316] Six of eight clones were inhibited by a target homozygous for *HLA-C2;Bw4* (**Fig. 5, Left**), consistent with the finding that clones with inhibitory KIR for self-HLA class I in most instances do not respond to HLA-C2 activation (**Table IV**). The same clones were tested on a target homozygous for *HLA-C2* but lacking the HLA-Bw4 ligand for 3DL1 (i.e., HLA genotype *C2:C2;Bw6:Bw6*). All eight clones displayed anti-HLA-C2 cytotoxicity, demonstrating the combined activating effect of 2DS1 signaling and recognition of “missing self-HLA class I” (**Fig. 5, Center**). Finally, the effect of lack of HLA-C2 ligand and absence of HLA-Bw4 on target cells (i.e., no stimulation of 2DS1 but recognition of “missing self-HLA class I”) is shown. Three clones were cytolytic, while 5 were inhibited (**Fig. 5, Right**). Therefore, “missing self-HLA class I” recognition in the absence of 2DS1 activation provides a variable activation signal.

25 *Tolerance of 2DS1^{SP} clones to cognate ligand in HLA-C2 homozygous donors is not dependent upon ligand-mediated down-regulation of the 2DS1 receptor*

[00317] Studies in transgenic mice have demonstrated ligand-mediated down-regulation of the activating NK receptor (Sun, J. C., and L. L. Lanier. 2008. J. Exp. Med. 205:1819-1828; Tripathy, S. K., et al. 2008. J. Exp. Med. 205:1829-1841). A similar mechanism could be involved in 2DS1 tolerance to *HLA-C2*. MFI of 2DS1 expression was determined on the eight *C2:C2* 2DS1^{SP} clones displaying anti-HLA-

- C2 cytotoxicity, and on the nineteen C2:C2 2DS1^{SP} clones lacking anti-HLA-C2 cytotoxicity to the *HLA-C2* homozygous target. 2DS1 expression levels were similar in these *HLA-C2* homozygous 2DS1^{SP} clones, irrespective of their anti-HLA-C2 responsiveness (**Fig. 6**). This figure shows 2DS1 surface expression on *HLA-C2* homozygous, 2DS1^{SP} clones. Left: anti-HLA-C2, cytolytic (n=8); Right: anti-HLA-C2, non-cytolytic (n=19). MFI was used to determine 2DS1 surface expression levels. Anti-HLA-C2 cytotoxicity is determined by reactivity against the *HLA-C2* homozygous EBV-BLCL (IHW 9036). Horizontal bars indicate medians. MFI levels of 2DS1 expression are compared between the two groups.
- 10 [00318] It has recently been proposed that NK activation is controlled by the localization of activating receptors in the NK plasma membrane. Presence or absence of inhibitory receptors with ligand specificity for self-HLA class I is in these studies suggested to regulate the activating receptor (Guia, S., et al. 2011. Sci. Signal. 4:ra21). It is possible that the 2DS1 receptor in IL-15 primed NK clones
- 15 could obtain a similar localization in the plasma membrane facilitating 2DS1 activation. Another possible mechanism for mediating self-tolerance to the HLA-C2 ligand in *HLA-C2* homozygous donors is *cis*-interactions between the 2DS1 receptor and the HLA-C2 ligand on the individual NK cell (Doucey, M. A., et al. 2004. Nat. Immunol. 5:328-336). The present study does not address this issue. Ongoing
- 20 studies with functional human NK cells in HLA class I transgenic mice may provide new insight on this issue (unpublished data).

Table V. 2DS1^{pos} C1:C1 NK clones co-expressing inhibitory KIR for self-HLA-class I

Donor <i>HLA-C</i> genotype		Target <i>HLA-C</i> genotype	n	Cytolytic		<i>p</i>
				Yes, <i>n</i> (%)	No, <i>n</i> (%)	
<i>C1:C1</i>	2DS1 <i>and</i>	<i>C2:C2;Bw4</i>	46	42 (91)	4 (9)	<0.0001 ^a
	2DL2/3	<i>C1:C2;Bw4</i>		1 (5)	18 (95)	
<i>C1:C1</i>	2DS1 <i>and</i> 3DL1	<i>C2:C2;Bw4</i>	2	0 (0)	2 (100)	

5

^a Frequency of cytolytic clones to targets with different *HLA-C* genotype is compared.

10

Table VI. Impact of 3DS1 expression on the anti-HLA-C2 cytotoxicity of 2DS1^{SP}

Donor <i>HLA-C</i> Genotype	KIR Phenotype	Target <i>HLA-C</i> Genotype	<i>n</i> ^a (%)	Cytolytic		<i>P</i> ^b
				Yes, <i>n</i> (%)	No, <i>n</i> (%)	
<i>C1:C1, C1:C2</i>	2DS1 ^{SP} /3DS1 ^{po} _s	<i>C2:C2;Bw4</i>	45 (70)	33 (73)	12 (27)	NS
	2DS1 ^{SP} /3DS1 ^{ne} _g		19 (30)	15 (79)	4 (21)	
<i>C2:C2</i>	2DS1 ^{SP} /3DS1 ^{po} _s	<i>C2:C2;Bw4</i>	20 (77)	7 (35)	13 (65)	NS
	2DS1 ^{SP} /3DS1 ^{ne} _g		6 (23)	1 (17)	5 (83)	

5

^a Clones are identical to those described in Fig. 2 and Table III. One *C2:C2* clone was not included in this analysis due to lack of cDNA for 3DS1 RT-qPCR amplification.

^b Frequency of anti-HLA-C2 cytolytic clones in each group is compared.

