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BRIEF REPORT

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CD25^{bright} NK cells display superior function and metabolic activity under regulatory T cell-mediated suppression

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ABSTRACT

Infusion of natural killer (NK) cells is an attractive therapeutic modality in patients with cancer. However, the activity of NK cells is regulated by several mechanisms operating within solid tumors. Regulatory T (Treg) cells suppress NK cell activity through various mechanisms including deprivation of IL-2 via the IL-2 receptor alpha (CD25). Here, we investigate CD25 expression on NK cells to confer persistence in Treg cells containing solid tumor models of renal cell carcinoma (RCC). Compared with IL-2, stimulation with IL-15 increases the expression of CD25 resulting in enhanced response to IL-2 as evidenced by increased phosphorylation of STAT5. Compared with CD25^{dim} NK cells, CD25^{bright} NK cells isolated from IL-15 primed NK cells display increased proliferative and metabolic activity as well as increased ability to persist in Treg cells containing RCC tumor spheroids. These results support strategies to enrich for or selectively expand CD25^{bright} NK cells for adoptive cellular therapy of NK cells.

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Introduction

Partial or complete loss of HLA class I expression is a frequent event by tumors to escape from T cell-mediated immunosurveillance.[1] However, such tumors may instead become sensitive to killing by natural killer (NK) cells. In recent years, adoptive transfer of NK cells has become an attractive therapeutic modality in patients with advanced cancer.[2] They play essential roles in tumor progression via the interaction of tumor cells with other cells within the tumor microenvironment (TME), and the functional state of tumor-infiltrating NK cells is influenced by several mechanisms operating within the TME. Tumor cells themselves as well as various cell types residing within the TME, including cancer-associated fibroblasts, myeloid-derived suppressor cells (MDSC), tumor-associated macrophages, and regulatory T (Treg) cells suppress NK cell activity.[3]

Treg cells play essential roles in immune homeostasis, autoimmunity, and anti-tumor immunity and can suppress NK cell activity through several different mechanisms. For instance, Treg cells produce transforming growth factor beta (TGF-β) to downregulate the expression of NKG2D and impair NK cell function. Also, Treg cells produce granzyme B and perforin to induce NK cell apoptosis, resulting in impaired tumor clearance.[4,5] Another potential mechanism whereby Treg cells inhibit NK cell function relies on the high expression of the IL-2α receptor (CD25) which Treg cells can use to deprive IL-2 and thereby inhibit IL - 2 - induced activation of NK cells. For example, during acute retroviral infection, suppression of NK cell activity depends on IL-2 consumption by Tregs.[6] Sitrin and colleagues showed that Treg cells suppress NK cells through IL-2 deprivation in a prediabetic murine model.[7] Furthermore, in a mouse model of inflammatory bowel disease, Treg cells consume IL-2 to induce apoptosis of CD4 T cells Treg cell-induced apoptosis.[8] In contrast, Oberle and colleagues reported that IL-2 depletion by human Treg alone is not sufficient to suppress proliferation of conventional T cells.[9] Thus, the role of depletion of IL-2 by Treg to inhibit NK cell activity needs further exploration.

IL-15 is another common γ-receptor cytokine family member that is important for NK cell differentiation, survival, and activation. We and others have previously reported that exposure to IL-15 significantly increases the expression of CD25 on human primary NK cells compared to IL-2.[10,11] We therefore investigated if IL-15 primed NK cells have improved ability to bind to IL-2 and hence maintain their activity upon exposure to Treg in a model of renal cell carcinoma (RCC). Compared to NK cells primed with IL-2, IL-15 primed are less susceptible to Treg-mediated suppression and shows improved proliferation in Treg-containing RCC tumor spheroids. We further show that the expression of CD25 defines improved NK cell functionality and metabolic fitness in the presence of Treg. Therefore, infusion of NK cells enriched for high CD25 expression might result in increased anti-tumor activity in patients with cancer.

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Materials and methods

Cell isolation

Human NK cells and Treg cells were purified from PBMCs using NK cell isolation kit, and $CD4^+$ $CD25^+$ $CD127^{dim/-}$ Regulatory T Cell Isolation Kit II respectively (Miltenyi Biotec). The purity of the isolated NK cells was above 90% as assessed by flow cytometry staining for CD56 and CD3. The NK cells were sorted after 2 d of cytokine activation (IL-15, 300 U/ml) into $CD25^{neg}$, $CD25^{dim}$, and $CD25^{bright}$ NK cell populations using BD FACSAria™ Fusion cell sorter.

