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# Genome-wide analyses and functional profiling of human NK cell lines

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ABSTRACT

Natural killer (NK) cell lines, including YTS, NK92, NK3.3, and NKL, represent excellent models for the study of human natural killer cells. While phenotypic and functional differences between these cell lines have been reported, a multi-parametric study, encompassing genomic, phenotypic, and functional assays, has not been performed. Here, using a combination of techniques including microarray and copy number analyses, flow cytometry, and functional assays, we provide in-depth genetic, functional, and phenotypic comparison of YTS, NK92, NK3.3, and NKL cell lines. Specifically, we found that while the cell lines shared similarities in enrichment of growth and survival pathways, they had differential expression of 557 genes, including genes related to NK cell development, survival, and function. In addition, we provide genetic and phenotypic analyses that demonstrate distinct developmental origins of NK92, YTS, and NKL cell lines. Specifically, NK92 has a phenotype associated with the CD56$^{bright}$ NK cell subset, while both YTS and NKL appear more CD56$^{dim}$-like. Finally, by classifying cell lines based on their lytic potential, we identified genes differentially expressed between NK cell lines with high and low lytic function. Taken together, these data provide the first comprehensive genetic, phenotypic, and functional analyses of these commonly used NK cell lines and provides deeper understanding into their origins and function. This will ultimately improve their use as models for human NK cell biology.

## 1. Introduction

### 1.1. NK cells

Human natural killer (NK) cells are innate lymphoid cells that function as a critical first line of defense against viral infections and malignancies. NK cells represent 0.61–16.87% of human peripheral blood lymphocytes and are also found in bone marrow, spleen, liver, lung, uterus, and secondary lymphoid tissues (Angelo et al., 2015; Bjorkstrom et al., 2016). A primary function of NK cells is their ability to kill virally infected or malignant target cells in an antigen-independent manner using a process of cytotoxic granule exocytosis (Vivier et al., 2008; Bryceson et al., 2005; Long et al., 2013). NK cells also secrete cytokines and have been shown to produce co-stimulatory signals for activation of other immune cell types (Vivier et al., 2008). NK cell lytic function is regulated by germline-encoded activating and inhibitory receptors that recognize ligands up-regulated on stressed or virally transformed cells and self MHC class I, respectively (Bryceson et al., 2005; Long et al., 2013). The importance of human NK cell function in health and disease is demonstrated in patients with primary immunodeficiencies, who as a result of NK cell deficiency, are highly susceptible to viral infections (Mace et al., 2013; Gineau et al., 2012; Hughes et al., 2012; Hanna et al., 2015; Grier et al., 2012; Cottineau et al., 2017; Mace et al., 2017).

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### 1.2. NK cell development and subsets

Like other lymphoid cells of the immune system, NK cells originate from $CD34^+$ common lymphoid precursors derived from self-renewing hematopoietic stem cells of the bone marrow (Yu et al., 2013). Human NK development is defined by six distinct phenotypic stages of NK cell precursors found in secondary lymphoid tissues (Freud et al., 2006; Scoville et al., 2017). As maturing NK precursors progress from Stage 1 ($CD34^+CD117^-$IL-$1R1^-CD94^-CD16^-$) to Stage 3 ($CD34^-CD117^+$IL-$1R1^+CD94^-CD16^-$), they lose the potential to differentiate into other cell types, including T cells and dendritic cells (Freud and Caligiuri, 2006). NK cell maturation is marked by the expression of transcription factors, including Eomes and T-Bet, and the acquisition of surface markers CD56, CD16 and CD57. $CD56^{bright}$ Stage 4b NK cells progress to Stage 5 ($CD56^{dim}$), which is accompanied by CD16 expression and further develop into Stage 6 NK cells, defined by acquisition of CD57 (Scoville et al., 2017; Freud and Caligiuri, 2006).

Phenotypically, human NK cells are classically defined as $CD56^+CD3^-$ innate lymphoid cells. Within peripheral blood, human NK cells can be divided into two distinct populations defined by their density of CD56, $CD56^{bright}$ and $CD56^{dim}$, with each subset expressing a discrete combination of receptors that reflect their unique phenotype and function. $CD56^{bright}$ cells comprise between 0.6–38.2% of the total NK population in peripheral blood while $CD56^{dim}$ cells make up the majority, with a range of 61.7–98.3% (Angelo et al., 2015). $CD56^{bright}$ NK cells, found primarily in secondary lymphoid tissues, are characterized as having low cytotoxic potential, consistent with low expression of lytic granules. However, they can produce abundant cytokines (IFN-γ, TNFα) in response to stimulation. $CD56^{bright}$ NK cells express the high-affinity IL-2 receptor alpha chain, CD25, and have low expression of killer immunoglobulin-like receptors (KIR) (Michel et al., 2016).

$CD56^{bright}$ NK cells are thought to be the direct precursor of $CD56^{dim}$ NK cells. During in vitro differentiation of human NK cells, the appearance of $CD56^{bright}$ cells occurs prior to $CD56^{dim}$ cells, suggesting $CD56^{dim}$ cells may arise from $CD56^{bright}$ cells (Grzywacz et al., 2006; Cooley et al., 2007). KIR expression correlates with a linear $CD56^{bright}$ to $CD56^{dim}$ transition, in that $CD56^{bright}$ NK cells have low KIR expression while $CD56^{dim}$ cells have higher expression of KIR and only express the heterodimeric (IL-2Rβ/IL-2Rγ; CD122/132) intermediate affinity IL-2 receptor (Angelo et al., 2015; Freud and Caligiuri, 2006; Montaldo et al., 2013). $CD56^{dim}$ NK cells are terminally differentiated and kill virally infected or tumorigenic cells through the directed release of lytic granules (Vivier et al., 2008; Cichocki et al., 2014). Receptor crosslinking can also cause $CD56^{dim}$ NK cells to produce IFN-γ and TNFα, however, this is generally not as potent when compared to the production of cytokines by $CD56^{bright}$ NK cells (Anfossi et al., 2006; Fauriat et al., 2010; De Maria et al., 2011).

Studies involving purified human NK cell intermediates also reveal the differentiation of terminally mature $CD16^+CD57^+KIR^+$ NK cells from less mature NK cell subsets in the presence of either supportive cell lines or in humanized mice treated with recombinant human IL-15 (Chan et al., 2007; Bjorkstrom et al., 2010; Lopez-Verges et al., 2010; Herndler-Brandstetter et al., 2017; Huntington et al., 2009). Despite this evidence for a terminal transition from $CD56^{bright}$ NK cells to $CD56^{dim}$ cells, the exact mechanism for this progression is still unknown.

### 1.3. NK cell lines

Three commonly used cell lines (NK92, YTS, and NKL) all originate from malignant expansions of NK cell leukemia/lymphoma. A fourth line, NK3.3, was generated by in vitro NK cell cloning from the blood of a healthy donor (Kornbluth et al., 1982). The NK92 cell line is derived from the peripheral blood of a male patient with large granular lymphocyte (LGL)-non-Hodgkins lymphoma and is IL-2 dependent (Gong and Maki, 1994). NK92 cells are positive for cell surface receptors CD56, CD2, CD7, CD11a, CD28, and CD45 but are CD16 negative (Gong and Maki, 1994; Drexler and Matsuo, 2000; Maki et al., 2001). NK92 also have germline configuration for beta and gamma genes of the T cell receptor (TCR) (Gong and Maki, 1994). While NK92 cells express few KIRs, they do have a relatively diverse activating receptor repertoire including expression of NKp30, NKp46, NKG2D, CD28, and 2B4 (Drexler and Matsuo, 2000; Maki et al., 2001). NK92 cells also have the potential to kill through lytic granule-independent pathways as is indicated by their expression of FasL, TRAIL, and TNFα (Maki et al., 2001). NK92 cells show high cytotoxic potential against susceptible target cells including K562 and 721.221 (Gong and Maki, 1994; Chen et al., 2007).