Table VII. Donor HLA class I and KIR and Recipient HLA class I

Donor KIR Phenotype	Donor HLA-KIR Ligands	Recipient HLA-KIR Ligands
KIR2DL1 ^{pos} /KIR2DL2-3 ^{neg} /KIR3DL1 ^{neg}	C1:C2 or C2:C2	C1:C1
KIR2DL1 ^{neg} /KIR2DL2-3 ^{pos} /KIR3DL1 ^{neg}	C1:C2 or C1:C1	C2:C2
KIR2DL1 ^{neg} /KIR2DL2-3 ^{neg} /KIR3DL1 ^{pos}	Bw4	Bw6
KIR2DL1 ^{pos} /KIR2DL2-3 ^{neg} /KIR3DL1 ^{pos}	C2:C2;Bw4 or C1:C2;Bw4	C1:C1;Bw6
KIR2DL1 ^{neg} /KIR2DL2-3 ^{pos} /KIR3DL1 ^{pos}	C1:C1;Bw4 or C1:C2;Bw4	C2:C2;Bw6

Table VIII. Clonogenic NK Cell Populations and Selected Clinical Applications

NK Cell Phenotype	Clinical Application
KIR2DS1 ^{pos} ; Inhibitory KIR ^{neg}	AML ^b ; HCT ^c
KIR2DS2 ^{SP}	AML; HCT
KIR3DS1 ^{pos}	AML; HCT
KIR3DS1 ^{pos}	HIV-I ^d
Activating KIR ^{pos}	Post-HCT CMV reactivation
NKG2D ^{pos} ± Inhibitory KIR ^{neg}	MICA/MICB ^{pos} or ULBP ^{pos} cancer or viral infection
NCR ^{pos} ± Inhibitory KIR ^{neg}	NCR ligand-positive cancer or viral infection
NKG2D CAR ^e	MICA/MICB ^{pos} or ULBP ^{pos} cancer or viral infection
CD19 CAR	ALL ^f
G(D2) CAR	Neuroblastoma
CS1 CAR	Multiple myeloma
Anti-WT1/HLA complex	AML

^a Donor HLA-C genotype: C1:C1 or C1:C2;

^b Acute myeloid leukemia;

^c Hematopoietic stem cell transplantation;

^d Recipient genotype: HLA-Bw4-80I;

^e Chimeric antigen receptor;

^f Acute lymphoblastic leukemia

Abbreviations used in this Application

[00319] 2DL1, inhibitory killer cell Ig-like receptor 2DL1; 2DL2, inhibitory
killer cell Ig-like receptor 2DL2; 2DL3, inhibitory killer cell Ig-like receptor 2DL3;
2DS1, activating killer cell Ig-like receptor 2DS1; 2DS1^{SP}, 2DS1 single positive;
5 2DS2, activating killer cell Ig-like receptor 2DS1; 3DL1, inhibitory killer cell Ig-
like receptor 3DL1; 3DS1, activating killer cell Ig-like receptor 3DS1; LILRB1,
leukocyte Ig-like receptor, subfamily B, member 1; AML, acute myeloid leukemia;
BLCL, B lymphoblastoid cell line; Bw4, HLA-KIR ligand group Bw4; Bw6, HLA-
KIR ligand group Bw6; C1, HLA-KIR ligand group C1; C2, HLA-KIR ligand group
10 C2; HCT, hematopoietic stem cell transplantation; IHWG, International
Histocompatibility Working Group; KIR, killer cell Ig-like receptor; MFI, mean
fluorescence intensity; RT-qPCR, quantitative RT-PCR.

[00320] All references cited and/or discussed in this specification are
incorporated herein by reference in their entireties and to the same extent as if each
15 reference was individually incorporated by reference.

SEQUENCE LISTING

SEQ ID NO:1

gccgccaccatggccttaccagtgaccgaactgggtgaatgtaataagtgatttgaaaaaattgaagatcttattcaatct
atgcatattgatgctactttatatacggaaagtgatgttcaccccagttgcaaagtaacagcaatgaagtgccttctctggag
ttacaagttatttcacttgagtcgggagatgcaagtattcatgatacagtagaaaatctgatcctctagcaaacaacagtttg
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agttttgtacatattgtccaaatgttcacacacttctga

SEQ ID NO:2

Gccgccaccatggccttaccagtgaccgatcacgtgccctcccccatgtccgtggaacacgcagacatctgggtcaa
gagctacagcttgactccaggagcggtacattgtaactctggttcaagcgtaaagccggcacgtccagcctgacgg
agtgcgtgttgacaaggccacgaatgtcgccactggacaacccccagttcctcaaatgcattaagccgcagcttcatct
cccagctcaaacaacacagcggccacaacagcagctattgtccgggctcccagctgatgccttcaaaatcacctcca
caggaaccacagagataagcagtcattgagtcctcccacggcaccctctcagacaacagccaagaactgggaactc
acagcatccgcctcccaccagccgaggtgtgtatccacagggccacagcgacaccactgtggctatctccacgtcc
actgtcctgtgtgtgggctgagcgtgtgtctctctggcatgctacctcaagtcaaggcaaactccccgctggccag
cgttgaaatggaagccatggaggctctgccggtgactgggggaccagcagcagagatgaagacttgaaaactgctc
tcaccacctatga

What is claimed is:

1. An *in vitro* method for producing a population of clonogenic natural killer (NK) cells, said method comprising:
 - a. culturing a human NK cell clone (i) in the presence of a feeder cell, wherein said feeder cell trans-presents human interleukin-15 (IL-15), and (ii) in a culture medium that is without added IL-2; and
 - b. maintaining said culture (i) under conditions of temperature, humidity, and CO₂ that support the proliferation of said human NK cell clone and (ii) for a period of time sufficient to achieve expansion of said human NK cell into said clonogenic NK cell population.
2. The *in vitro* method of claim 1 wherein said NK cell clone is a naturally-occurring NK cell isolated from a human donor.
3. The *in vitro* method of claim 1 wherein said NK cell is genetically modified.
4. The *in vitro* method of claim 1 wherein said human NK cell clone expresses a nucleic acid encoding one or more cell surface receptors that modulate an NK cell function and/or mediate NK cell cytotoxicity and/or mediate cytolytic activity.
5. The method of claim 4 wherein each of said one or more receptors is independently selected from an inhibitory receptor and an activating receptor.
6. The method of claim 4 wherein each of said one or more receptors is independently selected from the group consisting of a killer immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), and a chimeric antigen receptor (CAR).
7. The method of claim 4 wherein at least one of said receptors is a KIR wherein said KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2.