Cell culture

NK cells were seeded in 48-well flat-bottom plate (TPP techno) in X–vivo 20 medium (Lonza) supplemented with 10% heat-inactivated human AB serum and 300 U/ml of IL-2 or 300 U/ml IL-15. Purified Treg cells were cultured with 300 U/ml IL-2 and human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific) for 2 d prior to analysis. The renal carcinoma cell lines A498 and 786-O were maintained in RPMI1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and 1% antibiotics (penicillin, streptomycin).

Flow cytometry

Detailed information about the antibodies used in this study is summarized in Supplementary Table 1. Briefly, $1–2 \times 10^5$ cells were washed twice with FACS buffer (PBS with 2% FBS) and then resuspended in 20 µl PBS containing the appropriate antibody cocktails for extracellular antigens and incubated at 4° for 30 mins. Following staining of cell surface antigens, cells were fixed in BD Phosflow Fix Buffer for 10 min and washed twice with PBS. Then, cells were incubated on ice with BD Phosflow Perm Buffer III for 30 mins, followed by three times washing prior staining for intracellular antigens. For intracellular staining IFNγ, NK cells were incubated with an equal number of K562 cells for 4 hours in a medium containing Golgiplug (BD), and Golgistop (BD). Stained cells were washed twice with FACS buffer and acquired on a Novocyte flow cytometer (ACEA biosciences) and analyzed using Flowjo software (BD). Analysis was performed on live single cells. NK cells were gated as $CD4^-CD56^+$ $Foxp3^-$ or $CD3^-Foxp3^-$ subsets, Treg cells were gated as $CD4^+$ $Foxp3^+$ or $CD3^+$ $Foxp3^+$ cells.

3D tumor spheroid infiltration assays

For the analysis of NK cell infiltration into tumor spheroids, 5,000 or 10,000 786-O or A498 cells were seeded in Nunclon Sphera 96-well Round (U) bottom plate (Thermo Fisher). On day 5, 10,000 Treg cells were added in the presence of Ultra-LEAF™ purified anti-human CD3 antibody (Biolegend) and 300 U/ml IL-2 to tumor spheroids initially seeded at 10,000 cells. For Incucyte imaging, cytokine-activated NK cells were labeled with IncuCyte® NucLight Rapid Red Reagent (Essen BioScience) and added at 10,000 cells on day 7 (Figure 2g). Alternatively, 25,000 Treg cells were added on day 5 to tumor spheroids initially seeded at 5,000 cells, and cytokine-activated NK cells were added at 25,000 cells on day 7.

On day 8, spheroids were gently resuspended in 1 ml PBS and left to sediment to the bottom of a 5-ml eppendorf tube. This washing step was repeated twice. After each washing step, supernatants were collected as non-tumor-infiltrating NK cells. Spheroids containing tumor-infiltrating NK cells were harvested and trypsinized to obtain a single-cell suspension and thereafter analyzed by flow cytometry. Single-cell suspensions of spheroids and non-tumor infiltrating cells were stained with Live/Dead cell marker and anti-CD45 antibody to distinguish NK cells from tumor cells.

Immunofluorescence microscopy and image analysis

For intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set in accordance with manufacturer's procedures (eBioscience). The cells were then incubated with primary antibodies and fluorescence-conjugated secondary antibodies. The nucleus was stained by Fluoroshield™ with DAPI (Sigma). For mitochondria staining, sorted cells were stained with Mitotracker Red FM according to the manufacturer's guidelines (ThermoFisher). Slides of stained cells were prepared with cytospin 2 (SHANDON). Immunofluorescent staining was analyzed using the confocal laser scanning microscope LSM 700 system (Zeiss).

Real-time glycolytic and mitochondrial respiration rate measurement

NK cells were sorted by BD FACSAria™ Fusion cell sorter based on the expression of CD25 and seeded in a 24-well plate at 120,000 cells. The oxygen consumption rate (OCR) was measured under basal and stress conditions using the Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) with sequential injections of 1 mM of oligomycin, 1.6 mM of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 mM of antimycin/rotenone, following the manufacturer's instructions of the XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA). All metabolic assays were normalized to the number of cells.

