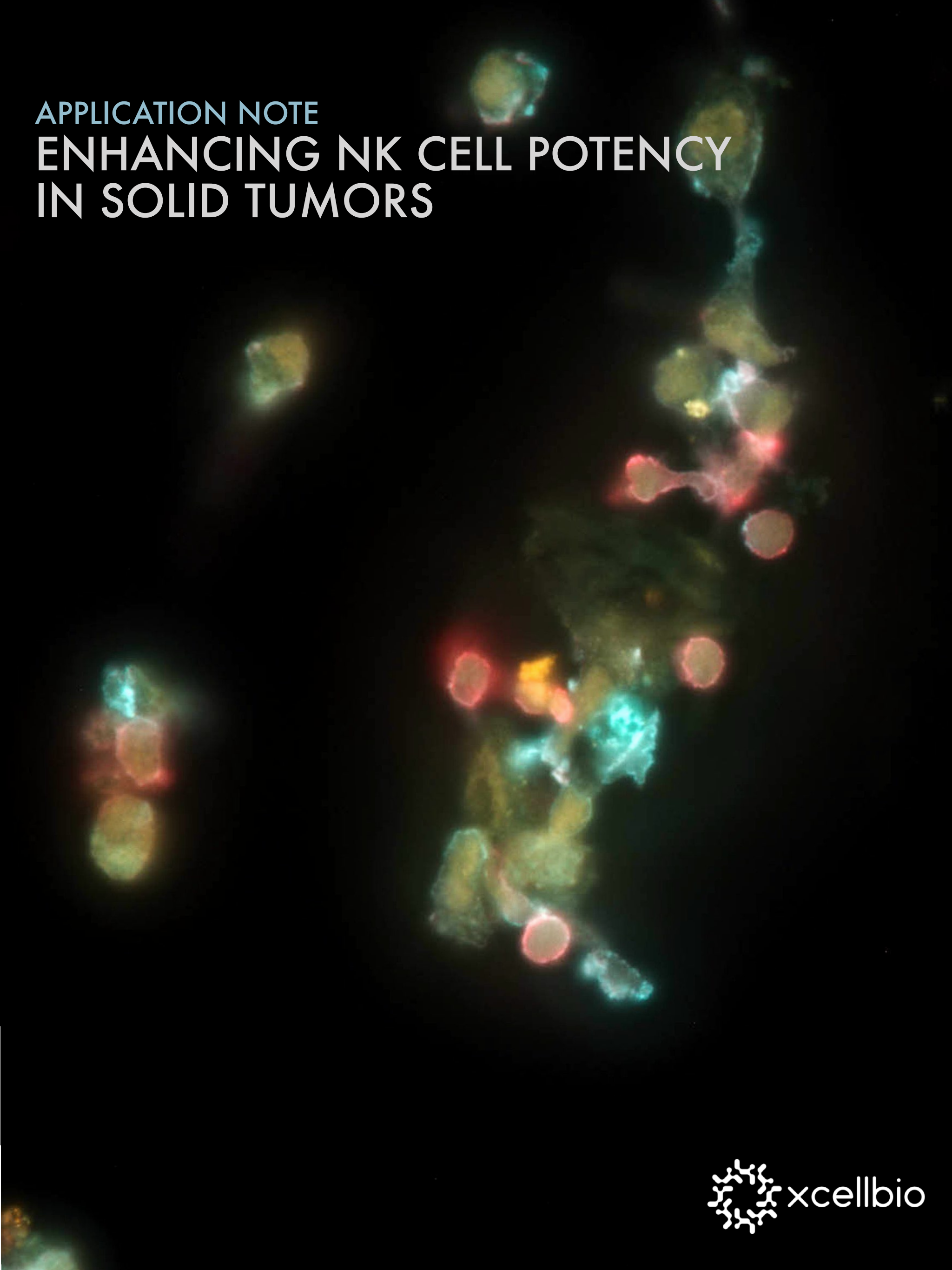
APPLICATION NOTE

ENHANCING NK CELL POTENCY IN SOLID TUMORS



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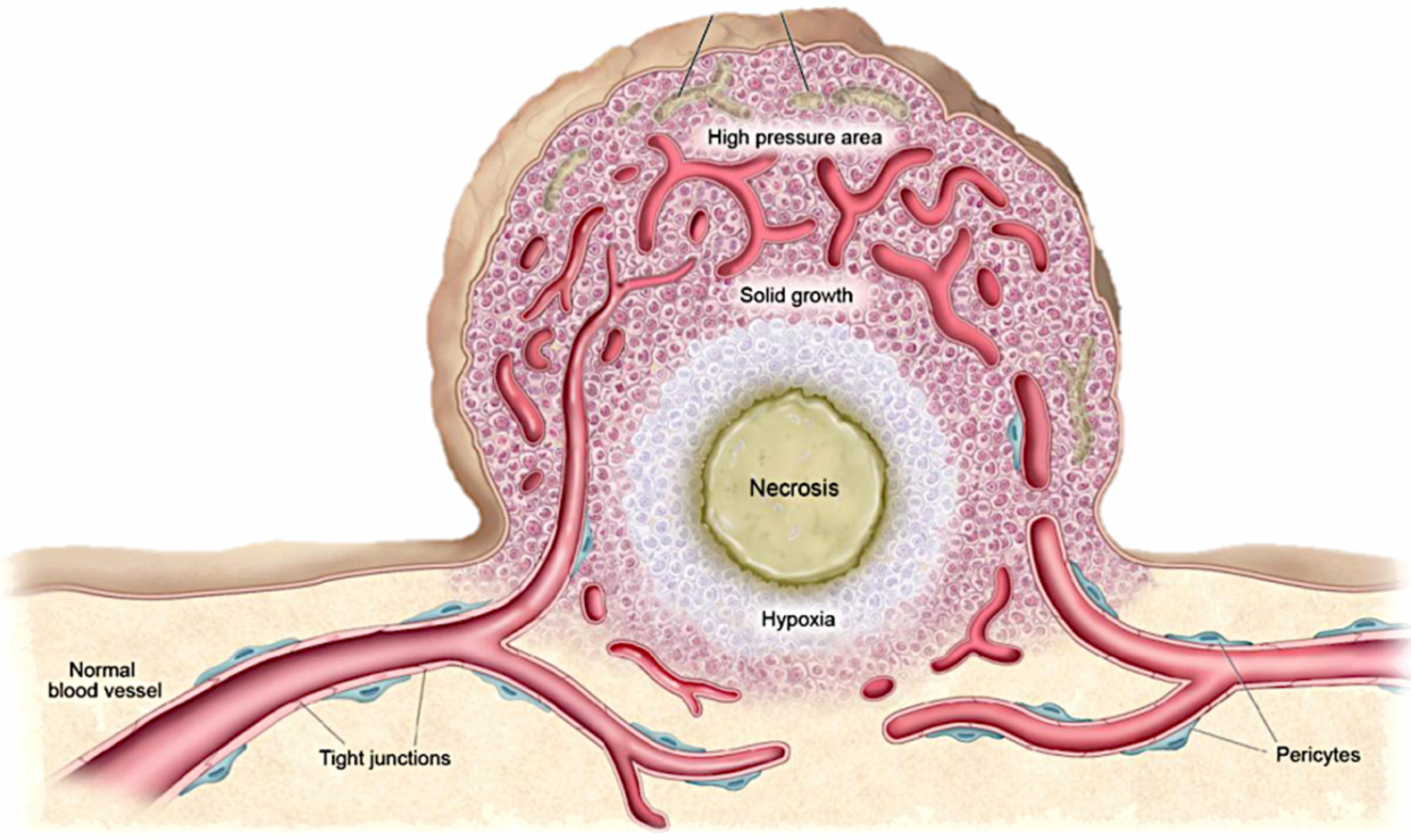
INTRODUCTION

In this application note, we showcase the effects of short- and long-term culture of NK-92 cells under tumor microenvironment-like conditions in the AVATAR System, prior to real-time cell killing analysis under these conditions using the AVATAR AI. These tools enable novel discoveries into the cellular dynamics of effector and tumor cells in the solid tumor environment and the prospect of developing improved therapeutics.

Harnessing NK cells is proving to be an attractive strategy for cancer immunotherapy, either by activating endogenous NK cells or through adoptive cell transfer

Natural killer (NK) cells are innate effector lymphocytes that play a critical role in clearing infected and transformed cells. In contrast to T and B cells, NK cell recognition is not dependent on antigen specificity and does not require prior sensitization. Instead, NK cell activity is controlled by the integration of signals from NK cell surface inhibitory and activating receptors (1). NK cells can directly kill infected and transformed cells through several processes. One cytotoxic mechanism involves the targeted release of cytolytic granules containing perforin and granzyme proteins, which function by forming pores in the membrane of target cells and through activation of the apoptosis pathway, respectively (2). NK cells also mediate target cell killing via engagement of death receptors. Binding of death ligands on NK cells, such as FAS ligand (FasL) and TRAIL, to their cognate receptors on target cells results in target cell apoptosis. NK cells can also induce antibody-dependent cell-mediated cytotoxicity via CD16 receptors.

NK cells for adoptive cell therapy can be derived or generated from sources like peripheral blood and hematopoietic stem cells, but challenges exist in isolating, expanding, and engineering NK cells from these sources. NK cells can also be sourced from immortalized cell lines like NK-92, which proliferate easily in culture and can provide a steady supply of off-the-shelf NK cells (3). Phase I trials using NK-92 cells have shown clinically significant results in patients with melanoma, lung cancer, and kidney cancer, yet challenges remain, many of which are associated with the immunosuppressive tumor microenvironment (TME).The TME is a unique milieu composed of tumor cells, stroma, infiltrating immune cells, cytokines, scarce nutrients, low oxygen concentrations and high interstitial pressure (4). The latter two characteristics are the focus of this work.