8. The method of claim 4 wherein at least one of said receptors is an NCR wherein said NCR is selected from the group consisting of NKp46, NKp44, and NKp30.
9. The method of claim 4 wherein at least one of said receptors is NKG2D or NKG2D-DAP10-CD3 ζ .
10. The method of claim 4 wherein at least one of said receptors is a chimeric receptor comprising one or more peptide selected from the group consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.
11. The *in vitro* method of claim 1 wherein said culture is established with a feeder cell density of from about 10⁴/mL to about 10⁶/mL.
12. The method of claim 11 wherein said feeder cell is engineered to trans-present said human interleukin-15 (IL-15).
13. The method of claim 12 wherein said engineering comprises an exogenously introduced nucleic acid encoding human IL-15R α , which nucleic acid is configured for expression and production of said human IL-15R α .
14. The method of claim 12 wherein said engineering comprises an exogenously introduced nucleic acid encoding human IL-15, which nucleic acid is configured for expression and production of said human IL-15.
15. The method of claim 12 wherein said expression is constitutive or inducible.
16. The method of claim 12 wherein said culture further comprises one or more additional feeder cell selected from the group consisting of a peripheral blood mononuclear cell (PBMC), an EBV-B lymphoblastoid cell (EBV-BLCL), and an RPMI8866 lymphoblastoid cell.
17. The *in vitro* method of claim 1 wherein said feeder cells are non-proliferative.

18. The *in vitro* method of claim 1 wherein said feeder cells are HLA class I negative cells.
19. The method of claim 18 wherein said HLA class I negative cells are selected from the group consisting of a pre-B-lymphocyte cell line, a bone marrow stromal cell line, an erythroleukemia cell line, a B lymphoblastoid cell line, a Burkitt lymphoma cell, and a Wilms tumor cell.
20. The method of claim 19 wherein said feeder cell is selected from the group consisting of: BaF/3, OP9, K562, , 721.221, a Daudi cell; an HFWT cell, and an HLA class I positive cell.
21. The *in vitro* method of claim 1 wherein said feeder cell is surface antigen mismatched relative to said an inhibitory surface KIR receptor(s) on said NK cell clone.
22. The *in vitro* method of claim 1 wherein said culture medium is without added IL-15.
23. The method of claim 22 wherein said culture medium is without any added cytokine.
24. The *in vitro* method of claim 1 wherein said culture medium comprises from about 0% to about 20% human serum.
25. The *in vitro* method of claim 1 wherein said period of time is from about 10 days to about 35 days or from about 15 days to about 30 days or from about 20 days to about 25 days.
26. The *in vitro* method of claim 1 further comprising harvesting said clonogenic NK cell population.
27. The *in vitro* method of claim 1 wherein said wherein said clonogenic NK cell population comprises from about 1×10^5 NK cells to about 5×10^6 NK cells or

from about 2.5×10^5 NK cells to about 2.5×10^6 NK cells or from about 5×10^5 NK cells to about 1.5×10^6 NK cells.

28. The *in vitro* method of claim 1 wherein the cloning efficiency for the clonogenic NK cell populations is at least about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50%.
29. The method of claim 4 wherein the expanded NK cell population contains at least about 50% NK cells expressing said one or more cell surface receptor.
30. The *in vitro* method of claim 1 wherein at least 90% of said NK cell population is viable.
31. The *in vitro* method of claim 1 wherein said NK cell population exhibits NK cell functions, e.g., cytotoxicity and/or cytolytic activity.
32. An *in vitro* method for producing a population of clonogenic NK cells expressing a phenotype of interest, and reacting to a specific HLA class I genotype of a recipient, said method comprising:
 - a. selecting at least one NK cell clone from a donor whose HLA class I genotype mismatches that of a recipient;
 - b. culturing said at least one NK cell clone in the presence of a feeder cell, wherein said feeder cell trans-presents human interleukin-15 (IL-15) and wherein said culturing is performed by addition of a culture medium that is without added IL-2; and
 - c. maintaining said culture (i) under conditions of temperature, humidity, and CO₂ that support the proliferation of said human NK cell clone and (ii) for a period of time sufficient to achieve expansion of said human NK cell into said clonogenic NK cell population;wherein said phenotypic trait of interest of said NK clonogenic population comprises expression of at least one cell surface receptor having the property of detecting “missing self-HLA class I ligand” in said recipient.

33. The *in vitro* method of claim 32 wherein said phenotypic trait of interest comprises an expression of at least one cell surface receptor selected from the group consisting of inhibitory KIR with ligand specificity for HLA class I and, optionally selected from the group consisting of activating KIR, c-type lectin-like receptors, natural cytotoxicity receptors, and NK-activating chimeric receptors.
34. The *in vitro* method of claim 32 wherein said inhibitory KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1 and said at least one cell surface receptor is selected from the group consisting of KIR2DS1, KIR2DS2, NKG2D, NKp46, NKp44, and NKp30; NKG2D-DAP10-CD3 ζ , and a chimeric receptor comprising one or more peptide selected from the group consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.
35. The *in vitro* method of claim 32 wherein said phenotypic trait of interest and the corresponding said recipient HLA genotype are selected from a row in Table VII.
36. A cell genetically modified to co-express human IL-15 and IL-15R α on its surface, wherein said cell has the property of inducing NK cells to proliferate *in vitro*.
37. The cell according to claim 36 wherein said cell is immortalized.
38. The cell according to claim 36 wherein said expression of IL-15 and IL-15R α is constitutive or inducible.
39. The cell according to claim 36 wherein the IL-15 and IL-15R α are independently either native or genetically modified human IL-15 and native or genetically modified human IL-15R α , respectively.
40. The cell according to claim 36 wherein said cell is HLA class I negative.

41. The cell according to claim 36 wherein said HLA class I negative cells are selected from the group consisting of a pre-B-lymphocyte cell line, a bone marrow stromal cell line, an erythroleukemia cell line, a B lymphoblastoid cell line, a Burkitt lymphoma cell, and a Wilms tumor cell.
42. The cell according to claim 41 wherein the cell line is BaF/3, or OP9, or K562, or 721.221, or a Daudi cell, or an HFWT cell.
43. The cell according to claim 36 wherein said feeder cell is an HLA class I positive cell.
44. The cell according to claim 36 wherein said cell expresses an HLA surface antigen that mismatches with NK clone inhibitory surface KIR receptors, and said cell also expresses at least one HLA class I antigen.
45. The cell according to claim 36 wherein the cell lacks major histocompatibility complex class I molecules.
46. The cell according to claim 36 wherein the cell is of murine origin.
47. The cell according to claim 36 wherein the cell is of human origin.
48. An isolated, purified clonogenic NK cell population expressing one or more phenotypes of interest, wherein the number of cells in said NK cell population is at least of the order of 10^5 and said phenotype comprises expression of one or more cell surface receptors that modulate NK cell function and/or mediate NK cell cytotoxicity and/or cytolytic activity.
49. The cell population according to claim 48 wherein each of said one or more receptors is independently selected from an inhibitory receptor and an activating receptor.
50. The cell population according to claim 48 wherein each of said one or more receptors is independently selected from the group consisting of a killer

immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), and a chimeric antigen receptor (CAR).

