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TUMOR MARKERS AND SIGNATURES

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The tumor microenvironment drives NK cell metabolic dysfunction leading to impaired antitumor activity

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Abstract

NK cells represent key players capable of driving antitumor immune responses. However, the potent immunosuppressive activity of the tumor microenvironment (TME) may impair their effector function. Here, we strengthen the importance of metabolic interactions between NK cells and TME and propose metabolic dysfunction as one of the major mechanisms behind NK failure in cancer treatment. In particular, we described that TME has a direct negative impact on NK cell function by disrupting their mitochondrial integrity and function in pediatric and adult patients with primary and metastatic cancer. Our results will help to design new strategies aimed at increasing the NK cell antitumor efficacy by their metabolic reprogramming. In this regard, we reveal an unprecedented role of IL15 in the metabolic reprogramming of NK cells enhancing their antitumor functions. IL15 prevents the inhibitory effect of soluble factors present in TME and restores both the metabolic characteristics and the effector function of NK cells inhibited by exposure to malignant pleural fluid. Thus, we propose here that IL15 may be exploited as a new strategy to metabolically reprogram NK cells with the aim of increasing the efficacy of NK-based immunotherapy in a wide range of currently refractory adult and pediatric solid tumors.

KEYWORDS
metabolic dysfunction, NK cells, tumor immunology, tumor microenvironment

What's new

The tumor microenvironment may hamper the functional activities of tumor-infiltrating immune effector cells by affecting both their antitumor cytotoxicity and metabolic fitness. This study shows that the tumor microenvironment exerts a direct negative impact on NK cells in pediatric and adult patients with primary and metastatic cancer by disrupting the integrity and function of

Abbreviations: ASL, azienda sanitaria locale; ATP, adenosine triphosphate; ECAR, extracellular acidification rate; HD, healthy donors; IL, interleukin; MFI, mean fluorescence intensity; mPE, malignant pleural effusions; NK, natural killer cells; OCR, oxygen consumption rate; OXPHOS, mitochondrial oxidative phosphorylation; PBMC, peripheral blood mononuclear cells; TIGIT, T cell immune receptor with Ig and ITIM domains; TME, tumor microenvironment.

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NK cell mitochondria. The findings suggest that metabolic disruption is one of the key mechanisms driving NK cell exhaustion and support therapeutic interventions that exploit IL-15 to reprogram NK cell metabolism and thereby increase the efficacy of NK cell-based tumor immunotherapy.

1 | INTRODUCTION

The microenvironment (TME) of both solid and hematological pediatric and adult tumors contains malignant cells, a variety of different nonmalignant cell types, including a number of myeloid and mesenchymal cells, as well as soluble factors that contribute to the establishment of an immunosuppressive environment.[1,2] TME may hamper the functional activities of tumor-infiltrating immune effector cells by affecting both their antitumor cytotoxicity and metabolic fitness.[3,4] As a consequence, they fail to control tumor growth and metastatic spread. NK cells play an important role in innate defenses against tumors but their functional may be greatly compromised by TME. A mechanism by which tumors can escape NK-mediated control is the induction of the expression of inhibitory receptors as well as downregulation of activating receptors involved in tumor cell recognition and killing.[5,6] Recent evidences have revealed that one of the mechanisms by which TME drives NK dysfunction is the induction of major metabolic defects in NK cells[7,8] leading to loss of NK cells lose their metabolic fitness and antitumor activity. In this scenario, it is conceivable that metabolic reprogramming of NK cells could restore metabolic fitness in NK cells and restore their antitumor activity even in a hostile nutrient-deprived TME. Thus, it is fundamental to identify mechanisms responsible for the metabolic-driven NK cell dysfunction[9] in order to develop new strategies aimed at potentiating and widening the efficacy of NK-based tumor immunotherapies.