TCGA and single cell RNA-seq data analysis

Cellular Fraction Estimates of TCGA-KIRC generated by CIBERSORT were obtained from Genomic Data Commons (https://gdc.cancer.gov/).[12] CIBERSORT algorithm was employed to infer the relative abundance of Treg and NK cells, and the median values were used to define low and high infiltrations. Samples with differential infiltration were compared for survival differences. Patient survival data was downloaded from https://xenabrowser.net/. Survival analysis was performed on GraphPad Prism 9. Single-cell RNA-seq data were downloaded from the Gene Expression Omnibus (GSE121638). NK cells were extracted, and their expressions were submitted to GSVA packages for gene set enrichment analysis. Gene sets were downloaded from MSigDB (www.gsea-msigdb.org).

Statistical analysis

Unless otherwise stated, all results were collected from multiple experiments, and figures were prepared in a Prism 9.0 (GraphPad version 8). Paired Student's t-tests between the two groups were used to determine significance. All results are presented as mean ± SD and represented histograms or images were selected based on the average values, $p < .05$ was considered significant (* $p < .05$; ** $p < .01$; *** $p < .005$; **** $p < .0001$).

Results

IL-15 primed NK cells are less susceptible to Treg mediated suppression

In contrast to most solid tumors, the high densities of $CD8^+$ T cells in clear cell RCCs are associated with poor prognosis. Likewise, high levels of *FOXP3* transcripts correlate with tumor stage and incidence of metastasis, potentially indicating a poor prognostic value of Treg in RCC.[13,14] However, the clinical impact of tumor-infiltrating NK cells is less clear in patients with RCC. To investigate the prognostic value of Treg and NK cells in patients with RCC, transcriptomic data were stratified based on gene expression levels. While patients with low Treg gene expression showed significantly longer disease specific and overall survival, patients with high NK cell gene expression showed significantly longer disease specific, but not overall survival (Figure 1a **and Supplemental Figure 1A**). Thus, efforts to enhance NK cell or repress Treg cell activity might result in improved prognosis in RCC patients. The type i cytokines IL-2 and IL-15 activates NK cell functions. Phenotypically, IL-15 in known to induce a higher expression of CD25 than IL-2.[10,11] Since Treg cells express high levels of CD25 and can inhibit NK cell activity through deprivation of IL-2,[6] we explored whether IL-15 primed NK cells preserve their activity in the presence of Treg. Upon stimulation with IL-2, IL-15 primed NK cells show stronger phosphorylation of STAT5 than IL-2 primed NK cells (Figure 1b-d). Similar levels of STAT5 phosphorylation are observed in IL-15 and IL-2 primed NK cells when stimulated with 100 U/ml and 1000 U/ml of IL-2, respectively (Figure 1e and f). In contrast, no significant changes in the phosphorylation of STAT3, Akt, or ERK are observed (**Supplemental Figure 1B and C**).

Upon co-culture with Treg cells and in the presence of IL-2, the expression of CD25 and surface IL-2 is significantly higher in IL-15 primed NK cells compared with IL-2 primed NK cells (Figure 1g, h, i). In line with this, the proliferation of IL-15 primed NK cells is maintained, whereas the proliferation of IL-2 primed NK cells is significantly impaired in the presence of Treg (Figure 1j). In the presence of K562 cells, IL-15 primed NK cells display higher proliferation than IL-2-primed NK cells, whereas the proliferation of Treg cells did not differ, resulting in a significantly higher NK/Treg ratio (Figure 2a-e). Furthermore, IFNγ production against K562 cells of IL-2 primed NK cells is significantly inhibited in the presence of Treg (Figure 2f).

To test whether cytokine-primed NK cells differ in their ability to infiltrate RCC tumors, NK cells were primed with either IL-2 or IL-15 and then added to Treg containing RCC tumor spheroids generated from RCC cell lines (Figure 2g). While the infiltration of IL-15-primed NK cells into 786-O spheroids is not affected by Treg, the infiltration of IL-2 primed NK cells is significantly reduced in the presence of Treg (**Supplemental Figure 1D**). Adding a higher amount of NK cells (25,000) and Treg cells (25,000) to fewer tumor cells (5,000) results in an overall increased infiltration of both IL-2 and IL-15 primed NK cells and no difference in infiltration between IL-2 or IL-15-primed NK cells is observed in spheroids without Treg. Still, the infiltration of IL-2 primed NK cells is significantly reduced in the presence of Treg cells, whereas the infiltration of IL-15-primed NK cells into 786-O spheroids is not affected by Treg (**Supplemental Figure 1D**).