51. The cell population according to claim 48 wherein at least one of said receptors is a KIR wherein said KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2.
52. The cell population according to claim 48 wherein at least one of said receptors is an NCR wherein said NCR is selected from the group consisting of NKp46, NKp44, and NKp30.
53. The cell population according to claim 48 wherein at least one of said receptors is NKG2D or NKG2D-DAP10-CD3 ζ .
54. The cell population according to claim 48 wherein at least one of said receptors is a chimeric receptor comprising one or more peptide selected from the group consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.
55. The cell population according to claim 48 wherein said population is monoclonal and the number of cells in said population is of the order selected from the group consisting of 10^5 and 10^6 .
56. The cell population according to claim 48 wherein said population is polyclonal and the number of cells in said population is of the order selected from the group consisting of 10^6 , 10^7 , 10^8 , and 10^9 .
57. The cell population according to claim 48 wherein the NK cells contain at least about 50% cells expressing said receptor.
58. The cell population according to claim 48 wherein said NK cell phenotype comprises expression of KIR2DS1 but not co-expression of inhibitory KIR with ligand specificity for HLA class I antigens.

59. The cell population according to claim 48 wherein said cells are obtained from a *HLA-C1:C1* homozygous or *HLA-C1:C2* heterozygous, and *KIR2DS1* positive donor.
60. The cell population according to claim 48 wherein said cells are obtained from *HLA-C1:C1* homozygous or *HLA-C2:C2* heterozygous, and *KIR2DS1* positive donor.
61. A biologic composition suitable for human administration comprising an aliquot of an isolated, purified clonogenic human NK cell population expressing a phenotype of interest comprising a number of NK cells of the order of 10^5 to 10^7 cells per clone.
62. The composition according to claim 61 wherein said phenotypic trait of interest comprises an expression of one or more cell surface receptor independently selected from an inhibitory receptor and an activating receptor.
63. The composition according to claim 61 wherein each of said one or more receptors is independently selected from the group consisting of a killer immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), and a chimeric antigen receptor (CAR).
64. The composition according to claim 61 wherein at least one of said receptors is a KIR wherein said KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2.
65. The composition according to claim 61 wherein at least one of said receptors is an NCR wherein said NCR is selected from the group consisting of NKp46, NKp44, and NKp30.
66. The composition according to claim 61 wherein at least one of said receptors is NKG2D or NKG2D-DAP10-CD3 ζ .
67. The composition according to claim 61 wherein at least one of said receptors is a chimeric receptor comprising one or more peptide selected from the group

consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.

68. A method for treating a disease selected from the group consisting of, but not limited to AML, ALL, melanoma, MDS, non-Hodgkin's lymphoma, neuroblastoma, multiple myeloma, transplant rejection, and GvHD, the method comprising the administration of an effective amount of an isolated, purified clonogenic human NK cell population, wherein said population comprises of the order of 10^5 to 10^7 cells per clone, and wherein said population expresses a phenotype of interest relevant for said disease in a recipient individual in need thereof.
69. The method according to claim 68 wherein said phenotypic trait of interest comprises an expression of one or more cell surface receptor independently selected from an inhibitory receptor and an activating receptor.
70. The method according to claim 68 wherein each of said one or more receptors is independently selected from the group consisting of a killer immunoglobulin-like receptor (KIR), a C-type lectin-like receptor (CLLR), a natural cytotoxicity receptor (NCR), and a chimeric antigen receptor (CAR).
71. The method according to claim 68 wherein at least one of said receptors is a KIR wherein said KIR is selected from the group consisting of KIR2DL1, KIR2DL2/3, KIR3DL1, KIR2DS1, and KIR2DS2.
72. The method according to claim 68 wherein at least one of said receptors is an NCR wherein said NCR is selected from the group consisting of NKp46, NKp44, and NKp30.
73. The method according to claim 68 wherein at least one of said receptors is NKG2D or NKG2D-DAP10-CD3 ζ .
74. The method according to claim 68 wherein at least one of said receptors is a chimeric receptor comprising one or more peptide selected from the group

consisting of a CD19 peptide, a G(D2) peptide, a CS1 peptide, and a WT1 peptide.

75. The method according to claim 68 wherein both said phenotype comprises expression of at least one cell surface receptor and said disease are selected from a row in Table VIII.
76. The method according to claim 68 wherein said effective amount of clonogenic human NK cells is of the order of 10^5 to 10^7 cells per kilogram of body weight of the recipient individual.