YTS cells are a sub-clone of the YT NK cell line which originates from the pericardial fluid of a male patient with acute lymphoblastic lymphoma (Yodoi et al., 1985; Yoneda et al., 1992). YTS are positive for CD56, CD7, CD28, and CD45RO but negative for CD2 and CD16, with TCR genes in germline configuration (Yoneda et al., 1992). This cell line does not require exogenous IL-2 for maintenance in culture. Due to the high expression of CD28, YTS readily kill 721.221 target cells that express high levels of B7.1, but have reduced cytolytic potential for other common NK cell targets (Chen et al., 2006).

The NKL cell line is derived from the peripheral blood of a male patient with LGL-leukemia and, like NK92 cells, require IL-2 for survival (Robertson et al., 1996). They are CD2, CD6, CD11a, CD27, CD29, and CD94 positive (Robertson et al., 1996). Depending on their time in culture, NKL can rapidly lose expression of CD16, CD56, and CD57 resulting in cultures that are CD56 negative with minimally detectable CD16 (Robertson et al., 1996; Le Bouteiller et al., 2002). The lytic function of NKL cells can vary, with both high and low specific lysis of susceptible K562 and 721.221 cells being reported (Chen et al., 2007; Robertson et al., 1996; Le Bouteiller et al., 2002; Matsuo and Drexler, 2003). NKL cells can also mediate lysis of antibody-coated P815 target cells (Robertson et al., 1996).

The non-malignant cell line, NK3.3, originates from the peripheral blood of a normal donor expanded in mixed lymphocyte culture and are IL-2 dependent (Mahle et al., 1989). They are positive for CD2, CD11a, CD38, CD45, CD16, and CD56 (Kornbluth et al., 1982; Umehara et al., 1997), although CD56 expression can vary over time in culture with IL-2 (J. Kornbluth, personal communication, 2017). NK3.3 cells are dependent on IL-2 for prolonged survival (Kornbluth et al., 1982). NK3.3 have cytolytic activity against susceptible target cells (K562 and MOLT-4) with specific lysis activity plateauing after 1 h in co-culture with target cells (Kornbluth et al., 1982; Mahle et al., 1989).

Each of these cell lines have different origins and consequently different phenotypes (Drexler and Matsuo, 2000; Matsuo and Drexler, 2003). They do, however, retain important NK cell characteristics and have served as useful models for studying NK cell biology. They all display phenotypic and biological similarities to normal human NK cells and do not rearrange their TCR genes or express a TCR complex (Kornbluth et al., 1982; Gong and Maki, 1994; Yoneda et al., 1992; Robertson et al., 1996). Despite their many shared inherent properties of NK cells, important differences have been reported among these cell lines, including differential expression of some cell surface receptors (Drexler and Matsuo, 2000; Matsuo and Drexler, 2003). These differences could potentially be exploited experimentally in comparative studies to better understand the molecular pathways or functions in question. Thus, we pursued the goal of identifying similarities and differences among these cell lines so that a rational approach could be utilized to gain greater perspective and obtain insight into human NK cell biology. Although the functional features and utility of NK cells lines is well established, a genome-wide microarray evaluation has not been previously reported. We evaluated steady-state coding mRNA for resting NK92, YTS, NKL, and NK3.3 NK cell lines using microarray gene expression. We further supplemented this with high-resolution phenotyping by flow cytometry and functional analyses, which we have

combined with gene expression data to associate gene expression with lytic function. Together our data demonstrate key similarities and differences among these cell lines and underscore the importance of understanding these differences when choosing a cell line for biological studies.

## 2. Materials and methods

### 2.1. Cell lines

All NK cell lines were maintained in log phase, at an approximate concentration of 1–5 × $10^5$ cells/mL of media. NK92 cells were acquired from the ATCC. YTS cells were a kind gift from Dr. J. Strominger (Harvard Medical School); NKL cells were generously provided by Dr. Jerome Ritz (Harvard Medical School); NK3.3 cells were a kind gift from Dr. Jacki Kornbluth (St. Louis University School of Medicine). Cells were cultured at 37°C atmosphere with 5% $CO_2$. YTS cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) plus essential nutrients. The NK92, NK3.3, and NKL cell lines were maintained in Myelocult media (STEMCELL Technologies) supplemented with 100 U/ml of IL-2 (Roche) and 50U/ml of penicillin/streptomycin (Gibco). Target cell lines used in $^{51}$Cr-release assays were maintained in log phase growth. All target cell lines were grown in RPMI plus 10% FBS and essential nutrients and were routinely confirmed to be mycoplasma negative. Cell line identity was also routinely validated using flow cytometry of known differentially expressed cell surface receptors.

### 2.2. Primary NK cells

*Ex vivo* human NK cells were isolated by Rosette-Sep Human NK cell Enrichment Cocktail (STEMCELL Technologies) and Ficoll-Paque Plus density gradient centrifugation (Amersham Biosciences), then re-suspended in complete media for immediate experimental use. All samples were acquired under the guidelines of the Declaration of Helsinki and in accordance with the Institutional Review Board of the Children's Hospital of Philadelphia.

### 2.3. Flow cytometry

Comprehensive flow cytometric phenotyping of NK cell lines was performed on cells in log phase of growth as previously described (Mahapatra et al., 2017) (Supplemental Table 1). In brief, NK92, YTS, and NKL were suspended at a concentration of 2.5–5 × $10^6$ cells/ml. All antibody staining was performed at room temperature in the dark. For panels which did not require stimulation, cells were stained in a total volume of 200µl with antibodies diluted in PBS-2% FBS for 20–25 minutes. For activating receptor and cytokine profiles, cells were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) for 4 h at 37°C, 5% $CO_2$. Unstimulated control cells were also incubated for 4 h in parallel. For detection of intracellular cytokines, Brefeldin A (10µg/ml, Sigma-Aldrich) and anti-CD107a were added at the beginning of incubation for both stimulated and unstimulated cells. Cells were then permeabilized with BD Cytofix/Cytoperm (BD Biosciences), followed by antibody staining for 45–60 minutes in BD Fix/Perm buffer. Activated cells were stained for surface markers for 20–25 minutes following the 4-hour incubation. To detect transcription factors, cells were permeabilized for 1 h at room temperature with FoxP3 Buffer (Tonbo Biosciences), followed by washes with FoxP3 Wash Buffer (Tonbo Sciences). After antibody staining, cells for all panels were washed and surface receptor stained cells were fixed with 1X PBS containing 1% paraformaldehyde (Sigma-Aldrich) and kept at 4°C in the dark until processed. A modified LSR Fortessa (BD Biosciences) with the capacity to detect 18 fluorescent parameters was used to acquire 1000–10000 events per sample. Negative controls were acquired using cell line specific fluorescence minus one controls. For viSNE analysis, the following parameters were imported into Cytobank Premium (Fluidigm) for each cell line: CD56, CD2, CD28, CD16, CD8, 2B4, CD18, CD11a, CD11b, CD11c, and CD54. Heat map visualization was used to demonstrate intensity of expression. Quantification of mean fluorescence intensity was calculated using FlowJo X (TreeStar Inc.) and was exported to Prism 7.0 (GraphPad Software) for analysis and visualization.