On average, the infiltration of Treg and NK cells is lower in A498 tumor spheroids than in 786-O spheroids. Regardless, compared to IL-2 primed NK cells, IL-15 primed NK cells show a significant increase in infiltration into RCC spheroids generated from 786-O and A498 cell lines as measured by flow cytometry (Figure 2h) and Incucyte imaging (Figure 2i). Notably, the frequency of Treg is reduced in the presence of IL-15 primed NK cells resulting in a 3.0-fold higher NK-to-Treg ratio compared with that in the presence of IL-2 primed NK cells. Adding a higher number of Treg and NK cells to fewer tumor cells results in a greater infiltration of IL-15-primed NK cells. Similarly, the frequency of Treg is significantly higher in the presence of IL-2-primed NK cells compared with that in the presence of IL-15 primed NK cells (**Supplemental Figure 1E**). Moreover, IL-15 primed NK cells show significantly higher proliferation compared to the IL-2-primed NK cells (Figure 2j). Taken together, these results show that IL-15 primed NK cells respond to lower levels of IL-2 and are more resistant to Treg suppression than NK cells stimulated by IL-2.

CD25 expression defines NK cells with increased resistance to Treg mediated suppression

To investigate if the expression of CD25 delineates more activated NK cells, a single-cell RNA sequencing analysis of RCC tumors (GSE121618) was performed. Indeed, CD25 positive NK cells show higher enrichment scores of pathways related to NK cell activation (**Supplemental Figure 2A**). To further investigate the role of CD25 expression on NK cells in increased resistance to Treg-mediated suppression and distinguish from other potential effects, cytokine stimulation by IL-2 or IL-15, NK cells were activated with IL-15 and sorted for differential expression of CD25 and analyzed for their activity in the presence of Treg (**Supplemental Figure 2B**). In the presence of Treg, NK cells expressing high levels of CD25 ($CD25^{bright}$) display significantly higher levels of STAT5 phosphorylation and proliferation upon restimulation with IL-2 compared with $CD25^{dim}$ and $CD25^{neg}$ NK cells (Figure 3a, b). In contrast, STAT5 phosphorylation and proliferation remain unchanged in Treg regardless of the presence of subpopulations of NK cells (data not shown).

Since IL-15 can activate NK cells via metabolic reprogramming,[10,15,16] we next sought to investigate whether IL-15 induced CD25 expression defines NK cells with different metabolic potentials. Compared with $CD25^{neg}$ and $CD25^{dim}$