Sun et al., Acta Pharmacol Sin, 2020

WORKFLOW

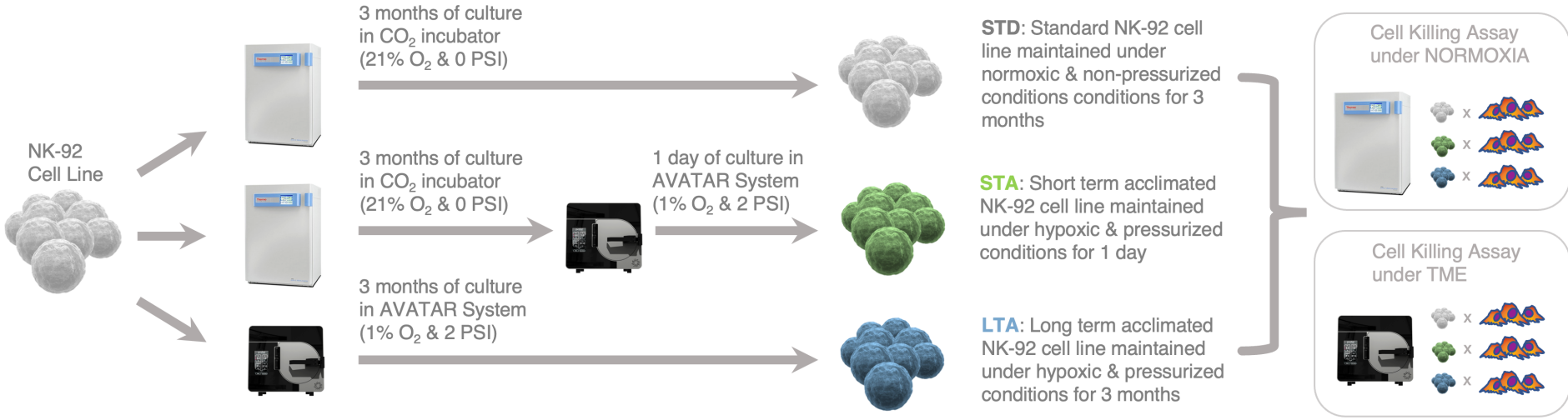


Figure 1. Experimental Setup. NK-92 cells were split amongst three culture conditions: STD cells were cultured for 3 months in a standard CO_2 incubator at normoxic, non-pressurized conditions, STA cells were cultured for 3 months in a standard CO_2 incubator at normoxic, non-pressurized conditions prior to 1 day of culture in the AVATAR system under hypoxic and pressurized conditions, LTA cells were cultured for 3 months in the AVATAR system under hypoxic and pressurized conditions. All three NK-92 cell populations were cocultured with A549 tumor cells and real-time killing as measured in the AVATAR AI system under normoxic or TME conditions.

Long-term acclimated NK-92 cells exhibit improved tumor killing activity in TME conditions. RNAseq and protein expression analysis further suggests that these adapted NK cells shift from perforin and granzyme B granule-mediated cytotoxicity to death-receptor mediated cytotoxicity. This initial study supports the hypothesis that immune cells can both react and adapt to different microenvironments. TME-adaptation strategies during cell expansion can enhance potency and efficacy of cell therapies designed to function in a solid tumor microenvironment

Though the effects of pressure on NK cell function is largely unexplored, several groups have studied the effects of hypoxia on NK cell function (5,6). Hypoxia affects the balance of activating and inhibitory receptor surface expression and activity on NK cells, downregulating NKp44, NKp46, NKp30 and NKG2D expression and function, and suppressing NK cytotoxicity against tumor targets (7). NK cells exposed to hypoxia further exhibit decreased expression of perforin and granzyme, decreased proliferation, and altered metabolism (8). A critical limitation of studies into the effects of hypoxia on NK cell function is that they have been limited to transient exposure to hypoxia, with hypoxia cultures lasting from 5 to 72 hours only. In this study we showcase how adapting NK-92 cells to a TME-like environment, with low oxygen and high pressure, for 3 months enriches for cells that metabolically adapt to pressurized and hypoxic culture conditions.

To investigate the short- and long-term effects of TME conditions on NK cell function, we cultured NK-92 cells under several environmental conditions and performed cytotoxic, transcriptional and protein expression analysis. NK-92 cells were either cultured in a conventional CO_2 incubator at 21% O_2 and 0 PSI (Standard, STD), or in an AVATAR System which can be precisely tuned to different O_2 and pressure levels that mimic TME conditions. For the TME conditions with decreased oxygen and increased pressure, NK-92 cells were conditioned for 24 hours to 1% O_2 and 2 PSI (Short Term Acclimated, STA) or conditioned for 3 months to 1% O_2 and 2 PSI (Long Term Acclimated, LTA) (experimental setup summarized in Figure 1).

We first assessed the effects of culture conditions on the ability of NK-92 cells to kill tumor cells in both normoxic and TME conditions. To this end we used the AVATAR AI instrument, which employs the environmental control of the AVATAR incubator paired with a specialized plate that enables real-time, label free cell killing analysis via electrical impedance. NK-92 cells were co-cultured with A549 tumor cells at an effector to target (E:T) ratio of 5:1 under normoxic (21% O_2 and 0 PSI) or TME (1% O_2 and 2 PSI) conditions. NK cell-mediated cytolysis was tracked over a 46-hour period.