### 2.4. Chromium release assays

NK cell cytotoxicity was evaluated by $^{51}$Cr-release assays using 721.221 Epstein-Barr virus-transformed B cells, Raji B cell lymphocytes derived from Burkitt's Lymphoma, and K562 erythroleukemia target cells as previously described (Orange et al., 2002). Briefly, target cells were incubated for an hour at 37 °C, 5% $CO_2$ with 100 µCi of $^{51}$Cr, then washed with RPMI complete media. Target cells were co-cultured with effector NK cell lines or fresh primary NK cells at 10:1, 5:1, 2.5:1, 1.25:1, 0.625:1 and 0.313:1 effector to target ratio for 4 h at 37°C, 5% $CO_2$. For Raji cells, co-culture with effector NK cell lines or *ex vivo* NK cells were performed with and without the addition of Rituximab at a concentration of 20 µg/ml. Total release wells were lysed with 100 µl of 1% IGEPAL (Sigma Aldrich) and then plates were centrifuged for 10 minutes at 1250 rpm with no brake. 100 µl from each well was transferred to a LUMA plate (Perkin Elmer) and dried overnight. Plates were read with a TopCount NXT. Percent specific lysis was calculated as (sample - average spontaneous release) / (average total release – average spontaneous release) x 100.

### 2.5. Cell isolation, RNA extraction, and DNA microarray analysis

The four human NK cell lines, YTS, NK92, NK3.3, and NKL, were kept in log phase of growth in culture to normalize cell cycle. Three independent experimental repeats for each cell line were generated. RNA was extracted from each cell line using the RNeasy isolation kits (Qiagen) and target cRNA for microarray experiments prepared using 1–5 µg of total RNA. RNA integrity was assessed by evaluating the ratio of optical absorbance for 260 and 280 nm using an Agilent Bioanalyzer 2100. Samples with $A_{260}$:$A_{280}$ between 1.8 and 2.0 were used for first strand cDNA synthesis using reverse transcriptase and a T7-$(dT)_{24}$ primer. The double-stranded cDNA was purified from reactants using the Affymetrix Cleanup Module (Affymetrix) following RNA degradation using RNAse H and second strand cDNA synthesis with DNA polymerase I. Further sample preparation, hybridization, and processing were performed using standard techniques. The Affymetrix Human Genome U133 A 2.0 Plus GeneChip Array was used to analyze expression levels for a genome-wide collection of mRNA transcripts and variants in each cell line.

### 2.6. Statistical analysis

CEL files generated from the Affymetrix Gene Chip experiments were imported into the analysis program GeneSpring GX (Agilent Technologies). Individual genes were linked with Affymetrix probe set identification numbers and gene ontologies, as per the Affymetrix website. Gene Chip Robust Multiarray Averaging (GC-RMA) was performed on all sets for quantile normalization. Using Partek Genomics Suite (Partek), the GC-RMA values were then log2 transformed. A 1-way ANOVA on the entire collection of data was performed to identify gene expression values that were significantly different across the four cell lines. Those expression values with a p-value of $< 0.001$ were imported back into GeneSpring. Cell lines with high cytotoxic function (YTS and NK92) were directly compared to expression values from cell lines with low cytotoxic function (NK3.3 and NKL) by a 1-way nested ANOVA. Significant values were imported into SpotFire software (SpotFire, Somerville, MA). Array comparative genomic hybridization was used for identifying copy number variants. Grouped flow cytometry

data was compared by ordinary 1-way ANOVA using Prism 7.0 (GraphPad Software). PCA analysis was performed with Orange 3.5 (Demsar et al., 2013). Briefly, the mean of log2 transformed data with > 50-fold difference in expression between at least two cell lines were imported into Orange 3.5. Data was decomposed to principal components and graphed using the Scatter Plot feature.

## 3. Results

### 3.1. Analysis of gene expression defines cell-line specific differences

Genome-wide analysis of gene expression was performed in triplicate from 4 NK cell lines (NK92, NKL, NK3.3, and YTS). A heat map of gene profiles across the four lines demonstrated a striking pattern of similarities in expression among the cells (Supplemental Fig. 1). The corresponding dendrogram suggested a closer lineage relationship between YTS and NK92 cell lines, with deviation first from NK3.3 and then NKL. Ingenuity Pathway Analysis (IPA) was used to identify signaling pathways predicted to be activated in NK cell lines. Significantly enriched pathways included many expected to be related to transformation of cell lines, including PI3K/AKT, MTOR and markers of metastasis (Supplemental Fig. 2). In addition, many genes identified as highly expressed in NK cell lines were linked to lymphocyte signaling and function, including integrins, CD28, JAK/STAT, IL-2, Homeobox, leukocyte extravasation and CXCR4 signaling pathways. Altered function-associated pathways included actin, phospholipase, ERK/MAPK, PAK, and Rho signaling.

GC-RMA and $\log_2$ transformed gene expression values for the entire genome of each NK cell line were tested by 1-way ANOVA analysis to determine values that significantly varied between cell lines. Despite the high degree of similarity between the cell lines, we identified 557 genes with > 50-fold difference in expression between at least two of the cell lines (Fig. 1A, Supplemental Table 2). NK cell-related genes with highly differential expression included *NCAM1*, *CD28*, *EOMES*, *GZMK*, *FCGR3A*, *KIT*, *CXCR4*, *CCL4*, *IL2RA, STAT4, CISH, SLAMF1-7 and -8, EAT2, SELL, NCR3,* and *IRF5* (Fig. 1B). Other differentially expressed genes of interest were more broadly related to cytotoxic function, including *UNC13C*, *VAMP8*, *ITGA2*, *FN1*, *WASF1*, *TIAM1*, and *CTLA4* (Fig. 1B). Unsurprisingly, several differentially expressed genes were linked to HLA haplotypes. Principal component analysis demonstrated that each cell line segregated appropriately with its replicates, validating the reproducibility of technical repeats (Fig. 1C). When differentially expressed pathways were considered by IPA comparison analysis, differences between the cell lines, including AhR, cyclin, G1/S checkpoint, and PTEN pathways were identified (Fig. 1D). Together, these data demonstrate that despite a high degree of genetic similarity, significant differences among NK cell lines could be identified.

### 3.2. Flow cytometric analysis confirms unique phenotypes of NK cell lines

Multiparametric flow cytometry was performed to measure 41 parameters related to NK cell phenotype and function using five panels (Mahapatra et al., 2017). Panels included surface receptors required for NK cell adhesion, activation, inhibition, and development. NK3.3 proved difficult to culture long term, therefore phenotypic expression by flow cytometry was only performed on NK92, NKL, and YTS cell lines. Functional capacity was measured with immunostaining for cytokines and lytic effector molecules, perforin, and granzymes. Given the clonal origin of cell lines and the resulting uniformity of frequency of receptor expression on a particular cell line, we analyzed data by comparing the mean fluorescence intensity (MFI) of parameters of interest acquired in the same experiment using the same settings. This approach enabled us to directly compare the relative density of marker expression between the cell lines of interest. Fluorescence minus one controls were used to establish thresholds of intensity for each fluorophore on each individual cell line.

NK92, NKL, and YTS cells can be distinguished from one another by CD2, CD28, and CD56 expression (Yodoi et al., 1985; Robertson et al., 1996). Cell lines had unique distributions of surface markers when we considered their expression by viSNE unsupervised clustering (Fig. 2A). In addition, we confirmed the differential expression of previously described markers by demonstrating that NK92 cells are $CD2^+CD28^+CD56^+$, while NKL cells are $CD2^+CD28^-CD56^-$ and YTS are $CD2^-CD28^+CD56^+$ (Fig. 2A, B). CD28 expression was the highest in YTS (Fig. 2B). NK92, NKL, and YTS had variegated expression of CD11c (Fig. 2A), making CD11c another cell surface receptor that could be used to distinguish the cell lines from one another. Both NK92 and NKL cells expressed the high affinity IL-2 receptor CD25, with NKL cells having a significantly greater density (Fig. 2B).The activating natural cytotoxicity receptors NKp46, NKp44, and NKp30 play an important role in both viral and tumor control (Kruse et al., 2014). While all cell lines expressed NKp46 (Fig. 2B), they were negative for NKp44. NK92 cells solely expressed NKp30 (Fig. 2B). When considering killer lectin-like receptors, only NKL cells expressed NKG2C and NKG2D (Fig. 2B).