In our study, we analyzed malignant pleural effusions (mPE). Notably, PE represents an easily accessible surrogate which may be informative, at least in part, on the effect of the inhibitory cells and soluble factors composing the TME. In previous studies, we showed that NK cells present in mPE upon short activation with cytokines were able to release cytokines and efficiently killed autologous and allogenic tumor cells, in particular IL2 and/or IL15 were able to rescue/potentiate NK cell antitumor activity. These results suggested the possibility of treating patients with primary or metastatic pleural tumors with infusion of IL2 or IL15 or NK cells preactivated with these cytokines in vitro. Considering the complex interactions occurring in the TME and the finding that infusion of IL2 can induce an expansion/activation of regulatory T cells,[10] it appears that the use of IL15 in the clinical treatment of cancer patients could represent a more safe and promising approach. However, data present in literature indicate that the continuous stimulation with IL15 may induce an exhaustion of NK cells.[11] As a consequence, it appeared important to define the optimal infusion/treatment setting to activate NK cells without inducing their exhaustion.

Here, we show that soluble components of TME negatively impact on the metabolic fitness of NK cells and resulting in impairment of their antitumor activity. Moreover, we identify IL15 as a strong candidate able to restore NK metabolic plasticity and thus their antitumor activity. Indeed, we identified a suitable activation protocol allowing IL15 to prevent and/or restore NK cell proliferation and antitumor activity by limiting cellular and metabolic exhaustion. Our results suggest that metabolic reprogramming of NK cells may represent a strategy to promote their activity and survival in the metabolically hostile TME as part of an approach to enhance the clinical efficacy of NK-based immunotherapy applied to a broad variety of pediatric and adult primary and metastatic tumors.

2 | MATERIALS AND METHODS

2.1 | Samples

Thirteen patients with primary or metastatic lung tumors were enrolled at ASL3, Ospedale Villa Scassi, Genoa, Italy and mPE obtained from thoracentesis. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors (HD) (IRCCS Bambino Gesù Children's Hospital, Rome) and isolated after density gradient centrifugation (Ficoll-Lympholyte, Cederlane). HD-NK cells were purified using NK isolation kit II (Miltenyi) and shortly activated (72 h) with IL15 (20 ng/mL, Miltenyi). Subsequently NK cells were cultured in the absence or in the presence of mPE for further 3 days with or without IL15. A549 adenocarcinoma cell line (RRID:CVCL_0023) were authenticated using STR profiling within the last 3 years. All experiments were performed with mycoplasma-free cells.

2.2 | Seahorse extracellular flux analysis

Seahorse experiments were performed on isolated NK cells using XF Cell Mito Stress kit (Seahorse Bioscience). OCR and ECAR were measured with XF96 Extracellular Flux Analyzers (Seahorse Bioscience). Briefly, cells were plated on poly-D-lysine-coated 96-well polystyrene Seahorse plates (50 000 NK cells/well), equilibrated for 1 hour at 37°C, and assayed for OCR (pmol/min) and ECAR (mpH/min) in basal conditions and after addition of oligomycin (1 μM), carbonyl cyanide-4-phenylhydrazone (1.5 μM), and antimycin A/rotenone (1 μM/0.1 μM). All of the SeaHorse experiments were performed using the manufacturer's recommended media (pH 7.4), without phenol red in order to standardize the pH conditions in all samples.