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RESULTS

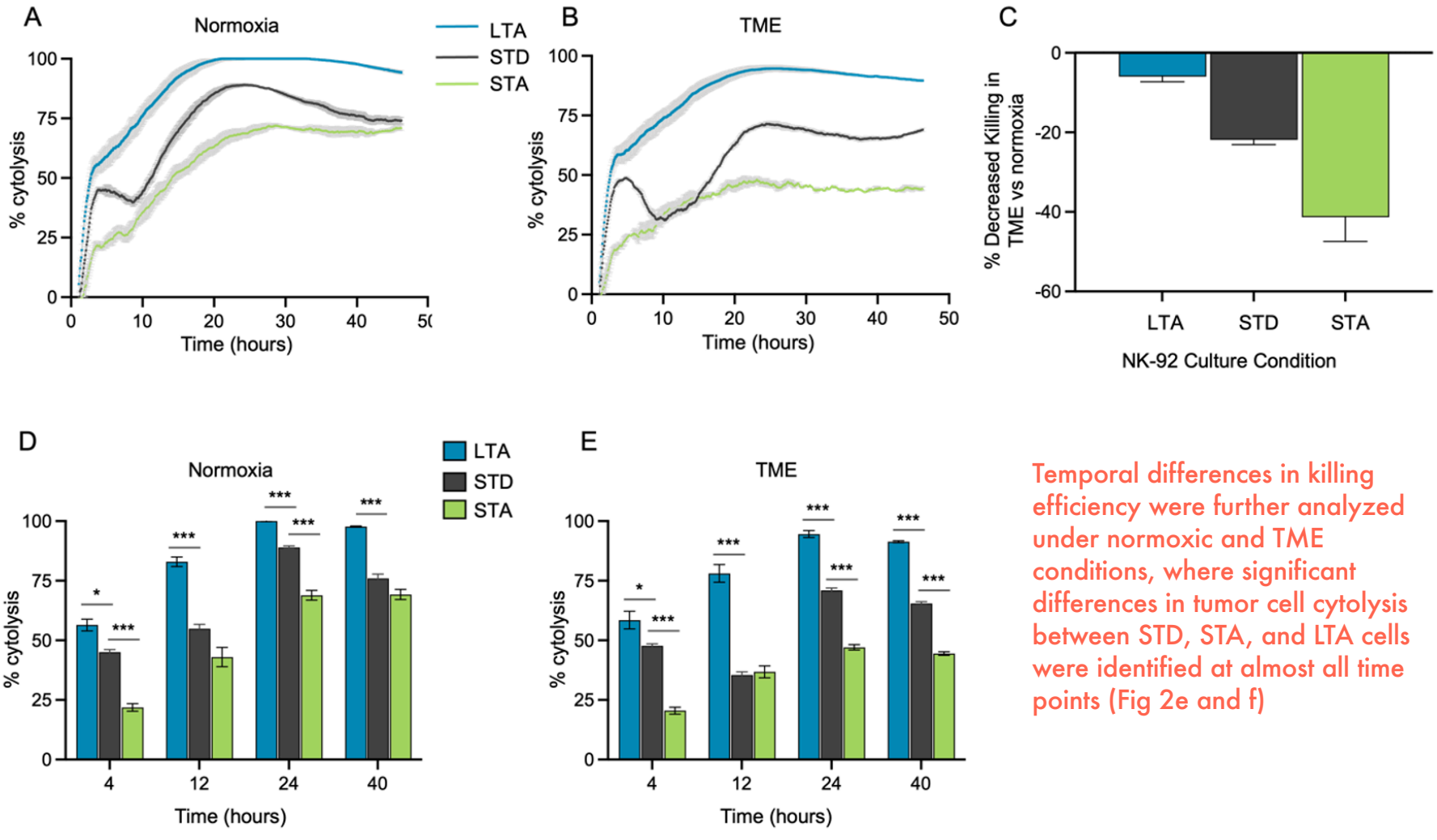

Figure 2. TME Acclimated NK-92 cells exhibit increased cytotoxicity. A) Real-time analysis of A549 cytolysis in coculture with NK-92 cells in normoxic conditions. Y-axis denotes percentage of killed cells. X-axis denotes time after addition of effector cells. Colored lines indicate culture condition of NK-92 effector cells. [AL1] B) Real-time analysis of A549 cytolysis in coculture with NK-92 cells in TME conditions. C) Bar graph summarizes the percent difference in the area under the curve between A549 cytolysis over 46 hours measured under TME versus normoxic conditions. D) Bar graph summarizes the percent difference in the area under the curve between A549 cytolysis over 46 hours measured under normoxic and TME conditions in STA and LTA cells versus STD cells. E) Bar graph summarizes cytolysis of A549 cells in culture with STD, STA, and LTA NK-92 cells in normoxic conditions over time. F) Bar graph summarizes cytolysis of A549 cells in culture with STD, STA, and LTA NK-92 cells in TME conditions over time. Error bars indicated standard error of mean[AL2] . Statistics represent unpaired t-tests relative to STD cells. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Temporal differences in killing efficiency were further analyzed under normoxic and TME conditions, where significant differences in tumor cell cytolysis between STD, STA, and LTA cells were identified at almost all time points (Fig 2e and f)

In normoxic conditions, NK-92 cells that had been exposed to TME conditions for 24 hours (STA) exhibited reduced killing, as compared to standard cells (STD), with slower kinetics of killing and lower total cytolysis (Fig 2a). Interestingly, cells that had been adapted to TME conditions for 3 months (LTA) exhibited increased cytotoxic activity, with faster killing kinetics and higher total cytolysis. When experiments were repeated under TME conditions, the differences between NK-92 culture conditions were even more stark (Fig 2b). To quantify these differences, we calculated the area under the curve (AUC) across the three NK-92 culture conditions. In Figure 2c, we calculated a percent difference in total killing capacity over 46 hours between NK-92 cells in normoxic and TME conditions.

While STD cells exhibited an average 21% reduction in killing activity under TME conditions, STA cells showed even lower killing in TME conditions, with an average 41% lower killing relative to normoxia. LTA cells exhibited only slightly reduced killing in TME conditions, with an average 6% reduction relative to normoxia. We further quantified the difference in total killing in STA and LTA cells as compared to STD cells over 46 hours. STA cells exhibited a 21% and 41% reduction in killing relative to STD cells, under normoxic and TME conditions, respectively (Fig 2d). In contrast LTA cells exhibited 28% and 55% increased killing efficiency relative to STD cells, under normoxic and TME conditions, respectively.