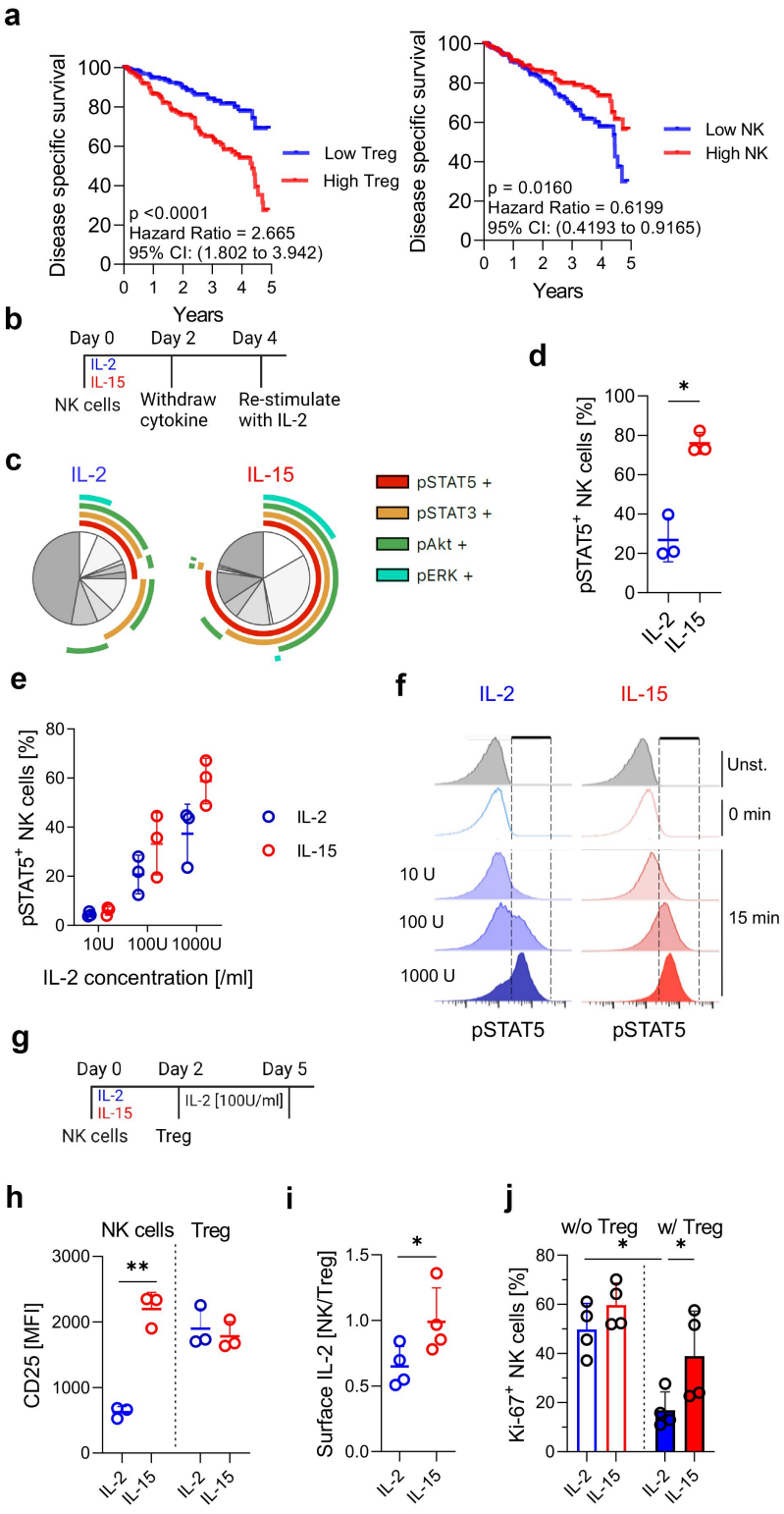

Figure 1. IL-15 primed NK cells show increased pSTAT5 levels and are less susceptible to Treg-mediated suppression (a) Kaplan–Meier survival curves showing disease specific survival in patients with clear cell renal cell carcinoma partitioned by transcript abundance of Treg or NK gene signature from KIRC-TCGA cohort. Survival curve differences were analyzed using Kaplan–Meier log-rank test. (b) Experimental setup of IL-2 re-stimulation. (c) Spice chart showing phosphorylation markers of NK cells primed with IL-2 or IL-15 analyzed 15 minutes after re-stimulation with 100 U/ml IL-2 ($n = 3$). (d) Frequency of pSTAT5+ NK cells responding to 100 U/ml IL-2 ($n = 3$). (e) Flow cytometry quantification of pSTAT5 positive NK cells primed with IL-2 or IL-15 ($n = 3$) following re-stimulation at different doses of IL-2. (f) Representative flow chart showing the phosphorylation of STAT5 (Y694) at different doses of IL-2 re-stimulation comparing IL-2 and IL-15 activated NK cells. (g) Experimental setup of NK and Treg co-culture. Isolated and activated Treg were added at 1:1 ratio to NK cells. (h) CD25 expression of NK cells and Treg in co-cultures ($n = 3$). (i) NK/Treg ratio of surface IL-2 expression of after 4-hour co-culture ($n = 4$). (j) Frequency of Ki-67 positive NK cells in the absence or presence of Treg ($n = 4$).