To assess NK cell functional markers and cytokine profiles, NK cell lines were stimulated with PMA/ionomycin. NKL and YTS both had higher intensity of the activation receptor, CD69, at rest, and following stimulation when compared to NK92 (Fig. 2C). As expected, NK cell activation led to a relative increase in surface LAMP-1 (CD107a) when compared to unstimulated cells on all cell lines following PMA/ionomycin stimulation, demonstrating their capacity for lytic granule exocytosis (Alter et al., 2004) (Fig. 2C). In addition, all cell lines produced IFNγ (Fig. 2C), with YTS having the greatest capacity for IFNγ production. NK92, NKL, and YTS cells did not produce TNFα, IL-5, and IL-13 in response to PMA/ionomycin stimulation (data not shown).

### 3.3. NK cell lines have gene and protein expression suggestive of developmental differences

The differential expression of receptors associated with NK cell maturation led us to hypothesize that NK cell lines, particularly NK92 and YTS, could represent distinct stages of NK cell development. Using flow cytometry data, the MFI of previously described signifiers of $CD56^{bright}$ and $CD56^{dim}$ subsets (Fig. 3A) were graphed with differences between cell lines determined by 1-way ANOVA with Tukey's multiple comparisons. Molecules associated with human $CD56^{bright}$ NK cells, including CD94, andNKG2A had significantly higher MFI on NK92 cells compared with YTS (Fig. 3B). Conversely, markers of mature $CD56^{dim}$ NK cells, including the KIRs, KIR2DL2/L3 (CD158b) and KIR3DL1 (CD158e), and the activating receptor, DNAM-1, were expressed with greater intensity on YTS than NK92 cells (Fig. 3B). While not statistically significant, the trend of higher expression of CD158b and CD158e in YTS compared to NK92 cells was consistent throughout experimental repeats. KIR2DL1 (CD158a) and KIR2DS4 (CD158i) were not expressed in any of the NK cell lines assessed (data not shown). The MFI of intracellular perforin and granzyme B, molecules associated with effector functions of $CD56^{dim}$ NK cells were higher in YTS compared to NK92 (Fig. 3C), further suggesting that YTS have a $CD56^{dim}$-like phenotype. CD16 expression has previously been shown to be low and/or absent in both NK92 and YTS cell lines (Gong and Maki, 1994; Yoneda et al., 1992), which was reflected by the lack of CD16 signal on both cell lines relative to previously described levels on primary human NK cells (Fig. 3B) (Mahapatra et al., 2017). Functionally, NK92 and YTS cells stimulated with PMA/ionomycin expressed IFN-γ, with YTS cells having a significantly higher MFI (Fig. 3C).

NKL cells displayed a phenotype that was similar to both NK92 and YTS cells. NKG2A and CD94 expression was highest on NKL cells but so too was DNAM-1 (Fig. 3B). NKL cells had very low expression of CD16 but had comparable expression of both perforin and granzyme B to that of NK92 cells (Fig. 3B, C). IFN-γ expression after PMA/ionomycin stimulation was significantly lower compared to NK92 and YTS (Fig. 2C). Unlike NK92 and YTS which highly expressed CD56 (Fig. 2A, B), NKL

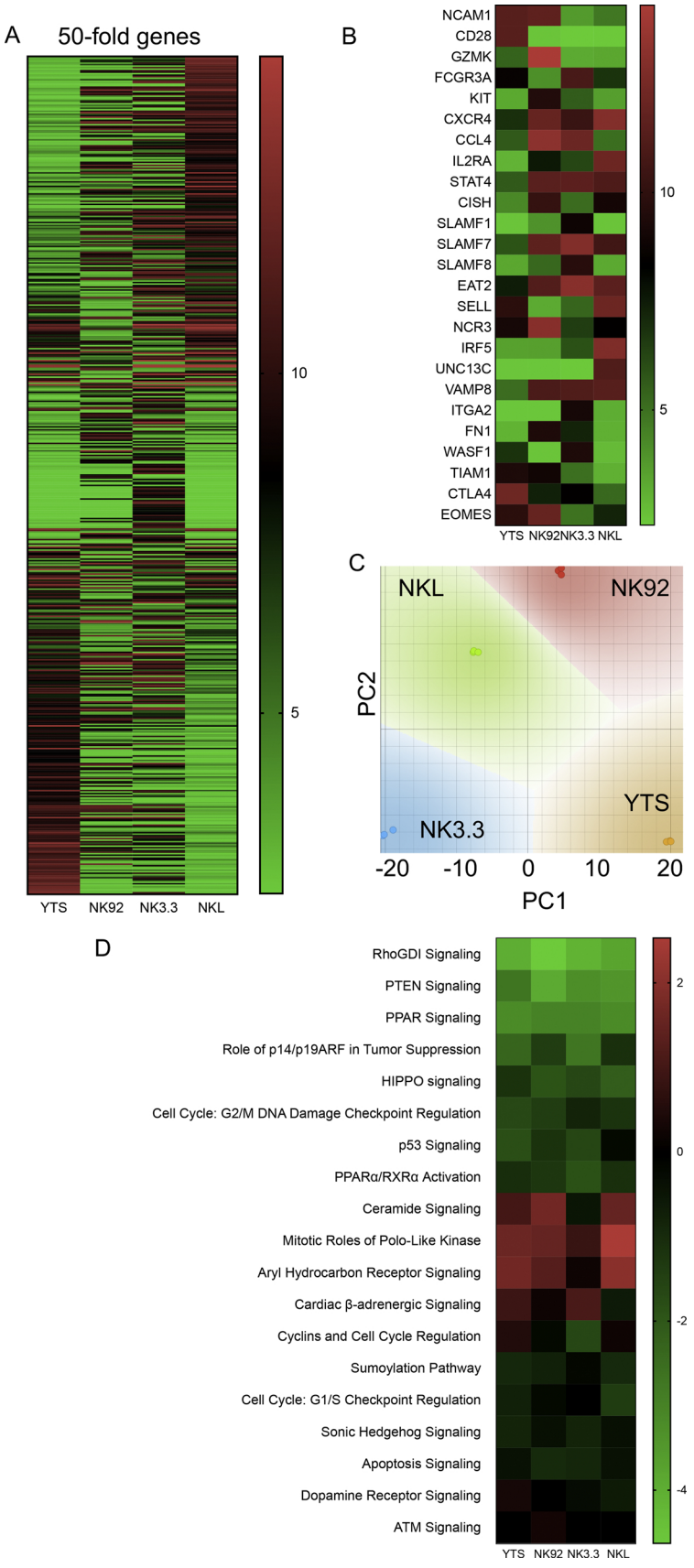



**Fig. 1. Heat map of genes differentially expressed among NK cell lines.** Microarray data was acquired from NK92, YTS, NK3.3, and NKL cell lines in triplicate as described in Materials and Methods. 1-way ANOVA was performed to identify significant differences in expression. A) Shown are 557 genes with > 50-fold difference in expression between at least two of the cell lines. Each represents the mean of data acquired in triplicate from each cell line. These genes are listed in Supplemental Table 2. B) Select genes with particular relevance to NK cell biology and function that are differentially expressed between NK cell lines. C) Principal component analysis of significantly differentially expressed genes shows clustering of triplicates from each group. D) Log2 transformed data was analyzed with IPA comparison analysis to identify gene enrichment predictive of canonical signaling pathways. Shown is a heat map of activation z-scores with $p < 0.05$. Positive and negative z-scores are indicative of up- and down-regulation of genes respectively.

cells did not, suggesting NKL cells could represent a $CD56^{dim}$-like population that has subsequently down-regulated CD56 expression.