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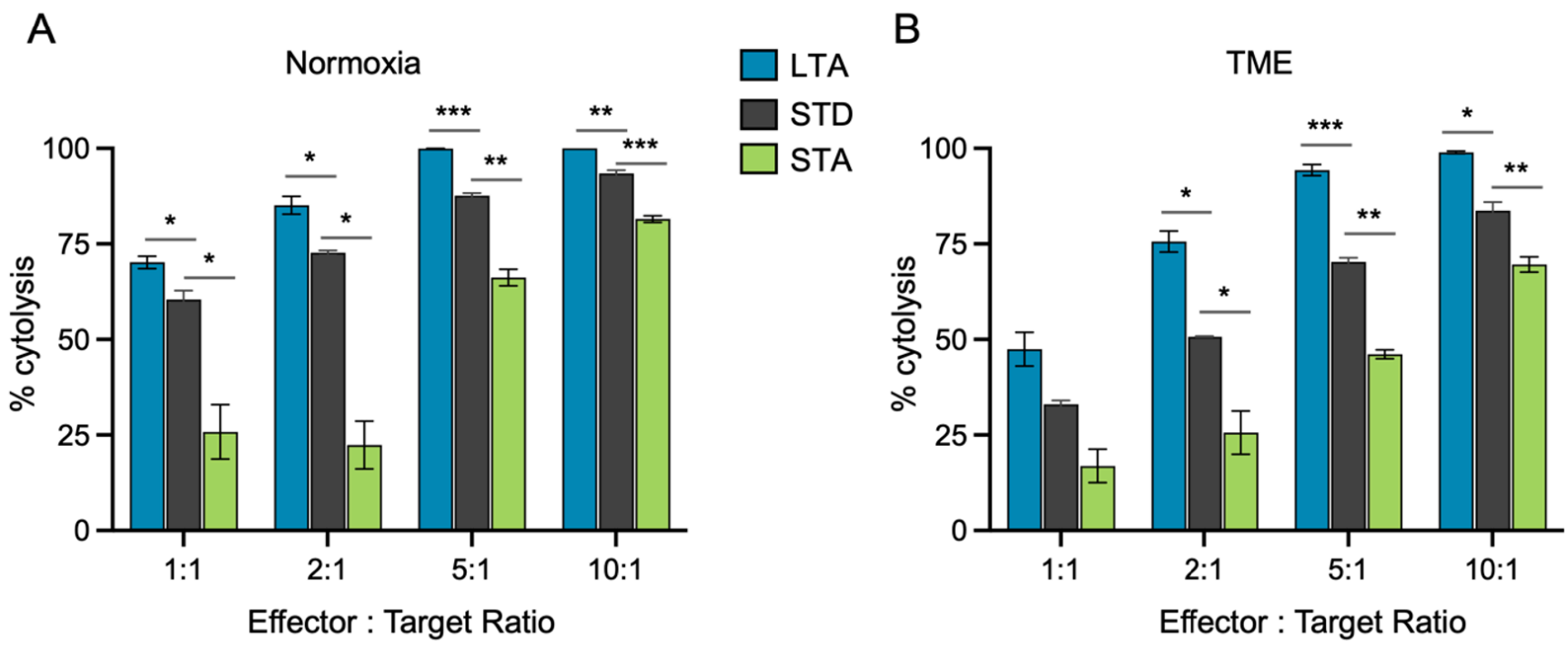

Figure 3. TME Acclimated NK-92 cells exhibit increased cytotoxicity across a range of E:T ratios. A) Bar graph summarizes cytolysis of A549 cells in culture with STD, STA, and LTA NK-92 cells across a range of E:T ratios in normoxic conditions. Y-axis denotes percentage of killed cells. Error bars indicated standard error of mean. B) Bar graph summarizes cytolysis of A549 cells in culture with STD, STA, and LTA NK-92 cells across a range of E:T ratios in TME conditions. Statistics represent unpaired t-tests relative to STD cells. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

We expanded this analysis to include a range of E:T ratios and measured NK-92-mediated cytolysis in normoxic versus TME conditions after 24 hours. In normoxic conditions, STA cells showed a significant defect in killing activity compared to STD cells at all E:T ratios (Figure 3a). This is consistent with previous work showing impaired cytotoxicity when NK cells are transiently cultured under TME conditions (Balsamo). In contrast LTA cells showed enhanced killing activity at all E:T ratios, as compared to STD cells. When this analysis was repeated under TME killing conditions, we again observed a killing defect in STA cells, as compared to STD cells, while LTA cells exhibited greater killing activity across all E:T ratios (Figure 3b). Thus, by culturing NK-92 cells in TME conditions for extended time periods, cells become adapted to the harsh environment and can sustain their potency.