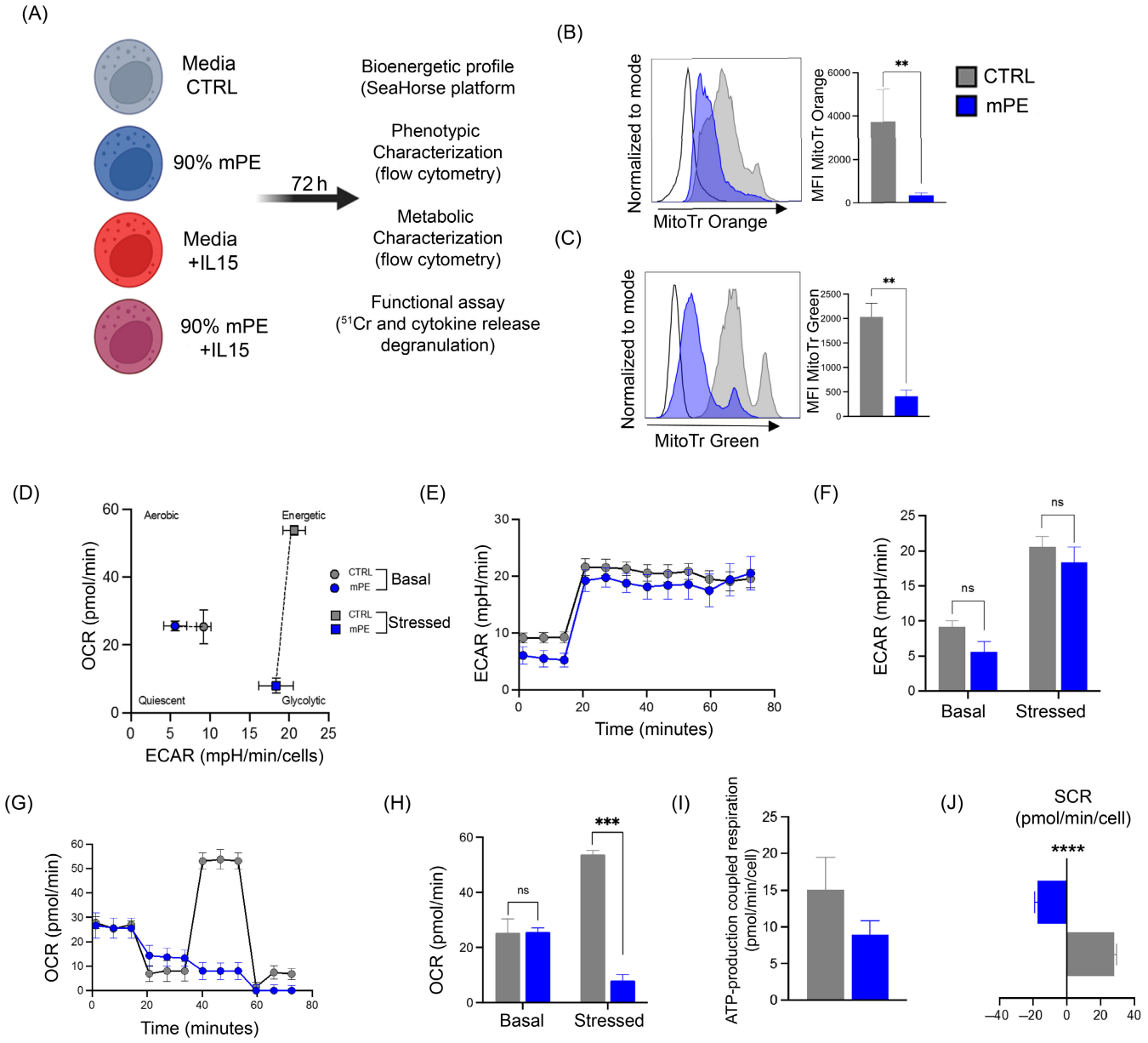

FIGURE 1 TME affects the metabolic features of NK cells. (A) Schematic treatment of HD-NK cells treated with cancer's patient-derived malignant pleural effusion (mPE). Briefly, purified HD-NK cells were kept in culture using 90% mPE in the presence (purple cell) or absence (blue cell) of IL15 for 72 hours. Control cells were kept in complete media (gray cell) or in culture media supplemented with IL15 (red cell). (B) Mitochondrial membrane potential (MitoTR Orange) and (C) mass (MitoTr Green) were assessed and quantified as MFI by flow cytometry. Metabolic landscape of NK cells was assessed using the SeaHorse platform. MitoStress Test was performed by the induction of mitochondrial perturbation using the sequential treatment with oligomycin (Oligo, 1 mM), FCCP (2 mM), and rotenone (Rotenone A + Antimycin, 0.5 mM). The representative energetic map was constructed using (D) OCR (Oxygen Consumption Rate) and (E) ECAR (Extracellular Acidification Rate) values registered during basal (before any perturbation) and stressed (after FCCP injection) conditions. (F) ECAR and (G) OCR was measured in real time by metabolic flux analysis; bars show the calculated basal and maximal (F) glycolysis and (H) respiration. (I) ATP-production coupled respiration and (J) spare respiratory capacity (SRC) were also calculated.