To further define these phenotypes, we mined our microarray data for genes associated with NK cell developmental subsets. Gene expression analysis revealed that many cell surface molecules and transcription factors important for human NK cell maturation were

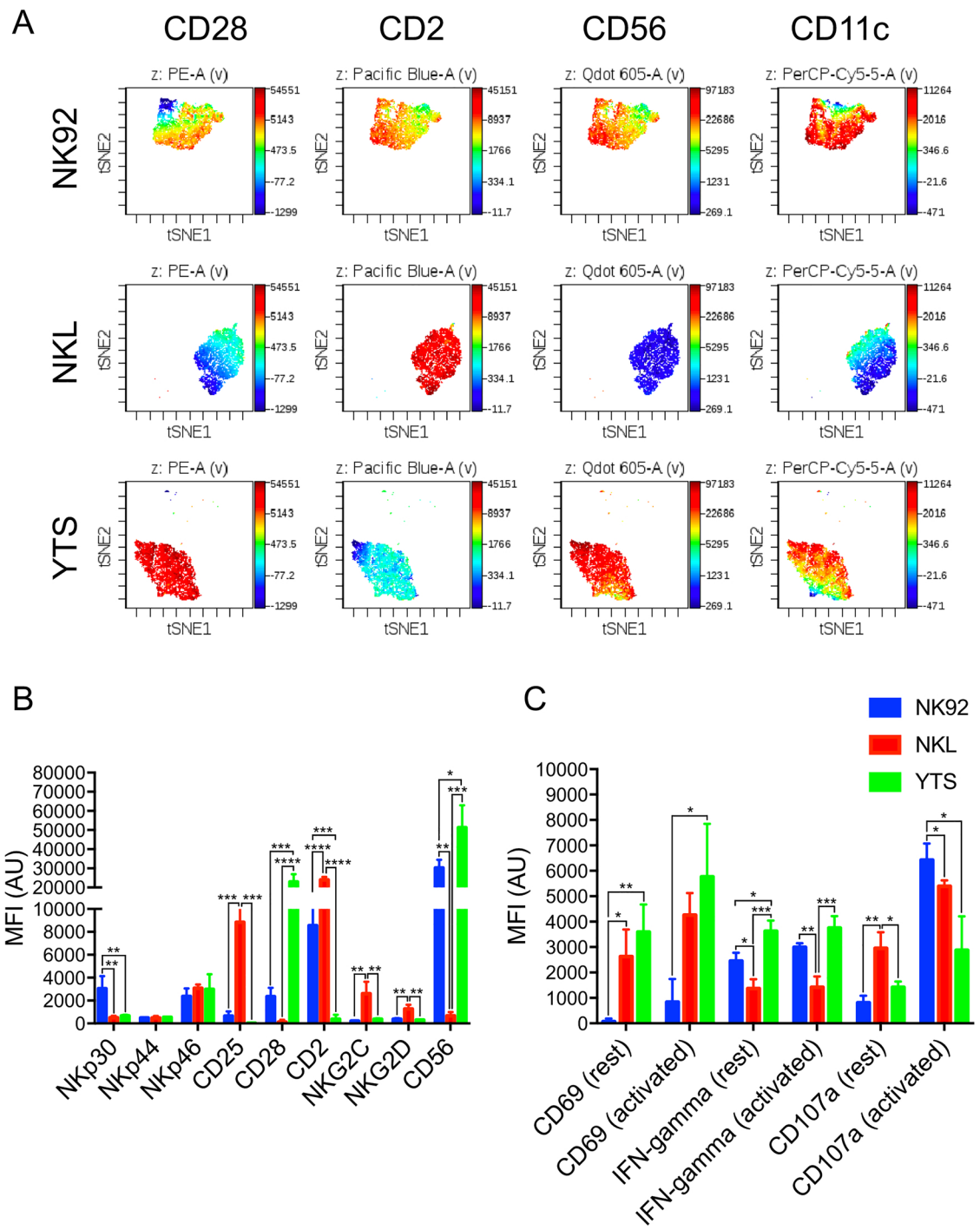



**Fig. 2. Flow cytometric analyses reveals differences between human NK cell lines.** Human NK cell lines were interrogated by NK cell phenotype panels as described in Materials and Methods. A) Density plots of tSNE1/2 fields comparing surface expression of activating receptors on NK92, NKL, and YTS cell lines. Heat maps reflect markers indicated. B) Mean fluorescence intensity (MFI) of known NK cell parameters and C) functional markers expressed on the NK cell lines using antibodies described in Supplemental Table 1. Where indicated, cells were activated with PMA/ionomycin or rested for 4 h prior to fixation and antibody incubation. Data shown is mean ± S.D. of three individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by 1-way ANOVA with Tukey's multiple comparisons.

similarly expressed amongst all cell lines, with the exception of *EOMES*, *TBX21, SELL, and FCGR3A* (Fig. 3D). *EOMES* and *TBX21* encode for Eomes and T-Bet transcription factors, respectively, and were more highly expressed in NK92 than in YTS (Fig. 3D). This observation was confirmed by intracellular flow cytometry (Fig. 3E), which showed significantly higher expression of Eomes in NK92 cells relative to YTS and NKL. Expression of Eomes is higher in immature human NK cells, including the $CD56^{bright}$ subset, whereas T-bet expression is up-regulated during development and is higher in $CD56^{dim}$ NK cells than $CD56^{bright}$ NK cells (Knox et al., 2014). Therefore, the differential expression of these transcription factors in NK cell lines is supportive of NK92 having a more $CD56^{bright}$ phenotype while YTS cells represent a mature, $CD56^{dim}$ phenotype.

*SELL* and *FCGR3A*, the genes encoding CD62L (L-selectin) and CD16, respectively, are differentially expressed among the cell lines with YTS and NKL having higher gene expression of both compared to