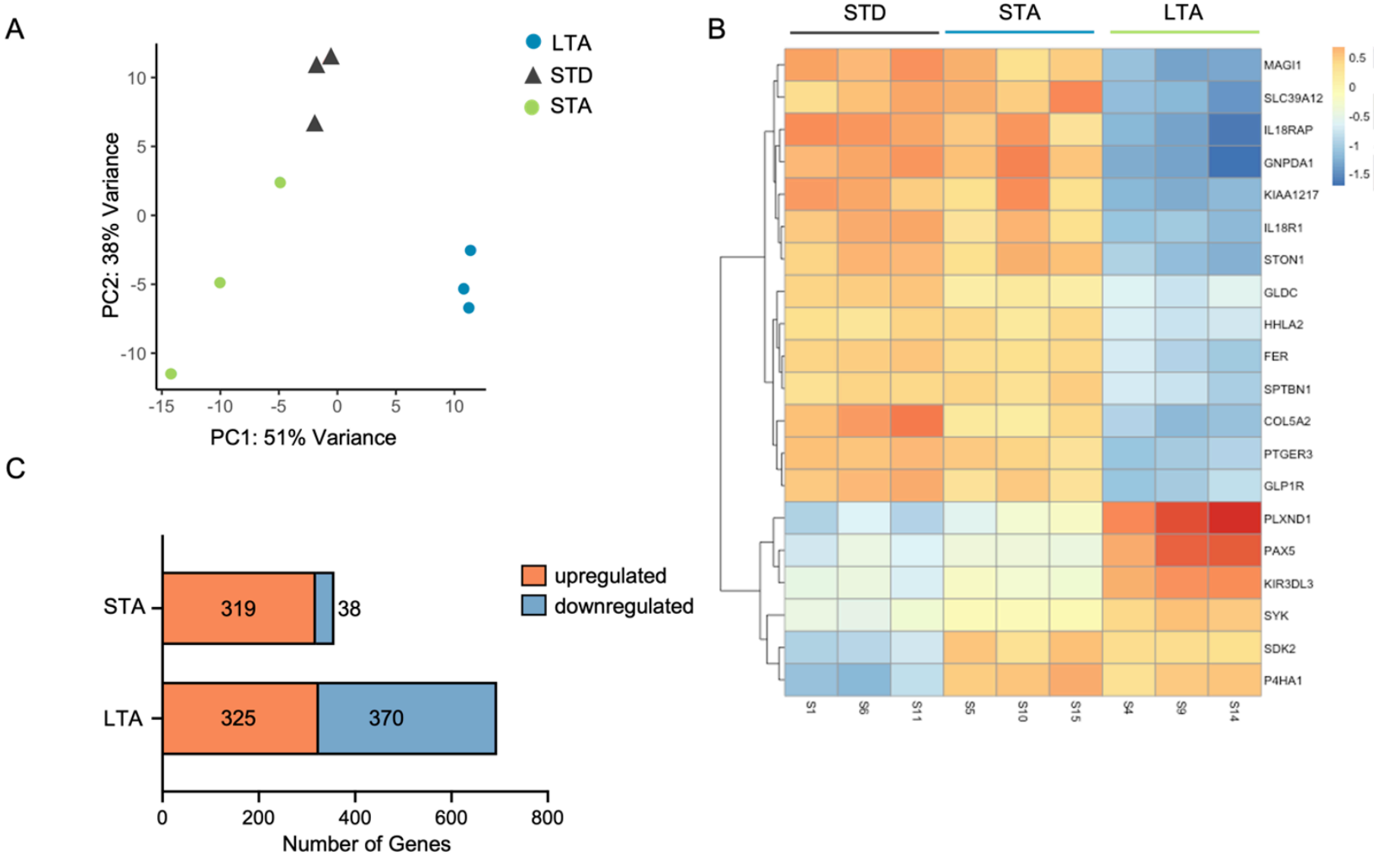

Figure 4. NK-92 cell adaptation to TME conditions modifies their transcriptome. A) Principal component analysis (PCA) of RNAseq data amongst the three NK-92 conditions. B) Heatmap of top 20 differentially expressed genes amongst NK-92 populations. C) Bar graphs summarize the number of genes upregulated and downregulated in STA and LTA cells, relative to STD cells.

To investigate the mechanisms underlying these cytotoxicity results, we performed RNA sequencing analysis of the three NK-92 populations (STD, STA, and LTA). Principal component analysis (PCA) uncovered significant differences in gene expression amongst these cells, with distinct clustering amongst NK-92 cells acclimated to TME conditions (Figure 4a). LTA cells exhibited a more dissimilar gene expression pattern than STA cells, as compared to STD cells.

These distinct gene expression patterns were further confirmed by performing differential gene expression analysis, with the top 20 differentially expressed genes highlighted in the heat map in Figure 4b. Differentially expressed genes include PLXND1, which has been implicated in NK cell function through interactions with Sema-3E, and the tyrosine kinase SYK, which is required for NK-mediated cytotoxicity (9) (10). As expected, LTA cells exhibited the greatest gene expression changes relative to STD cells, with 695 genes exhibiting greater than 2-fold differential expression in LTA cells and only 357 genes differentially expressed in STA cells (Figure 4c).

Because of the striking differences in killing potency observed in these NK-92 populations, we focused the remainder of our analysis on the expression of cytotoxic genes amongst the groups. When we analyzed the expression of 31 common cytotoxic genes, we observed that many genes commonly associated with NK cell killing were downregulated in LTA cells, as compared to STD cells (Figure 4a).