2.3 | Flow cytometry analyses and functional assays

Cells were stained with the following mAbs: CD335 (NKp46); NKG2D; CD16; CD107a; CD57 and PD-1 (Miltenyi); CD45; NKG2D; DNAM-1; CD57; CD96 and TIGIT (Biolegend); NKp44; NKp46; NKp30; CD56; CD14; CD19 and CD3 (Beckman Coulter). All samples were analyzed on BD-Celesta (BD) and CytoFlex LX Flow Cytometer (Beckman Coulter). Data analysis was done using FlowJo software (TreeStar Inc.) and CytExpert (Beckman Coulter). For mitochondrial studies, cells were incubated with 50 nM MitoTracker Green and/or 25 nM MitoTracker Orange for 30 min at 37°C before staining

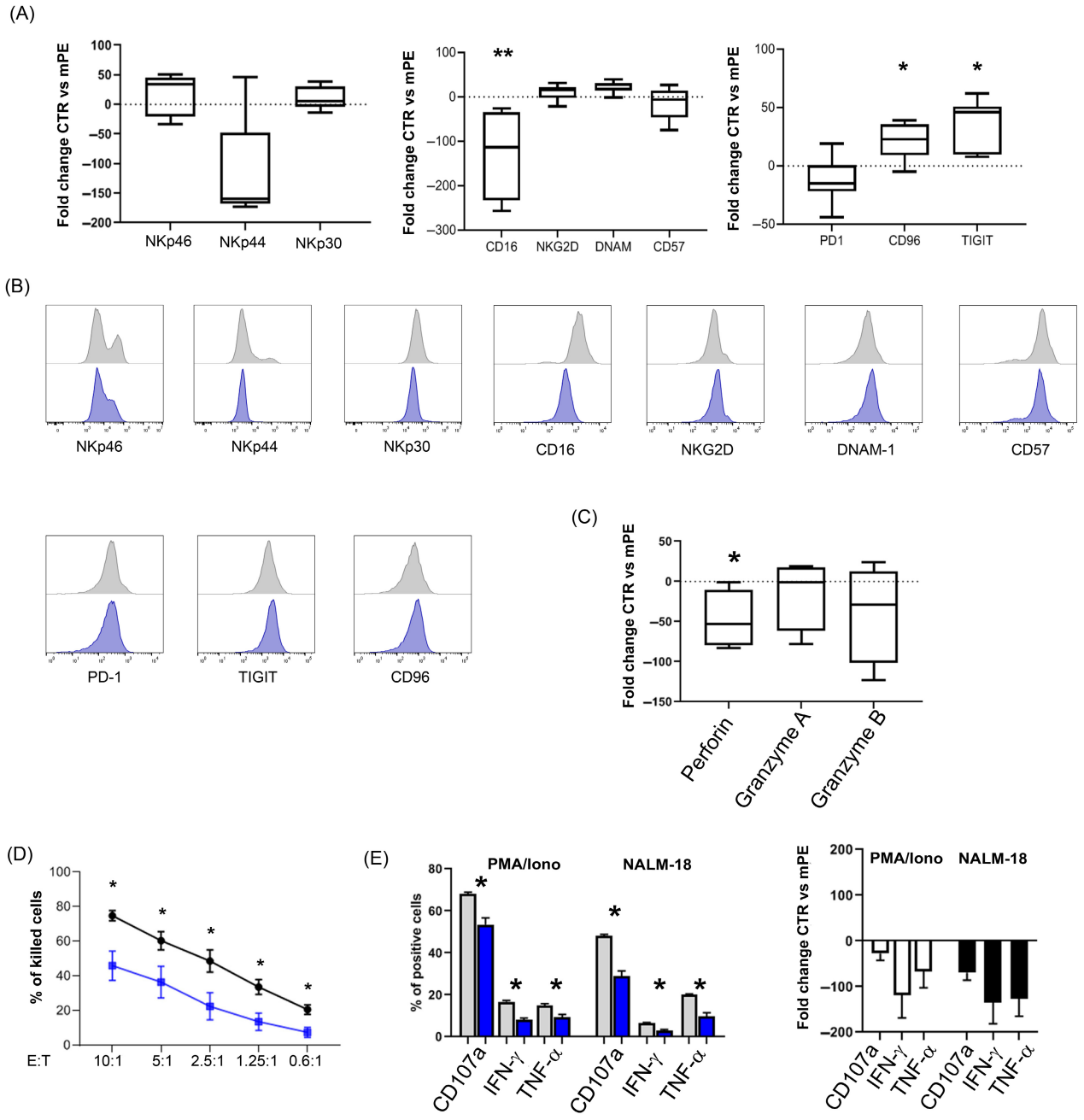

FIGURE 2 TME affects the functional features of NK cells. (A-C) Expression of the indicated markers evaluated by flow cytometry. (A) Fold change of the MFI of the indicated markers on HD-NK cells cultured alone vs HD-NK cells culture in the presence of mPE (n = 4-9) (B) Flow cytometry histograms of the indicated markers evaluated on HD-NK cells cultured alone (gray histograms) and on HD-NK cells culture in the presence of mPE (blue histograms). One representative experiments out of 9 performed. (C) Fold change of the MFI of the cytolytic granules in HD-NK cells cultured alone vs HD-NK cells culture in the presence of mPE (n = 6). (D, E) HD-NK cells that were shortly activated (72 hours) with IL15 and subsequently cultured for additional 3 days in the presence or in the absence of different mPE. (D) Cytolytic activity of HD-NK cells cultured in the presence (blue line) or in the absence (black line) of different mPE (n = 7). Mean of ^{51}Cr release ± SEM using A549 as target cells. The Effector:Target (E:T) ratios are indicated. (E) Percentage of positive cells and fold change (HD-NK cells (gray bars) cultured alone vs HD-NK cells culture in the presence of mPE (blue bars)) of degranulation (CD107a expression) and cytokine production (IFN-γ and TNF-α) in response to PMA and ionomicine stimuli or to tumor target cells (NALM-18) evaluated by flow cytometry (n = 4).

(Life Technologies). Intracellular staining was performed using FIX/PERM intracellular KIT (Miltenyi) after the manufacture instructions, and then stained with: IFN-γ (Miltenyi), TNF-α (eBioscience), granzyme A and B (BD) and perforin (Ancell). All panels were stained with LIVE/DEAD fixable Aqua stain (Invitrogen) or Ghost Dyes (TONBO) before surface or intracellular staining. UltraComp eBeads were used for all compensation. To perform cytotoxicity using the ^{51}Cr-release assay, NK cells cultured with different stimuli, were