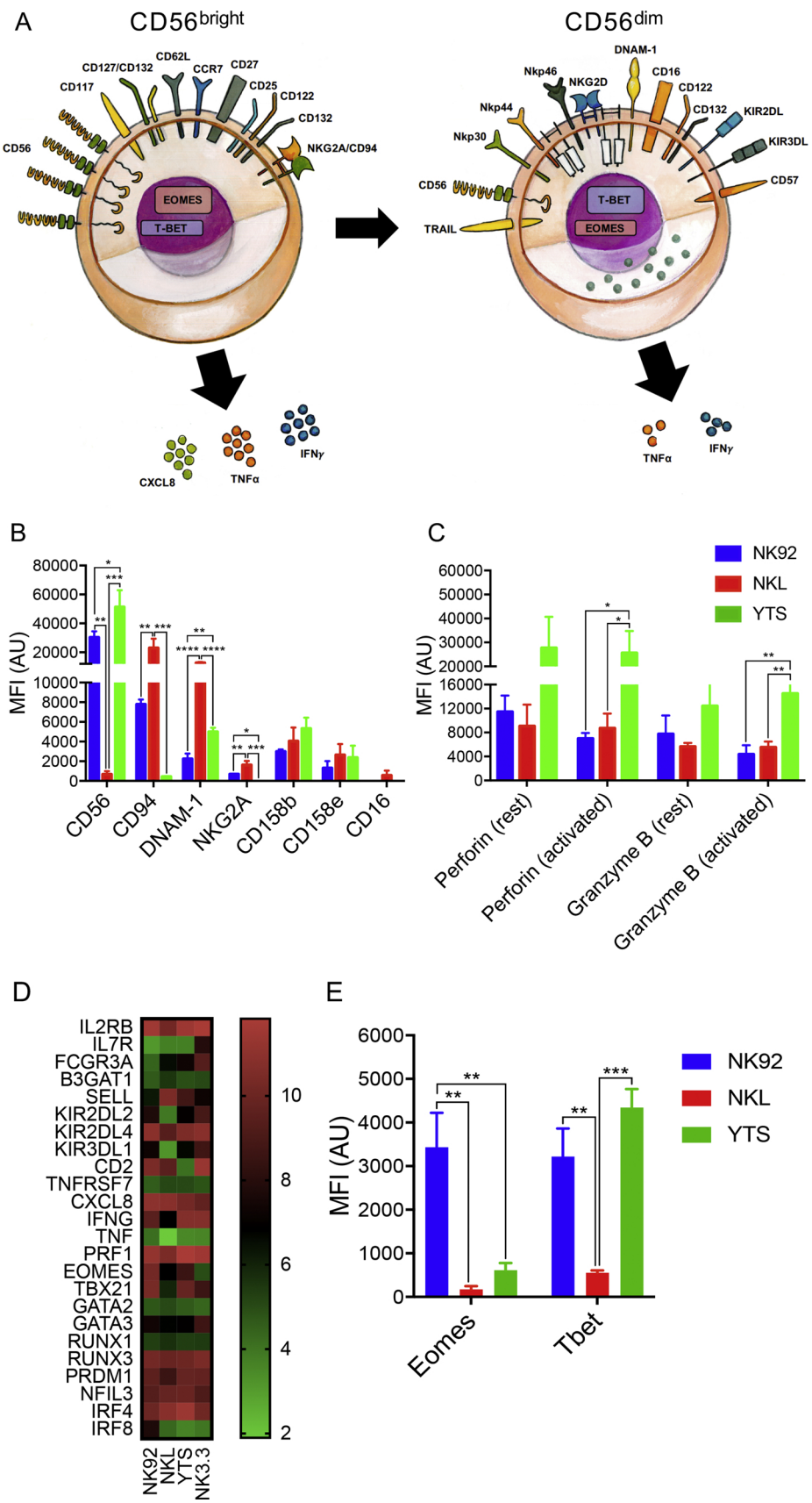



**Fig. 3. Multi-parametric flow cytometry and gene expression of developmental markers in human NK cell lines.** Cell lines were evaluated for specific NK cell markers that distinguish CD56$^{bright}$ from CD56$^{dim}$ NK cells by flow cytometry and gene expression. A) Diagram illustrating known differences in receptor and effector molecule expression between human CD56$^{bright}$ and CD56$^{dim}$ NK cell subsets. B) Mean fluorescence intensity (MFI) of distinguishing surface and C) intracellular molecules between CD56$^{bright}$ and CD56$^{dim}$, with or without stimulation with PMA/ionomycin using antibodies described in Supplemental Table 1. D) Expression of log2 transformed genes associated with discrete NK cell developmental stages extracted from microarray datasets. Data is representative of one independent experiment performed in triplicate. E) Intranuclear expression of transcription factors associated with NK cell development. Flow cytometry data presented reflects mean ± S.D. of three individual experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by 1-way ANOVA with Tukey's multiple comparisons.

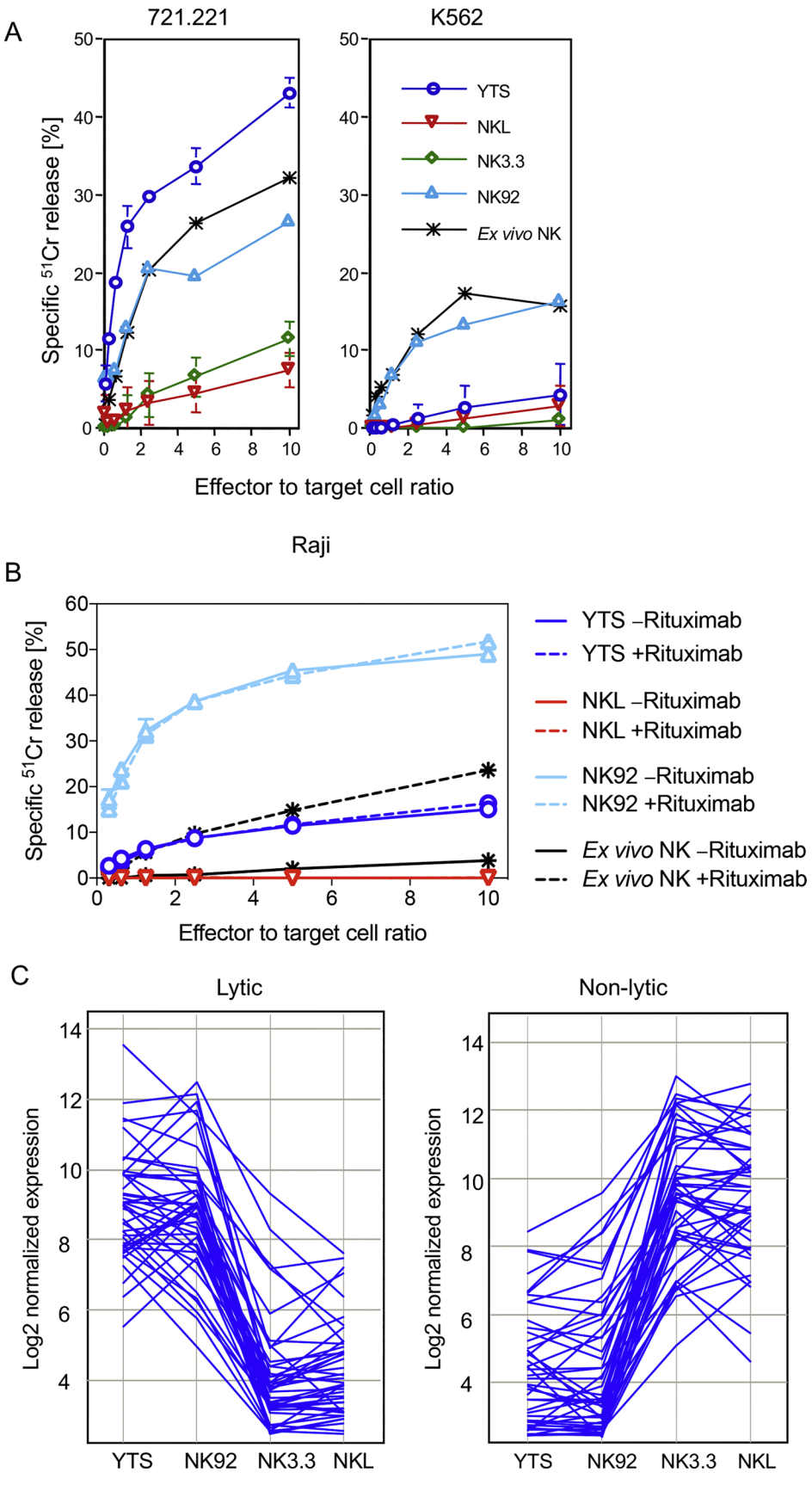



**Fig. 4. $^{51}$Chromium release assays.** NK cell cytotoxicity assays (4 h) were performed against standard targets. A) NK cell lines or *ex vivo* NK cells were incubated with 721.221 or K562 target cells labeled with $^{51}$Cr at the ratios indicated. B) Raji target cells with or without the addition of Rituximab; results are presented as mean ± S.D. and are representative of at least three individual experiments. C) Differential gene expression patterns among NK cell lines by killing activity. A nested 1-way ANOVA compared types, defined as "high killing" NK cell lines (YTS and NK92) and "low killing" lines (NK3.3 and NKL). Genes within the high killing and low killing type comparisons having differences in expression levels of > 8-fold with p-values < 0.0001 were selected. Out of this set, genes having within-type fold changes between -5 and 5 were selected. Those genes that demonstrated at least a 5-fold change difference between types are represented above. The y-axis represents the log2 normalized gene expression values corresponding to the original 1-way ANOVA analysis. A complete list of genes differentially expressed between these groups is provided in Supplemental Table 3.