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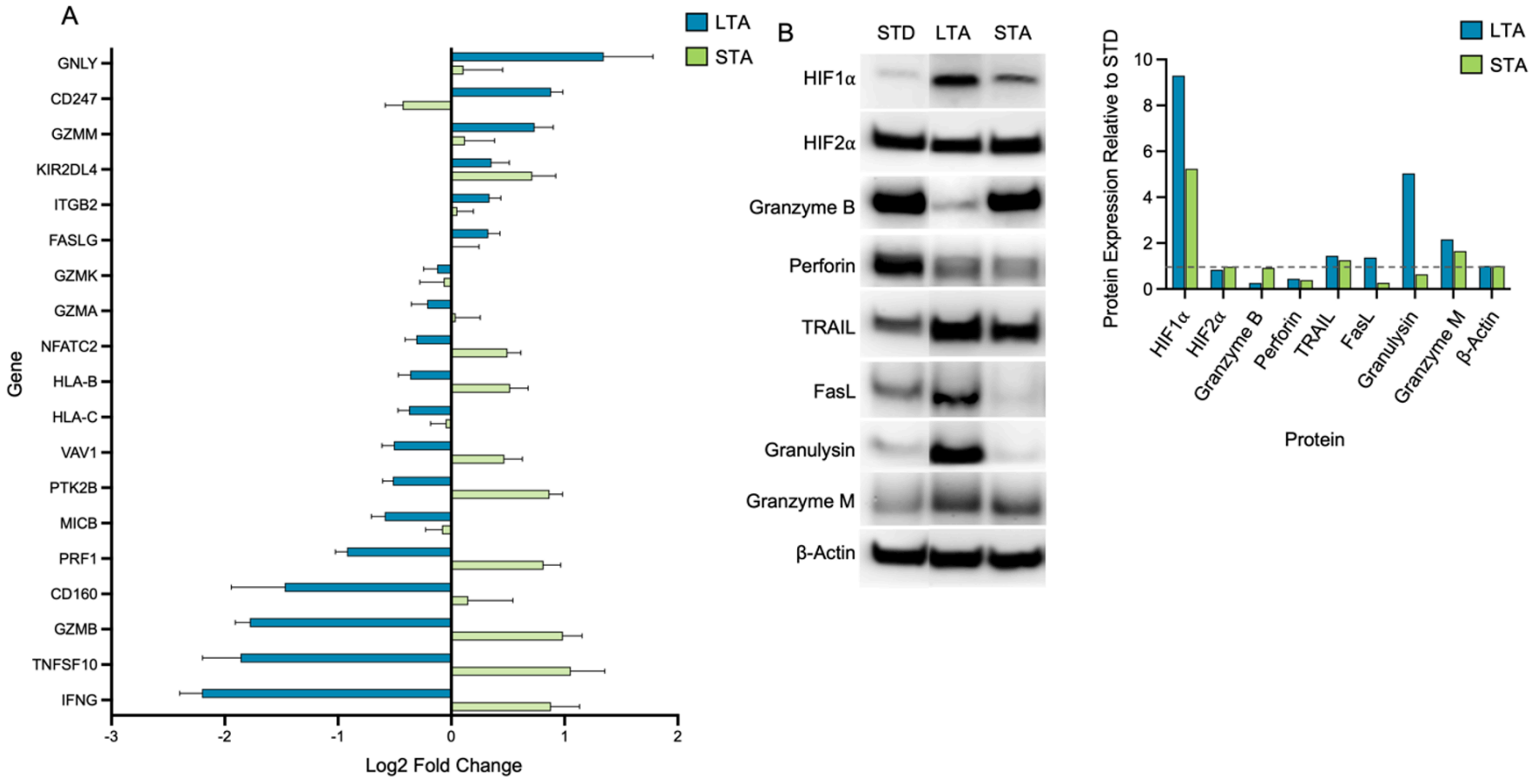

Figure 5. Long-term adaptation of NK-92 cells to TME conditions upregulates HIF1a and death receptor genes. A) Bar graphs indicated the Log2 Fold change in the expression of 19 cytotoxic genes in STA and LTA cells, relative to STD cells. B) Western blot shows the expression of hypoxia-related and cytotoxic proteins in STD, STA, and LTA cells. Bar graphs show density analysis of western blot, indicating relative expression of indicated proteins in STA and LTA cells, relative to STD cells. Dotted line indicates equivalent expression to STD.

These genes included IFNG, which encodes the cytokine IFN-g, GZMB, which encodes the serine protease Granzyme B, and PRF1, which encodes the pore-forming protein Perforin 1. This was surprising, as LTA cells exhibited the highest NK-mediated cytolysis in both normoxic and TME conditions. These genes were only moderately affected in STA cells. In contrast, LTA cells exhibited upregulation of GNLY, which encodes the cytotoxic protein granulysin, GZMM, the serine protease Granzyme M, and FASLG, which encodes the Fas Ligand death receptor. Next we performed protein analysis for some of these cytotoxic genes. As expected, we saw that the hypoxia-inducible transcription factor HIF1a was induced in STA cells and exhibited even higher expression in LTA cells (Figure 5b). As seen in the gene expression data, Granzyme B and Perforin proteins were expressed at much lower levels in LTA cells, while Granzyme M and Granulysin were overexpressed in LTA cells, relative to STD cells. Interestingly, LTA cells also exhibited increased expression of the death receptor pathway proteins TRAIL and Fas Ligand. Thus, we hypothesize that LTA cells mediate increased cytotoxicity under normoxic and TME conditions through upregulation of death receptor-mediated pathways.