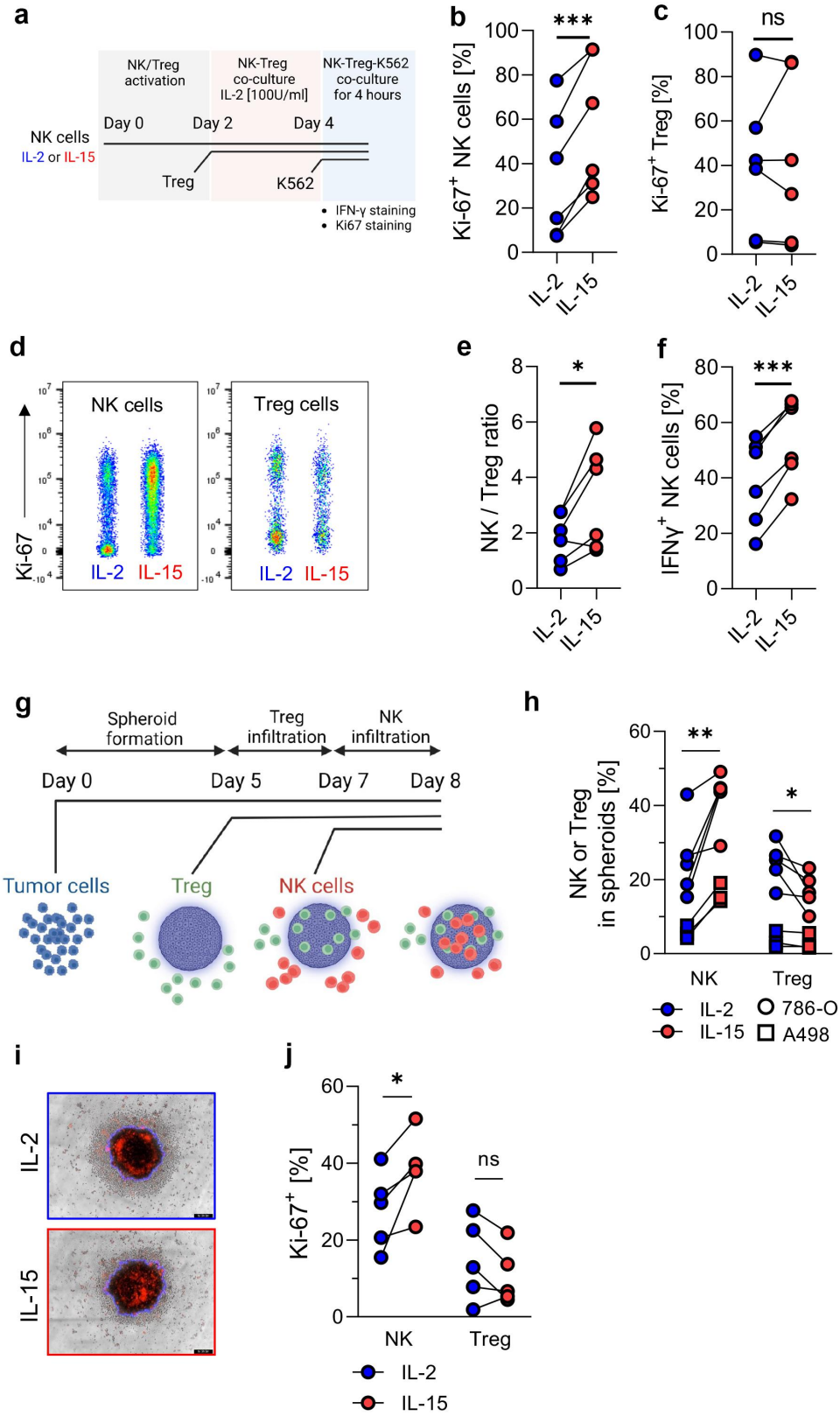

Figure 2. IL-15 primed NK cells show increased proliferative capacity and anti-tumor activity under Treg suppression. (a) Experimental setup of NK-Treg-K562 co-culture. Two-day activated NK cells and Tregs were co-cultured at a 1:1 ratio (25,000 NK cells to 25, 000 Treg) for two days. Thereafter, K562 cells (25,000) were added to NK:Treg cultures on day four for four hours for analysis of IFNγ production and proliferation. (b and c) Proliferation (Ki-67) of NK cells and Treg performed after two days Treg-NK cell co-culture and upon 4 hours exposure to K562 cells ($n = 6$). (d) Representative flow cytometry plots showing proliferation (Ki-67) of NK cells and Treg. (e) NK/Treg ratio on day 4 after 4 hours culture with K562 cells ($n = 6$). (f) IFN γ production by NK cells upon 4 hours exposure to K562 cells ($n = 6$). (g) Experimental setup of NK cell infiltration in NK-Treg-RCC spheroids. On day 0, tumor cells were seeded at either 5,000 or 10,000 cells. On day 5 and 7, activated Treg and cytokine-activated NK cells were added to RCC cell line spheroids respectively at a 1:1 ratio of either 25,000 or 10,000 cells. NK cell infiltration and proliferation were analyzed by flow cytometry on day 8. (h) Flow cytometry analysis of tumor-infiltrating NK cells and Treg into 786-O and A498 spheroids ($n = 3$ to 5). (i) Representative Incucyte image of NK cell infiltration (Red) into 786-O tumor spheroids on day 8. (j) Proliferation (Ki-67) of NK and Treg in 786-O RCC spheroids ($n = 5$). Results (h-j) are derived from experiments with seeding of 10,000 tumor cells on day 0, and addition of 10,000 Treg and 10,000 NK cells on days 5 and 7 respectively. Paired Student's t-test was used to determine significance.