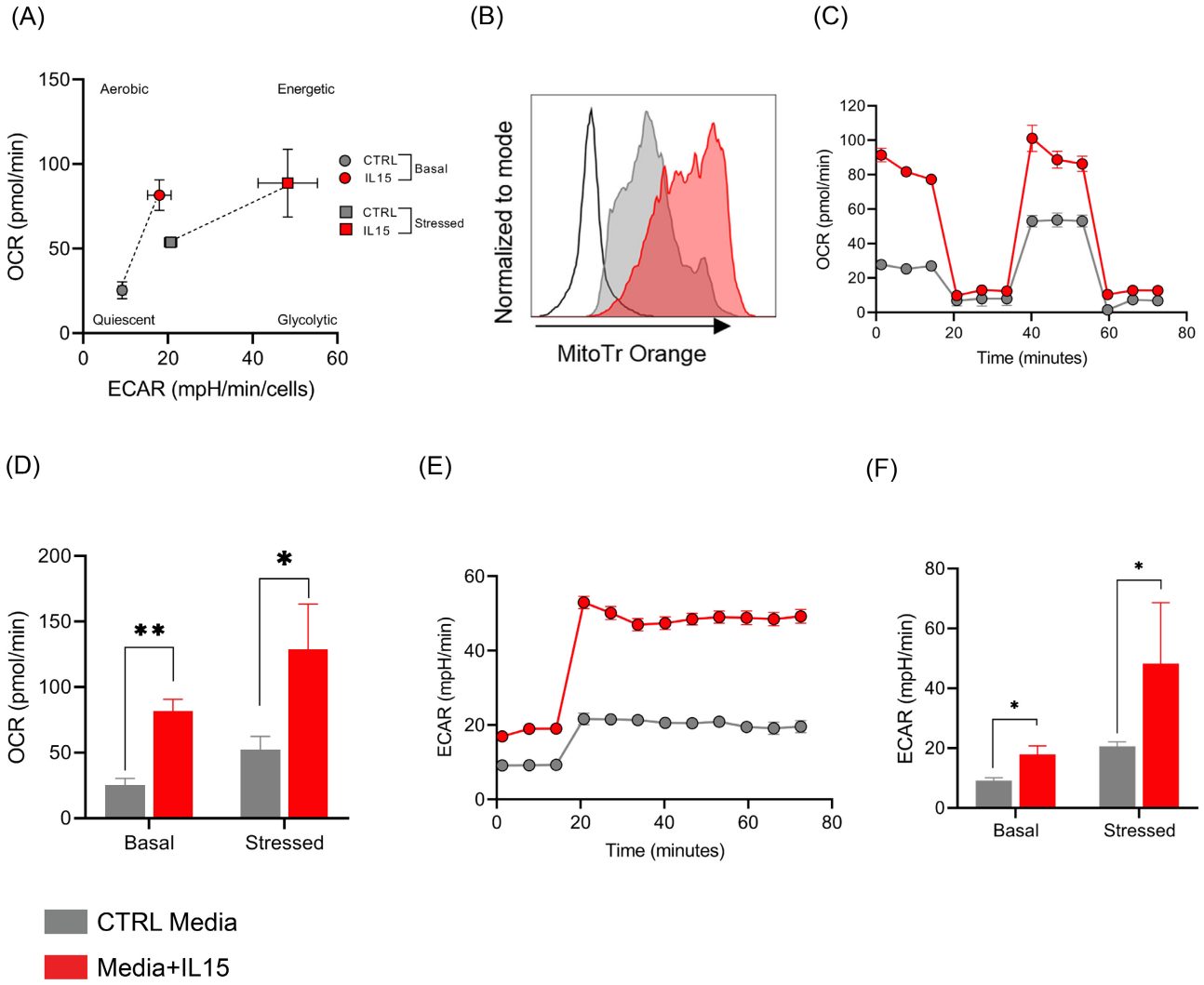

FIGURE 3 IL15 induces metabolic NK cell activation. Untreated NK cells were kept in culture in the presence or absence of IL15 and analyzed after 72 hours. Results are shown as representative (A) energetic map, (B) MitoTr Orange MFI quantification, (C) OCR flux analysis, (D) quantification under basal and stressed conditions, (E) ECAR real time flux, and (F) quantification under basal and stressed conditions (n = 4).

cocultured for 4 hours with ^{51}Cr-labeled A549 (RRID:CVCL_0023) adenocarcinoma target cell line at different E:T ratios in complete medium. Proliferation assays were performed using freshly isolated HD-NK cells labeled with CFSE (Invitrogen) after the manufacture instructions and cultured with different concentrations of IL15 (from 20 ng/mL to 1 ng/mL), and different proportions of mPE (from 90% to 10%). After 6 days, NK cell proliferation was analyzed by flow cytometry for CFSE stain intensity.

2.4 | Statistical analyses

Statistical analyses were performed with GraphPad Prism 8 software (La Jolla, CA). Differences between two groups were calculated by unpaired, two-tailed Student's *t*-test. Unless noted in the figure legend, all data are shown as mean SEM. For the statistical analysis were used: in Figures 1B,C,F,H,J, 2E, 3D,F and 4A-C unpaired Student's *t* test; Figures 2D and 4G RM one-way ANOVA Sidak's multiple comparisons test; Figure S1C Ordinary One-way ANOVA; Figure 2A,C, Figures S1D and S3A Wilcoxon matched-pairs test. We considered significant *P*-values of less than .05 (*), less than .01 (**), less than .001 (***), or less than .0001 (****). Where not indicated, data were not statistically significant.

3 | RESULTS

Our previous data showed that NK cells isolated from mPE of lung cancer patients display an impaired ability to kill tumor cells.[1,8,12,13] After exposure to mPE the metabolic status and bioenergetic profile of healthy donors (HD) NK cells was analyzed and matched with their activation/exhaustion phenotype and function (Figure 1A). First, NK cells were treated with different proportions (from 90% to 10%) of mPE and the proliferation rate of NK cells was analyzed. Since we did