FIGURE 1

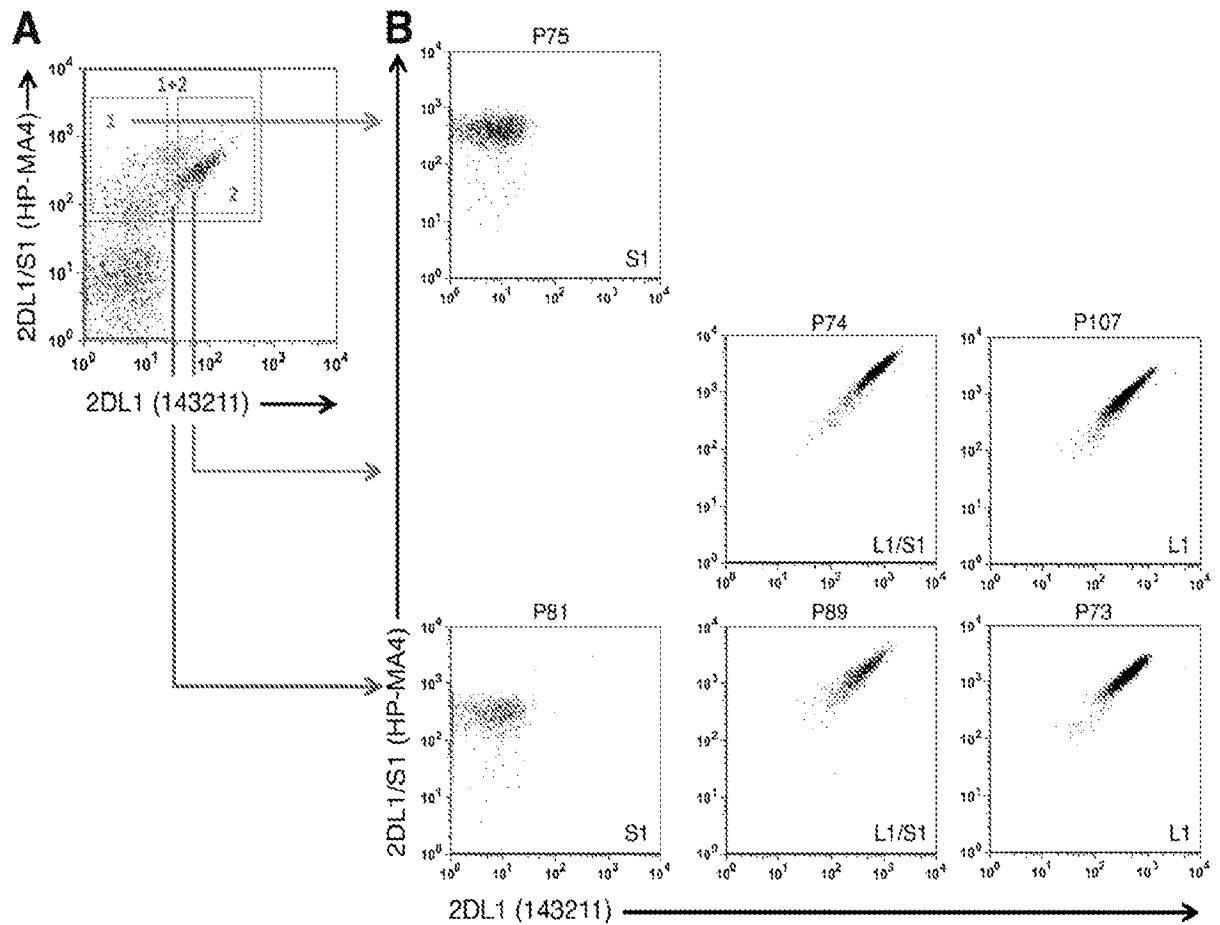


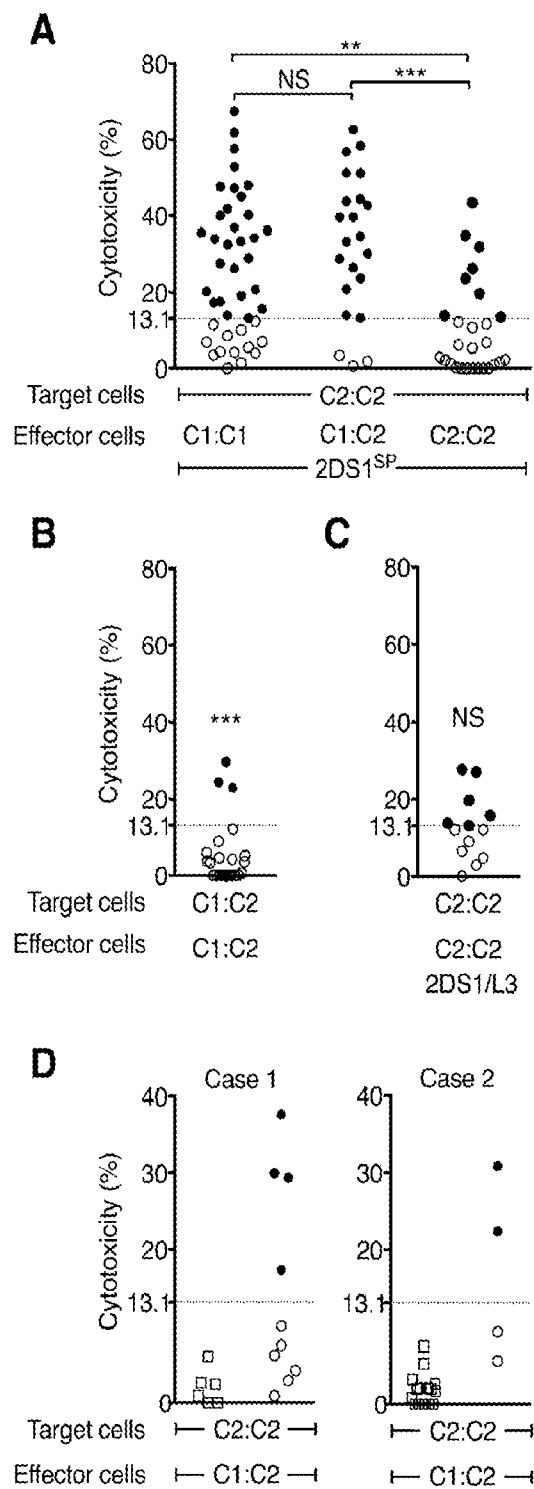