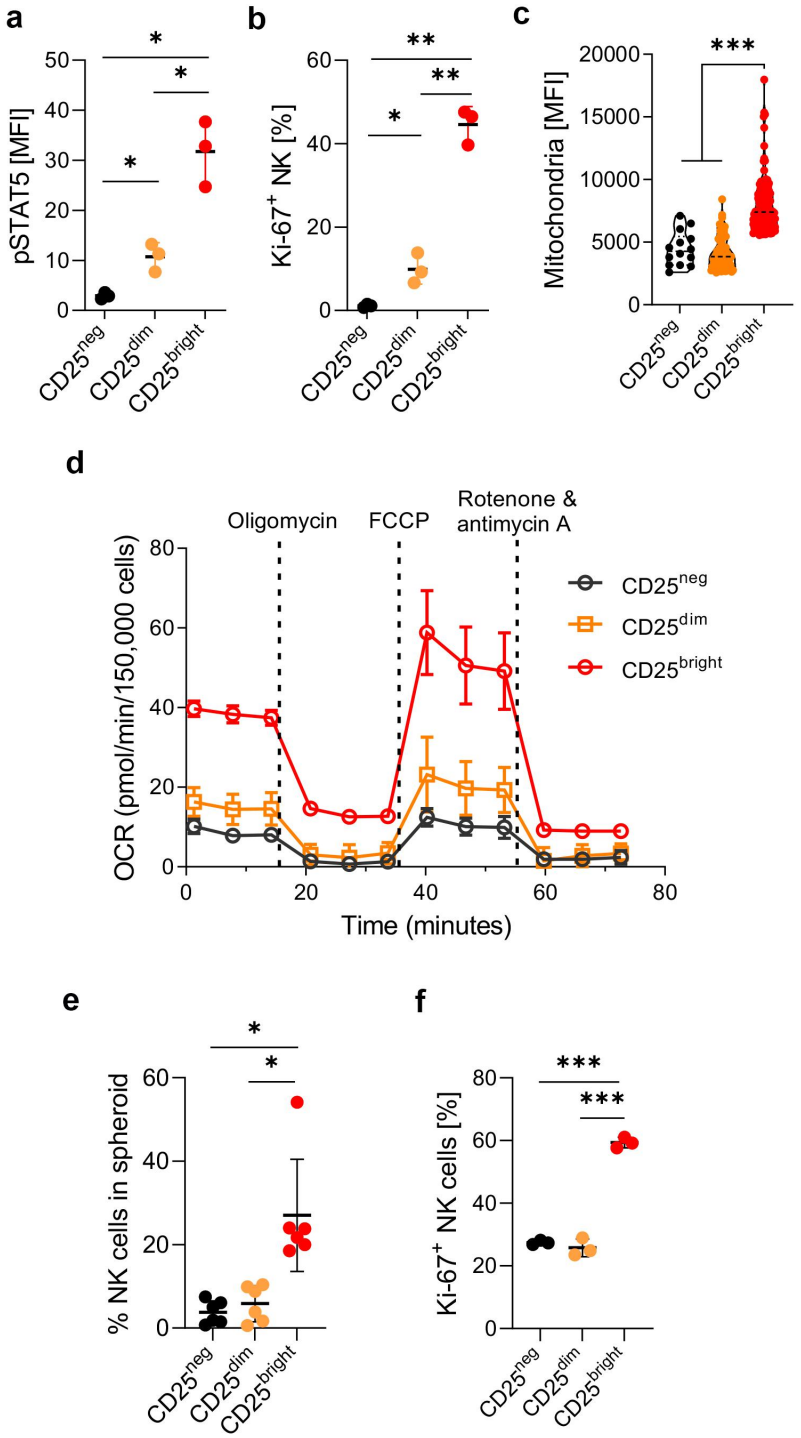

Figure 3. CD25 expression is associated with improved NK cell response to IL-2 re-stimulation and metabolic activity. (a) Phosphorylation of STAT5 and (b) proliferation among CD25 NK cell subsets ($n = 3$). Sorted NK cells were co-cultured with Treg for two days (100 U/ml IL-2) before flow cytometry analysis ($n = 3$). (c) Quantification of mitochondria membrane potential per cells via microscopy, each dot represents a single cell. (d) Oxygen consumption rate (OCR) was measured after injection of oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and Rotenone & antimycin A ($n = 4$). (e and f) Frequencies and proliferation of live NK cells in 786-O RCC spheroids as detected by flow cytometry ($n = 6$). Tumor cells (10,000) were seeded and day 0, followed by addition of Treg (10,000) on day 5, and NK cells (10,000) on day 7. NK cell infiltration (E, $n = 6$) and proliferation (F, $n = 3$) were analyzed by flow cytometry at day 8.