NK92 (Fig. 3D). In addition, expression of CXCL8, a chemokine produced by immature human NK cell intermediates (Montaldo et al., 2013), including CD56$^{bright}$ cells, was higher in NK92 than YTS (Fig. 3D). Collectively, differences in key developmental molecules CD94, NKG2A, CD158b, DNAM-1, the transcription factors Eomes and T-Bet, and functional molecules suggest that NK92 and YTS cell lines were derived from developmentally different NK cell subsets.

### 3.4. Analysis of NK cell lytic function identifies genes associated with differential killing potential

The functional capacity of NK cell lines was further tested by measuring NK cell cytotoxicity against canonical NK targets. YTS and NK92 cell lines efficiently killed HLA-null 721.221 target cells, whereas NKL and NK3.3 had lower killing capacity (Fig. 4A). Cytolytic activity against the prototypical NK cell target cell, the K562 erythroleukemia cell line, was compared and demonstrated that NK92 cells were efficient killers, but that none of the other lines were very effective. The inability of YTS cells to kill K562 has been previously described and attributed to a lack of CD80 or CD86 expression on K562 cells (Chen et al., 2006). Although NKL cells did not have demonstrable lytic function towards Raji B cells, NK92 and YTS did have measurable activity, with NK92 activity being the greatest (Fig. 4B). However, the lytic activity of the cell lines did not differ when Rituximab was added, indicating death of the Raji cells was not through antibody-dependent cell-mediated cytotoxicity (ADCC). This is in accordance with previously reported data, at least with regards to NK92 cells (Grier et al., 2012). As expected, *ex vivo* NK cells had no significant lytic function towards Raji targets in the absence of Rituximab, but efficiently mediated ADCC in its presence (Fig. 4B).

The lytic activity of NK92 and YTS cells was generally comparable to that mediated by *ex vivo* NK cells, whereas the other cell lines had minimal lytic function (Fig. 4A). Thus, over multiple experiments there was a consistent distinction in the cytolytic activity mediated by NK92 and YTS as compared to NKL and NK3.3 cells. Although there are likely unique properties of each individual cell line, there may be important common inherent similarities between YTS and NK92 cells that can provide insight into the cytolytic process when these cells are compared to NKL and NK3.3 cells.

To define differences in gene expression between the NK cell lines with high killing ability versus those that kill less effectively, we evaluated the p-values and fold change values generated by a 1-way nested ANOVA comparing cell type (high killing cell lines versus low killing cell lines). A total of 417 genes with significance within this type comparison were identified. To identify the most significant differences in gene expression between lytic and non-lytic cell lines, genes with at least 8-fold change difference between the lytic vs. non-lytic category, yet a no greater than 5-fold difference between the cell lines in each category with p-values $< 0.0001$ were selected (Fig. 4C). The genes associated with greater lytic function included few genes canonically associated with human NK cell function. The gene with the highest differential expression between lytic and non-lytic cell lines was *NCAM1*, which encodes CD56 (Supplemental Table 3). Another Ig superfamily protein, *LAIR2* (CD306), was also highly differentially expressed. Amongst genes significantly up-regulated by less lytic cell lines, butyrylcholinesterase (*BCHE*) had the highest fold-change difference, and there were few genes with previously described roles in NK cell function. These data identify potential candidates for new regulators of lytic function. They also demonstrate that lytic potential is not necessarily dictated by the expression levels of genes encoding lytic effector molecules or well-described NK cell activating/inhibitory receptors.

### 3.5. Copy number variation between NK cell lines

To further delineate differences amongst the four cell lines, gene copy number (CN) analysis was performed, using array comparative genomic hybridization. The total number of copy number zero (CN0) reads is summarized in Supplemental Table 4 and those with a confidence score of 10 or greater is summarized in Supplemental Table 5. In addition, there were regions of heterozygous deletion (CN1) and duplications (CN3 and CN4) for each of the cell lines (Supplemental Tables 6–8). Amongst the four cell lines, NK3.3 had the most CN0s (288), with NKL having the least (37) (Fig. 5B). NK92 in particular had a number of gene amplifications, in part due to a large duplication of the CN4 region (Supplemental Table 8). To determine the number of deleted genes within the CN0s shared among the cell lines, a Venn diagram was created using the R statistical software package. Of the over 500 deleted genes compared among the cell lines, only 27 are shared among all four (Fig. 5A). Each cell line has its own unique set of genes deleted within the CN0s, ranging from 2 (NKL) to 323 (NK3.3), with a variability in the number of deleted genes shared between one or two other cell lines (Fig. 5A, B). Analyses demonstrated variable distribution of regions of deletion between the cell lines (Supplemental Fig. 3). Interestingly, these again highlighted ontological relationships between the cell lines, with NK92 and YTS having similar patterns of deletions distinct from NKL and NK3.3. The mapping of these regions of deletion provides insight into the origin of genetic diversity in the cell lines and generates a useful tool for researchers seeking to understand this diversity and when choosing a cell line model.

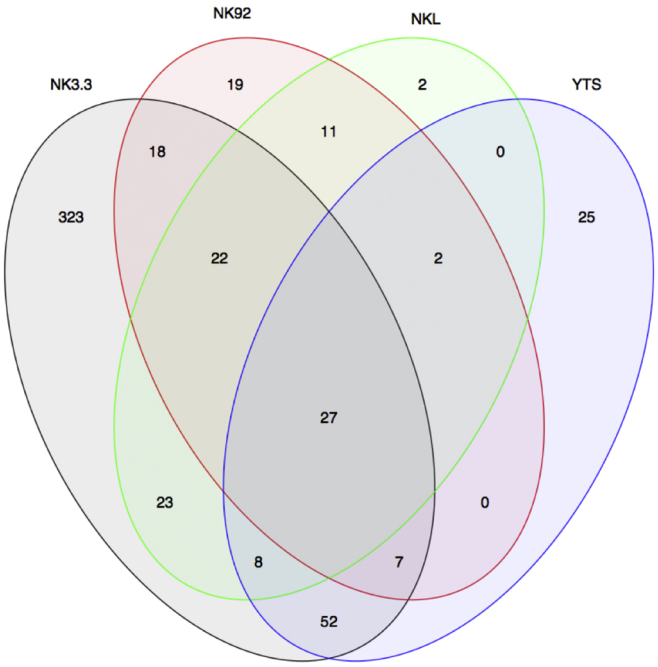
## 4. Discussion

Immortalized cell lines offer researchers the unique opportunity to model a variety of human disease states and dissect the mechanisms that give rise to clinical phenotypes. In that regard, human NK cell lines have been used to study the mechanisms that govern NK cell development, function and causes of dysregulation in various immunological deficiencies. However, despite the prevalence of NK cell lines in human NK cell research and establishment of their function and utility, a multidimensional characterization, encompassing genome-wide microarray, multi-parameter flow cytometry, and functional studies, has not been performed. Therefore, we sought to identify similarities and differences among four human NK cell lines: NK92, NKL, NK3.3, and YTS. To our knowledge, this is the first comprehensive study in which genotypic, phenotypic, and functional characteristics have been compared amongst these four cell lines. Given the importance of these cell lines in modeling and advancing human NK cell biology, understanding their similarities and differences will be instrumental in designing future experiments and in choosing the best cell line to model biological questions.