FIGURE 2

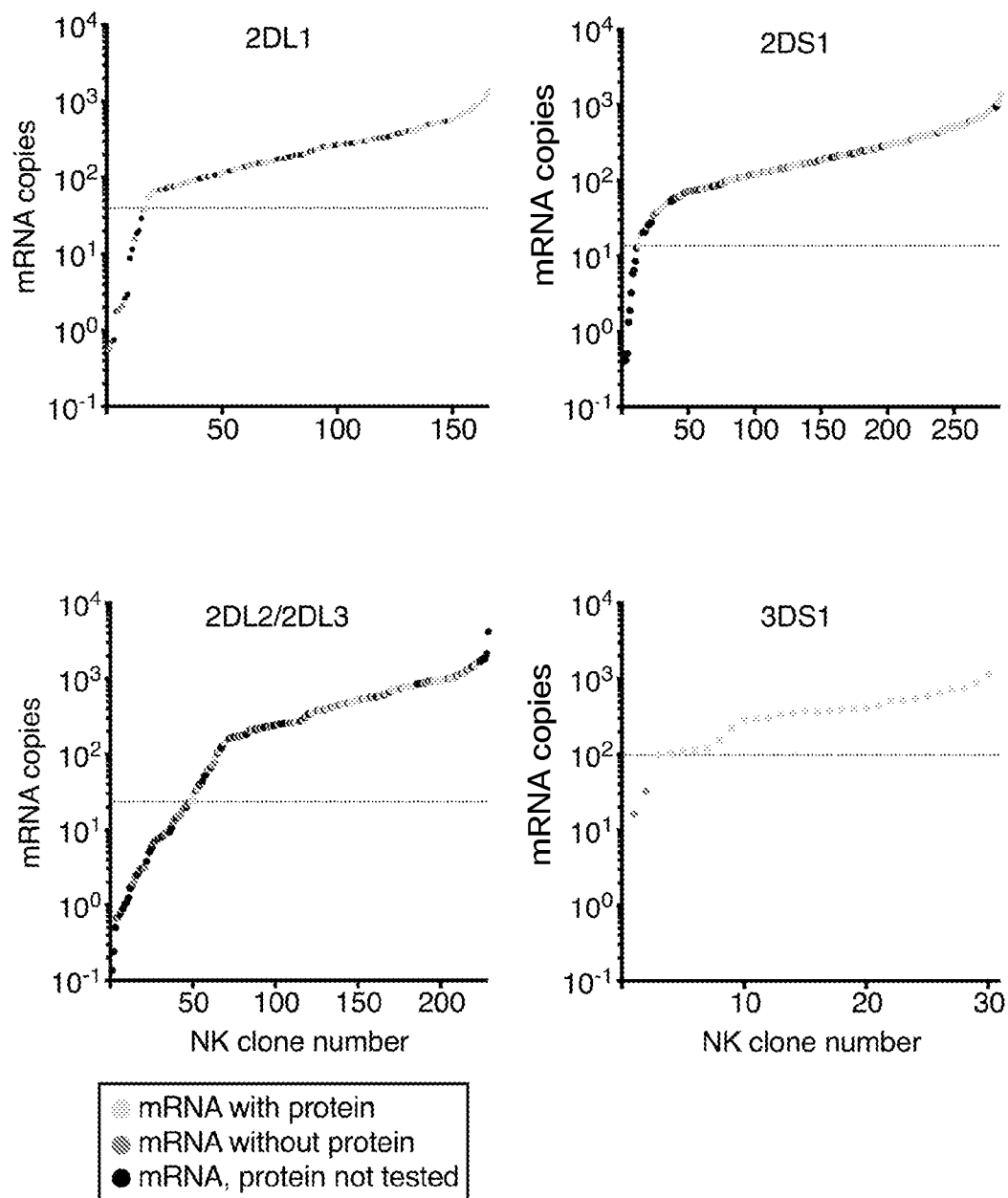
FIGURE 3

FIGURE 3 (Black & White)