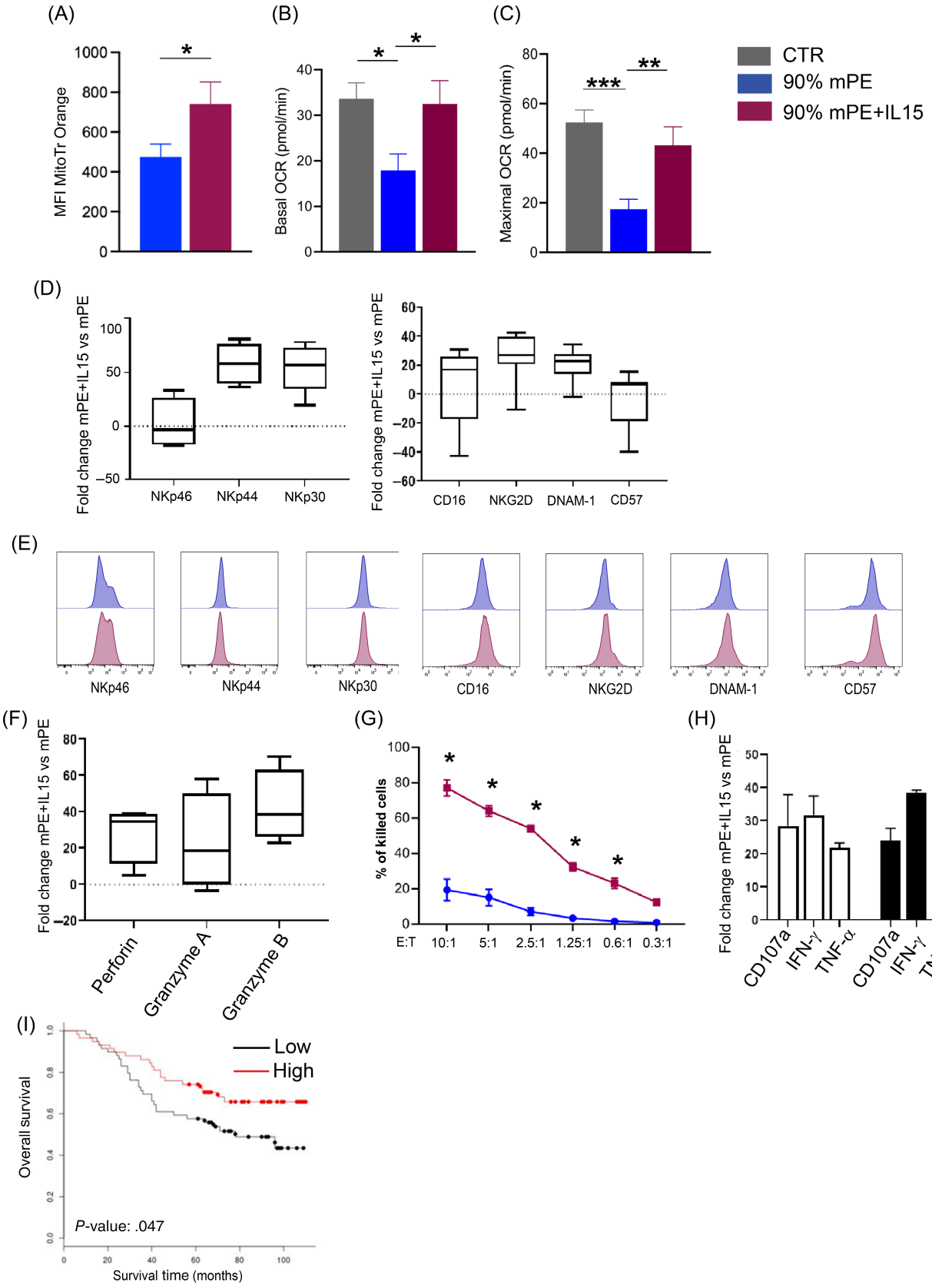

FIGURE 4 Legend on next page.

not observe substantial differences using different percentages of mPE (Figure S1A), we have chosen 90% of mPE to better mimic the unfavorable conditions of the TME. Subsequently, we analyzed the status of mitochondria and we observed that NK cells treated with mPE displayed a severe mitochondrial impairment as revealed by a decrease both in the mitochondrial functionality (MitoTracker Orange CMTMRos staining; Figure 1B) and mass (MitoTracker Green; FM; Figure 1C). These results were not due to NK cell death caused by mPE conditioned media, as shown by the similar viability detected in CTR and mPE-treated cells (Figure S1B). Regardless of similar viability frequencies, the mitochondrial functionality was significantly decreased by mPE treatment in a dose-dependent manner (Figure S1C).