As expected, genomic analysis of the NK cell lines revealed high expression of genes involved in growth and survival pathways, and lymphocyte signaling, including molecules involved in NK cell function and leukocyte extravasation. Despite their overall similarities, we identified 557 genes with significantly different expression among the cell lines. A number of these were associated with NK cell function, phenotype, or signaling, including *NCAM1*, *CISH*, *CD28*, *GZMK*, *CXCR4*, *FCG3RA,* and *SELL*. While differential expression of these genes may reflect their clonal origins, we also find that gene expression, and subsequently protein expression by flow cytometry, support the hypothesis that NK cell lines, particularly YTS and NK92, are derived from distinct developmental stages.

Utilizing both gene and protein expression, we found that NK92, YTS, and NKL can be distinguished by their similarities to either primary CD56$^{bright}$ or CD56$^{dim}$ human NK cells. NK92 are more CD56$^{bright}$-like while YTS have more of a CD56$^{dim}$-like phenotype. NKL cells share phenotypic similarities with both CD56$^{bright}$ and CD56$^{dim}$ NK cells, being highly positive for CD94, NKG2A, DNAM-1, and weakly positive for CD16. The similarity of NK92 cells to CD56$^{bright}$ NK cells is supported by their expression of CD94, and NKG2A. YTS cells have many phenotypic similarities to CD56$^{dim}$ NK cells, including high DNAM-1,

A




B

| Cell Line | Total Number of CN0s |
|---|---|
| YTS | 144 |
| NK92 | 112 |
| NK3.3 | 288 |
| NKL | 37 |

| Cell Line | Number of CN0s with Confidence Score >10 and 0 Distance from the Gene |
|---|---|
| YTS | 52 |
| NK92 | 38 |
| NK3.3 | 135 |
| NKL | 13 |

**Fig. 5. Copy number zero (CN0) analysis.** Cell lines were analyzed for gene copy number variants using array comparative genomic hybridization. A) Deleted genes that were shared amongst the four cell lines within CN0s that have a confidence score of 10 or greater are shown. B) Summary of copy number variations between the 4 cell lines tested showing both total CN0 (top) and those with a confidence score > 10 (bottom). A complete list of all genes is shown in Supplemental Tables 4 and 5.

KIR2DL2/L3, and KIR3DL1 expression with respect to NK92, and high intracellular perforin and granzyme B expression compared to NK92 and NKL cells. Protein expression of CD16 and CD62L were undetectable in both NK92 and YTS cells. However, gene expression of *FCGR3A* and *SELL* were greater in NK92 than YTS, further supporting the idea that NK92 are CD56$^{bright}$-like and YTS are CD56$^{dim}$-like. While the exact mechanism by which gene and protein expression are disconnected is not clear, matrix metalloproteases have been shown to cleave both CD16 and CD62 L in response to activation (Romee et al., 2013). This suggests that in YTS in particular, surface expression of CD16 and CD62L may be down-regulated by this or a similar mechanism.

Genetic and flow cytometric evaluations of known NK cell transcription factors also suggest NK92 are CD56$^{bright}$-like and YTS are CD56$^{dim}$-like. T-bet and Eomes expression have previously been linked to NK cell terminal maturation in both mouse and man, with down-regulation of Eomes and up-regulation of T-bet being associated with the most mature NK cell subsets (Knox et al., 2014; Daussy et al., 2014; Luetke-Eversloh et al., 2014; Simonetta et al., 2015). The significantly higher gene and protein expression of Eomes by NK92 cells suggests that they are derived from a less mature developmental subset than YTS. While both YTS and NK92 express T-bet, the higher trending expression of T-bet by YTS further supports this model. Expression of both Eomes and T-bet by NKL cells is low, however, our flow cytometry data indicates that the expression of T-bet is greater in NKL cells than Eomes, suggesting NKL cells may be more closely associated with CD56$^{dim}$ NK cells. Collectively, these data support the observation that NK92 cells are derived from CD56$^{bright}$ cells and further define YTS and NKL cells as likely originating from the CD56$^{dim}$ lineage. The key differences in gene and protein expression between these commonly used human NK cell lines are of particular relevance to researchers selecting an NK cell line to model a particular biological question.

Consistent with previous reports, we have shown that NK cell lines have distinct functional capacities. NK92 cells have high cytotoxicity against both K562 and 721.221 target cells (Gong and Maki, 1994; Matsuo and Drexler, 2003). Similarly, YTS cells induce lysis of 721.221 target cells but do not kill K562, likely due to a lack of CD80 or CD86 expression (Chen et al., 2006). In contrast, NKL and NK3.3 are weakly cytolytic against K562 and 721.221 when compared to NK92 and YTS cell lines. NK92 cells and to an extent, YTS, are able to induce lysis of Raji cells. This function is independent of ADCC, as the addition of Rituximab did not increase their ability to mediate target cell death. The similarities in killing Raji cells with or without the addition of Rituximab for each cell line is most likely due to the lack of CD16

expression on their surfaces, despite detectable gene expression.

Comparing gene expression patterns between lytic (NK92 and YTS) and less lytic (NKL and NK3.3) cell lines, we identified 417 genes that have significantly differential expression between these two groups. Perhaps surprisingly, genes associated with increased or decreased capacity for target cell lysis did not include lytic effector molecules such as perforin, granzymes, or cytokines. Many of the genes have poorly described roles in NK cell function but could be of relevance to target cell recognition and lysis, including *TIAM1*, *RASS4* and *PIK3AP1*.

The gene with the highest fold change difference in expression between lytic and less lytic cell lines was neural cell adhesion molecule (*NCAM1*), which encodes CD56, the predominant phenotypic marker of peripheral human NK cells (Lanier et al., 1991). While the role of CD56 in human NK cell cytotoxicity is unclear (Lanier et al., 1991; Nitta et al., 1989), there is increasing evidence for a requirement for CD56 in human NK cell function. During development, motility of NK cells and the formation of the developmental synapse has been shown to be dependent on CD56 (Mace et al., 2016). In addition, CD56 has been identified as a pathogen recognition receptor, mediating direct interaction with the fungus, *A. fumigatus* (Ziegler et al., 2017). Although *NCAM1* (CD56) has the highest fold change with genes associated with lytic function in our high killers, it's possible that *NCAM1* is simply associated with phenotype and plays no functional role in NK cell cytotoxicity. Regardless, it is interesting to note that the cell lines with the greatest lytic potency, NK92 and YTS, are highly positive for CD56 protein expression. In non-NK cells, CD56 has been implicated in cytokine induced killer-mediated lysis through *trans*-homophilic binding of CD56 molecules (Valgardsdottir et al., 2014). Additional studies are required to define the role of CD56 in human NK cell lytic function.

Finally, the generation of high-resolution copy number variation analysis provides additional insight into the origin and function of these commonly used NK cell lines. These data also demonstrate the chromosomal instability of these lines, with significant copy number variation in all cell lines, particularly large deletions in the NK3.3 line, and duplications in the NK92 cell line. These data may prove useful when considering an appropriate human NK cell line for modeling biological questions.

In conclusion, we have used microarray technology along with multi-parametric flow cytometry and functional assays to probe the similarities and differences among four NK cell lines, NK92, NKL, NK3.3, and YTS. Our analyses have revealed that while there are similarities among the cell lines, they can be separated based on their genomic and phenotypic differences. Distinctions in their functional ability have been correlated to genomic expression with various genes being either positively or negatively associated with cytotoxicity. This work builds upon previous studies using these NK cell lines and provides researchers with the tools necessary to make informative choices when choosing their model system.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.07.015.

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