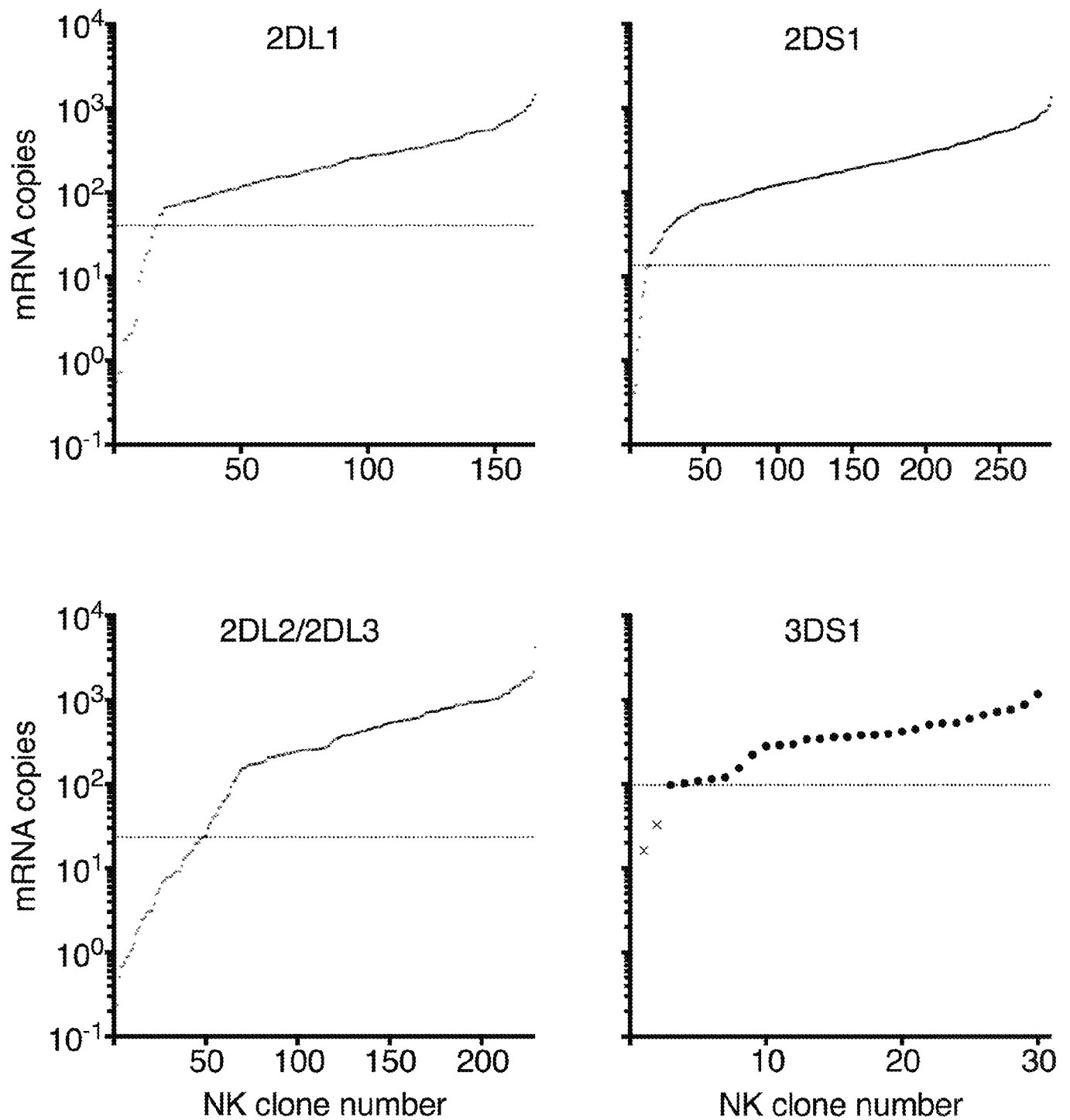
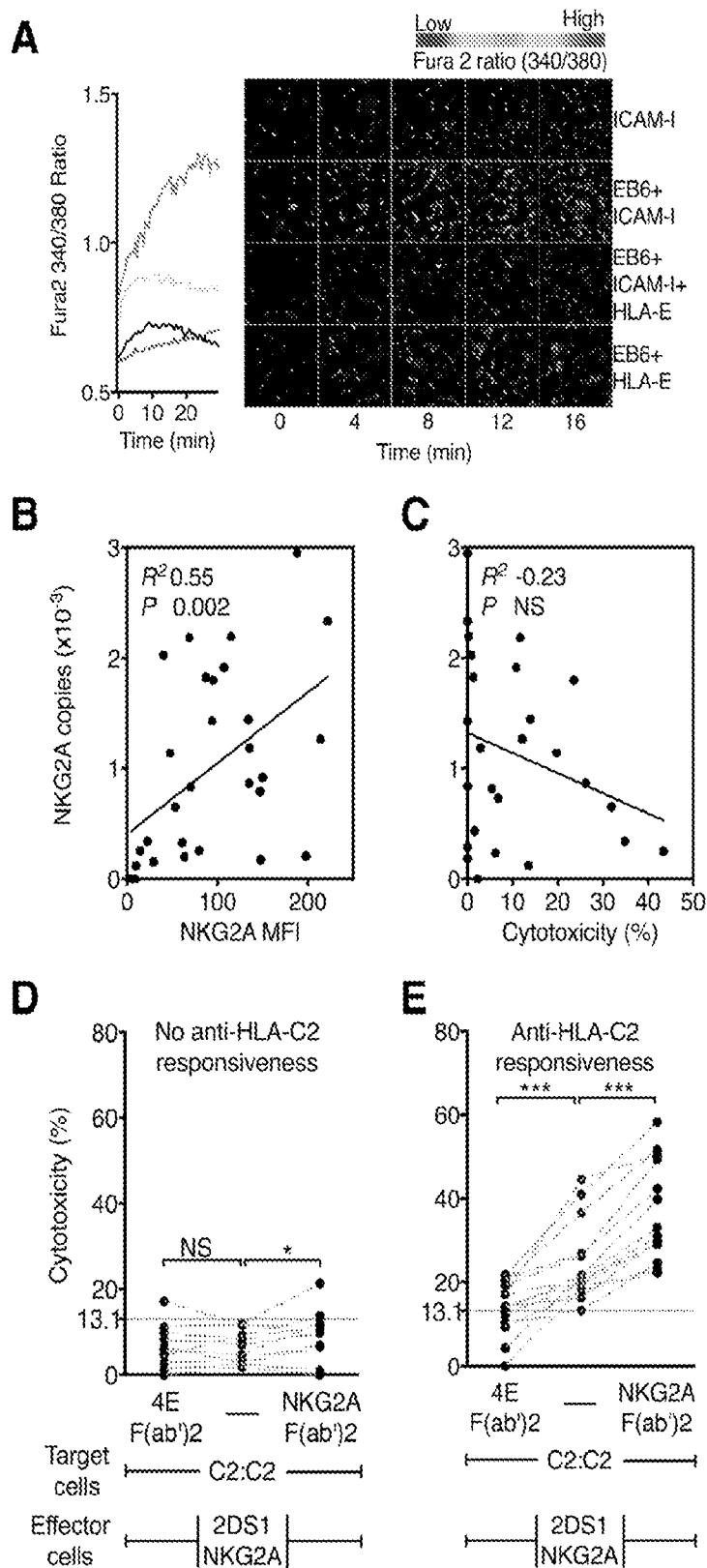