CONCLUSION

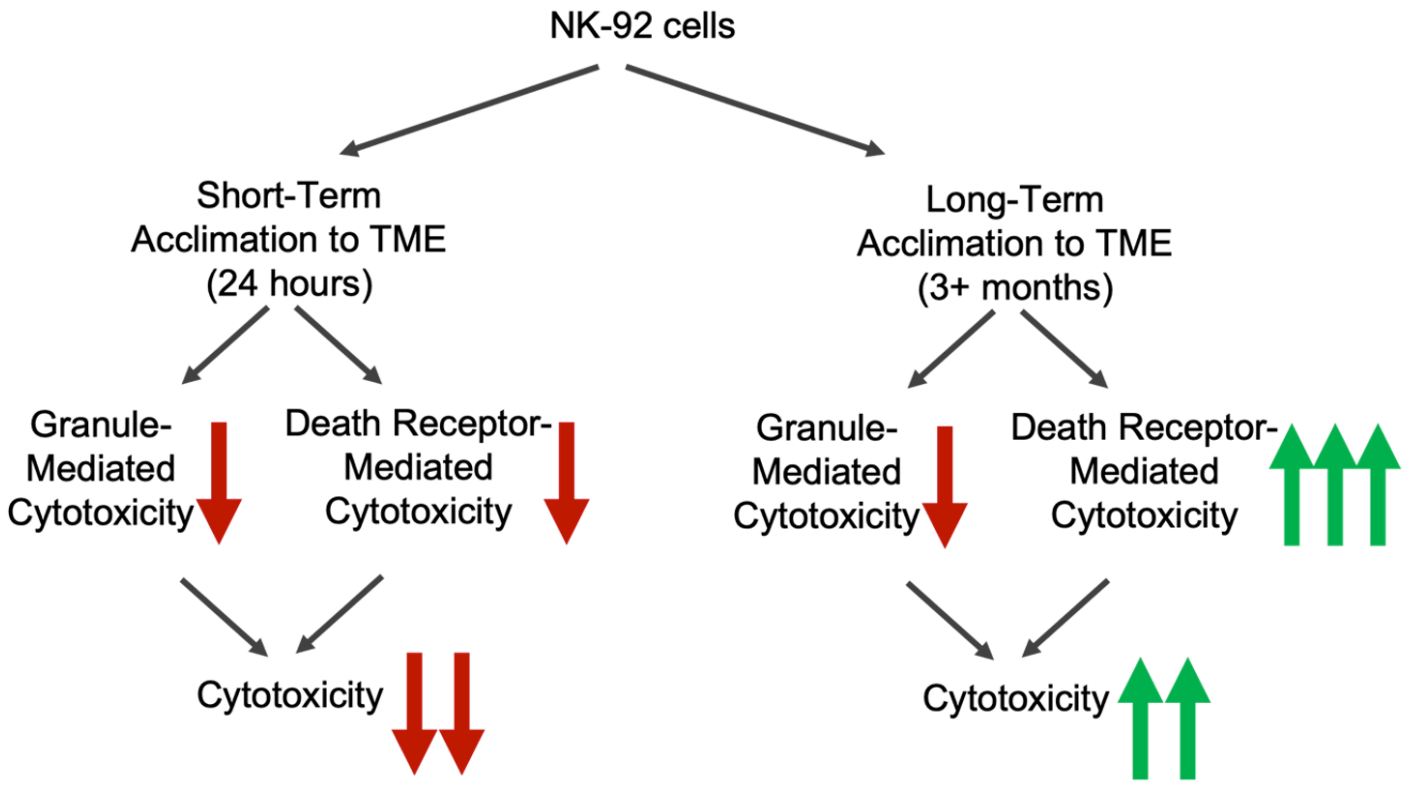

Figure 6. Model describing the effects of short- and long-term acclimation of NK-92 cells to TME conditions when compared against conventional culture conditions (normoxia).

The AVATAR instrument enables researchers to precisely control the environment of cells in culture to model TME-like conditions through modulation of oxygen and pressure. In conjunction, the AVATAR AI allows for real time, label-free cell killing analysis under these conditions, generating novel insights into cellular dynamics within the solid tumor environment. Utilizing this immunosuppressive modeling strategy in concert with tuning target and effector cell populations to optimally perform in TME conditions may lead to better comprehension and enable improved therapeutic development.

Based on the results showcased here, we propose a model wherein short-term exposure of NK-92 cells to low oxygen, high pressure culture conditions that mimic the TME leads to reduced expression of genes required for both granule-mediated and death receptor-mediated cytotoxicity and a resulting decrease in cytolysis compared to cells maintained under normoxic conditions (Figure 6). In contrast, long-term exposure enriches for cells which can metabolically adapt to these harsh conditions and are characterized by decreased expression of granule-mediated cytotoxic genes and increased expression of death receptor cytotoxic genes. This leads to enhanced cytolysis, relative to cells cultured under standard conditions.

Hypoxia plays a critical role in reprogramming cellular metabolism, with the most notable effects being a shift from mitochondrial respiration toward increased glycolysis to maintain ATP levels, known as the Warburg effect. Thus, it will be important to explore the metabolic effects of short- and long-term exposure of NK-92 cells to hypoxia. Furthermore, based on the significant role that the HIF1a plays in remodeling gene expression, in part through modulating the expression of histone modifying enzymes, it will also be interesting to investigate the epigenetic profile of our NK-92 populations.

METHODS

NK-92 Culture and acclimation

NK-92 cells were purchased from ATCC (CRL-2407) and maintained in medium containing MEM-α, no nucleosides (Gibco), 12.5% FBS (Gibco), 12.5% Horse Serum (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 0.2 mM myo-inositol (Sigma-Aldrich), 0.02 mM folic acid (Sigma-Aldrich) and 200 IU/mL IL-2 (Peprotech) in a 5% CO2 incubator.

For long term acclimation at TME conditions, NK-92 cells were maintained in the AVATAR system at 1% O_2 and 2 PSI for 3 months. For short term acclimation at TME, NK-92 cells were cultured in the AVATAR system at 1% O_2 and 2 PSI for 24 hours prior to initiation of cytotoxicity assays.

Cytotoxicity analysis of NK-92 cells using AVATAR AI

A549 cells were seeded in AVATAR AI E-plates and incubated for 24 hours in normoxic (21% O_2 and 0 PSI) or TME (1% O_2 and 2 PSI) conditions. XcellSoft software was used to monitor cell attachment and proliferation. On the next day, NK-92 cells were added to A549-containing wells at E:T ratios of 10:1, 5:1, 2:1 and 1:1. XcellSoft was used to monitor cell killing for 48 hours. Data was analyzed using XcellSoft and plot in Graphpad Prism.

RNA-seq and data analysis

NK-92 cells were collected by centrifugation and lysed in RLT Plus. Total RNA was isolated using the RNeasy® Plus Mini kit (Qiagen). RNA concentration and purity were measured by NanoDrop (Thermo Scientific) and RNA samples were sent to Novogene (Sacramento, CA) for mRNA-seq library preparation and sequencing. The reads were mapped to the reference human genome sequence (hg38) using Rsubread (v2.4.3). Gene read counts were quantified using featureCounts in paired-end mode. Principal component analysis was performed to calculate variance within each group, and differential expression of genes was identified using the DESeq2 v1.30.0.

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