NK cells, CD25^{bright} NK cells display a significantly increased mitochondrial membrane potential (Figure 3c **and Supplemental Figure 2C**). Furthermore, the baseline and stressed conditions of glycolysis and mitochondria respiration are significantly higher in CD25^{bright} NK cells compared with CD25^{dim} and CD25 negative NK cells (Figure 3d **and Supplemental Figure 2D**). To assess if the expression of CD25 influences the ability of IL-15-primed NK cells to infiltrate RCC tumors, NK cells with differential expression of CD25 were added to Treg containing 786-O tumor spheroids. While no difference in the intratumor frequencies of CD25^{neg} and CD25^{dim} is observed, CD25^{bright} NK cells show

significantly higher infiltration compared with CD25^{neg} NK cells (Figure 3e). The intratumor CD25 ^{bright} NK cells display significantly higher levels of proliferation in Treg containing tumor spheroids (figure 3f). Collectively, these results show that CD25 expression defines NK cells with increased resistance to Treg mediated suppression and increased metabolic capacity and ability to infiltrate solid tumors.

Discussion

Treg cells often accumulate in solid tumors and are present at higher frequencies in more aggressive and advanced tumors.[17] They actively inhibit T and NK cells through various mechanisms, including via the production of TGFβ or granzyme B and perforin to induce NK cell apoptosis.[4,5] Through their high expression of CD25, Treg can act as a "cytokine sink" to deprive IL-2 in local environments and induce apoptosis of CD4 T cells and dysfunction of NK cells.[7,8] Here, we confirm earlier findings that exposure to IL-15 results in a significant increase CD25 expression by NK cells compared with the exposure to IL-2.[10,11] Consequently, IL-15 primed NK cells responded better to IL-2 stimulation and are provided with increased resistance to Treg-mediated suppression.

To our knowledge, no other study has compared the impact of IL-2 and IL-15 to render NK cells resistant to Treg-mediated suppression. In an early study, Tao et al. showed that IL-15 cytokine-induced killer cell (CIK) cultures contained less Treg compared with the IL-2 cultures.[18] Rettinger and colleagues further showed that CD25 positive CIK cells were more effective at killing leukemia cells.[19] With respect to the NK cells, the expression of CD25 demarcates more functionally activated cells in different cirrhotic livers and decidua.[20,21] Curiously, in stage IV highly metastatic M1c melanoma patients, the frequency of CD25 positive NK cells is lower compared with M1a and M1b patients.[22] Here, we extend these observations by analyzing single-cell RNA sequencing data, demonstrating that CD25 positive NK cells show a heightened activation status in patients with RCC.

Several studies have demonstrated that IL-15 increase the metabolic activity of NK cells through increased mTOR activity.[10,15,16] It has previously been demonstrated that IL-15 activated NK cells are more resistant to various modes of suppression, including reactive oxygen species and prostaglandin-E2 governed through mTOR-dependent mechanisms.[23,24] It is important to consider the interval of IL-15 treatment of NK cells since chronic exposure to IL-15 can exhaust NK cells via metabolic defects.[25,26] Here, we show that upon a single stimulation with IL-15, cells expressing higher levels of CD25 showed improved metabolic activity and ability to infiltrate Treg containing RCC spheroids compared with NK cells with low or no expression of CD25. These results further support the role of CD25 expression to delineate NK cells with improved ability to infiltrate tumors and higher metabolic potential. Several features including hypoxia, necrosis, and pH are influenced by tumor spheroid size.[27] Although, such features might impact lymphocyte infiltration, we did not address whether CD25 positive NK cells remain superior at infiltrating tumors across different spheroid sizes than CD25 negative NK cells.

Altogether, our findings support the idea that NK cells primed with IL-15 better compete with Treg for IL-2 within tumors and that the expression of CD25 is associated with improved NK cell functionality, including metabolic potential. Therefore, strategies to enrich for or selectively expand CD25^{bright} NK cells should be considered for adoptive cellular therapy of NK cells.

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Disclosure statement

The authors have no relevant competing interests to declare.

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Data availability Statement

The data that support the findings of this study are available from the corresponding author, AL, upon reasonable request. Raw data of cellular fraction estimates, patient survival, and gene sets for scRNA-seq (GSE121638) were downloaded from https://gdc.cancer.gov, https://xenabrowser.net, and www.gsea-msigdb.org.

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