FIGURE 4



6/7

FIGURE 5

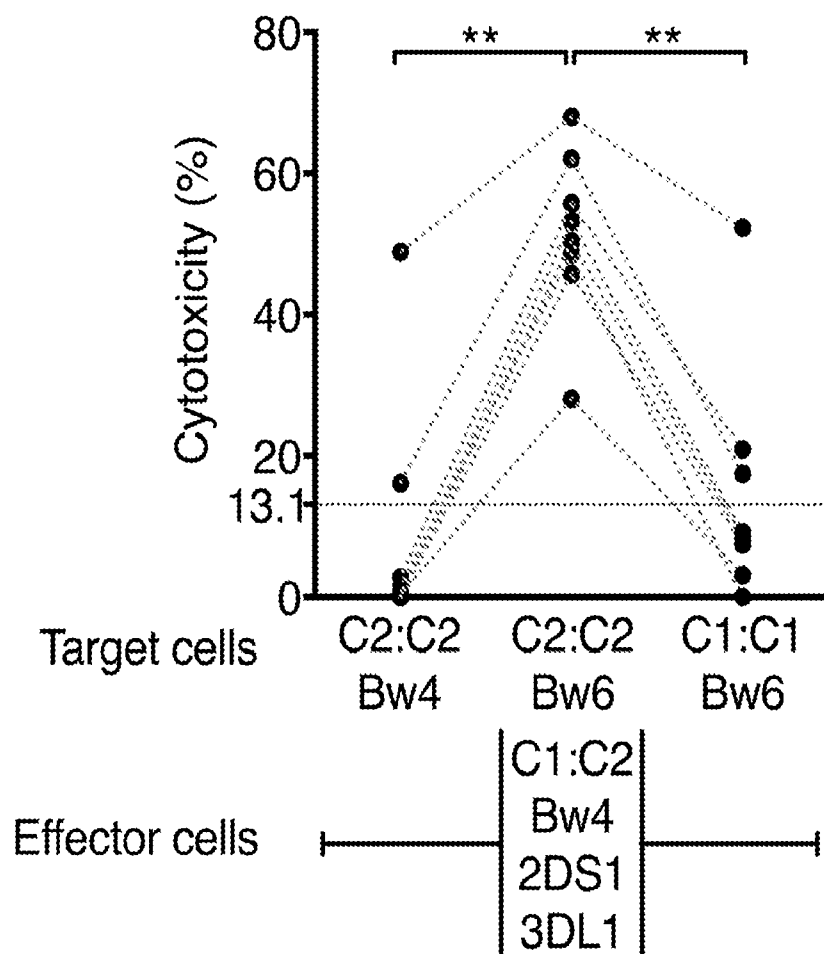
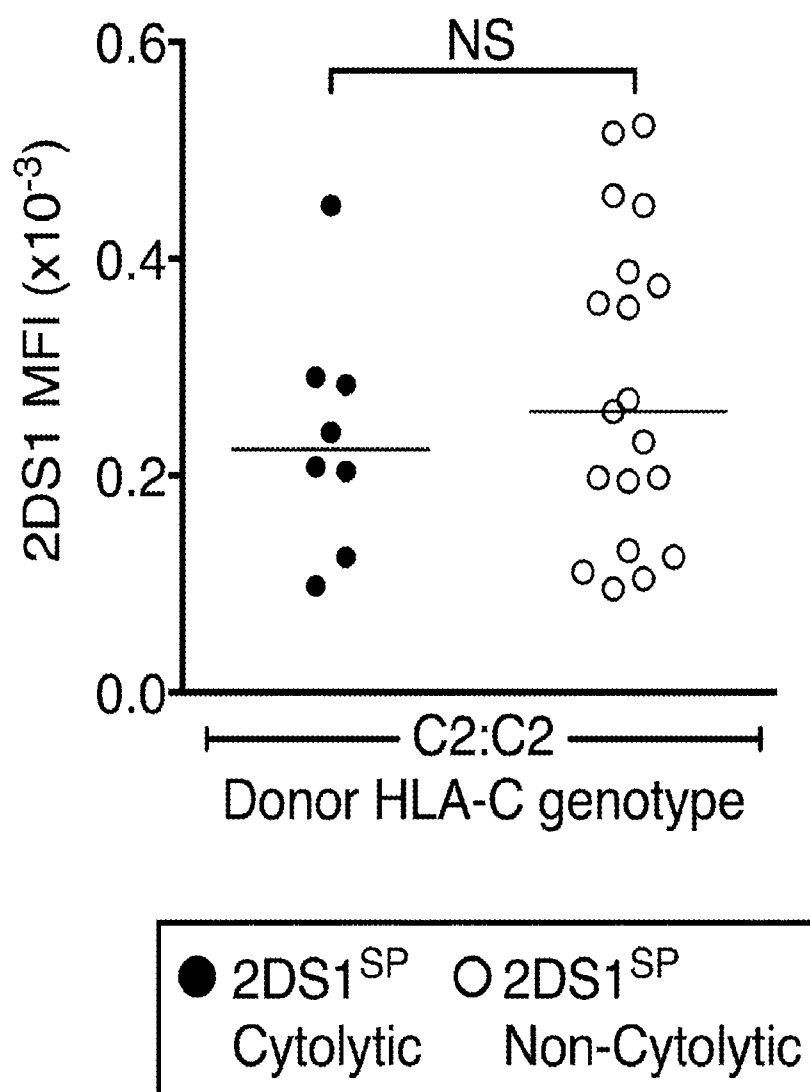


FIGURE 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/024315**A. CLASSIFICATION OF SUBJECT MATTER****C12N 5/0783(2010.01)i, C12N 5/10(2006.01)i, C07K 14/705(2006.01)i, C07K 14/54(2006.01)i, A61K 35/17(2014.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 5/0783; A61K 35/12; A61P 35/00; C12N 5/10; C07K 14/705; C07K 14/54; A61K 35/17

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords:clonogenic natural killer cell, interleukin-15, interleukin-2, culture medium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014-037422 A1 (INVEN2 AS) 13 March 2014 See abstract; page 1, lines 16-18; page 5, lines 33-34; page 13, lines 25-27; page 15, lines 1-4; page 16, lines 24-25; page 20, lines 11-17; page 21, lines 12-13 and 19-26; claims 1-2, 7-8, 16, 18 and 20.	48-57, 61-67
Y		1-34
A		35-47, 58-60
X	BURKETT, PATRICK R. et al., 'Coordinate Expression and Trans Presentation of Interleukin (IL)-15R α and IL-15 Supports Natural Killer Cell and Memory CD8 $^{+}$ T Cell Homeostasis', The Journal of Experimental Medicine, 4 October 2004, Vol. 200, No. 7, pp. 825-834. See abstract; page 829, right column, paragraph 2.	36-43, 46-47
Y		1-34
Y	FEHNIGER, TODD A. et al., 'Potential mechanisms of human natural killer cell expansion in vivo during low-dose IL-2 therapy', The Journal of Clinical Investigation, July 2000, Vol. 106, No. 1, pp. 117-124. See abstract; figures 1-3; table 1.	1-34
A	US 2011-0044962 A1 (BECK, ROSE C.) 24 February 2011 See abstract; claim 1.	1-67

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 July 2015 (29.07.2015)

Date of mailing of the international search report

29 July 2015 (29.07.2015)

Name and mailing address of the ISA/KR

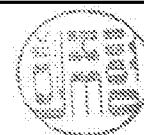
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/024315

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIAO, WEI et al., 'Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy' , Immunity, 24 January 2013, Vol. 38, No. 1, pp. 13-25. See the whole document .	1-67

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/024315

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 68-76
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 68-76 pertain to a method for treatment of the human by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/024315

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014-037422 A1	13/03/2014	None	
US 2011-0044962 A1	24/02/2011	US 2014-186319 A1 US 8518397 B2	03/07/2014 27/08/2013