Interestingly, the metabolic flux analysis revealed that NK cells, regardless of their exposure to mPE, have a similar glycolytic metabolic profile; both under basal and stressed conditions (Figure 1D-F). However, mPE-treated NK cells failed to effectively engage the OXPHOS pathway in response to stressed energetic conditions (Figure 1D,G-H), an obligate step to sustain their viability and their effector functions within a hostile TME. Indeed, mPE-NK cells displayed a significantly lower maximal OXPHOS (Figure 1H) as compared to control NK cells, along with a reduced rate of ATP coupled with mitochondrial respiration (Figure 1I). Accordingly, Spare Respiratory Capacity (SRC) of mPE-NK cells was negatively affected, thus reinforcing the idea that TME causes a remarkable disablement of mitochondrial function and fitness in NK cells (Figure 1J). Next, we sought to determine whether the TME-induced mitochondrial defects had an impact on NK cell function. We show that mPE-treated NK cells acquired features of functional exhaustion, as indicated by down-regulation of major triggering NK receptors such as NKp44 and CD16, a major triggering NK receptors, and upregulation of CD96 and TIGIT inhibitory receptors (Figure 2A,B and Figure S1D). In addition, the cytolytic granules, containing perforin, Granzymes A and B, were also modulated in the presence of mPE (Figure 2C). Considering these results, we evaluated the functional capability of NK cells conditioned with mPE. The modulation of activating and inhibitory receptors and the decrease of cytolytic granules can explain the impairment of NK cytotoxicity (Figure 2D), degranulation (CD107a) and cytokines release (IFN-γ and TNF-α, Figure 2E). All together, these results support the notion that mPE can prevent NK activity by impairing their mitochondrial function.

In view of the major role of IL15 in NK cell proliferation and effector function,[1] we further investigated the possible effect of IL15 on NK metabolism. First, we established the optimal concentrations of IL15 able to sustain NK cell viability, proliferation and effector function. Thus, NK cell absolute numbers, viability, and proliferation were analyzed upon culture with different concentrations of IL15 (from 20 ng/mL to 1 ng/mL). As show in the Figure S2A-B, we obtained similar results in the presence of 20, 10, and 5 ng/mL of IL15. At 1 ng/mL a slight reduction of cell proliferation and an increase of cell death were detected. Based on these results and on previous data[11] in the subsequent experiments 20 ng/mL of IL15 were used. Notably, we found that IL15 was able to metabolically reprogram NK cells which resulted in an enforcement of their antitumor activity. Indeed, NK cells expanded ex-vivo in the presence of IL15 exhibited a potent metabolic burst, which was associated to a higher bioenergetic profile (Figure 3A). In particular, IL15-treated NK cells exhibited a sustained increase in mitochondrial membrane's potential (Figure 3B), suggesting a highly improved NK metabolic fitness, which, in turn, results in a selective advantage in the metabolically hostile TME. Accordingly, IL15-treated NK cells exhibited a significant increase of basal OXPHOS (Figure 3C,D) and ECAR (Figure 3E,F) both in basal and after disruption of mitochondrial complexes. Taken together, these data indicate a highly improved metabolic fitness in IL15-treated NK cells, which could explain their sustained effector function.[14,15] Based on these results we analyzed whether IL15 could also overcome and rescue the inhibitory effect of TME on NK cells. Thus, we cultured HD-NK cells with IL15 in media containing different concentrations of mPE. The proliferation capability was not significantly affected even at the highest proportions of mPE (Figure S2C). These data, together with those shown in Figure S1A, confirmed the possibility of using 90% mPE in our experimental setting. NK cells exposed to mPE in the presence of IL15 were empowered with higher mitochondrial function (Figure 4A), which was accompanied by a higher bioenergetic profile under basal or stressed conditions. Indeed, by testing concomitantly the metabolic profiles of NK cells kept in culture with mPE or mPE + IL15, we found

FIGURE 4 IL15 prevents TME inhibitory effect. (A) mPE-treated NK cells were also cultured with IL15 supplement. Mitochondrial membrane potential was assessed by flow cytometry and quantified as MFI. (B, C) Metabolic landscape of HD-NK cells cultured in the presence or absence of IL15 and treated with mPE for 72 hours was assessed using the MitoStress test on the SeaHorse platform. Oxygen Consumption Rate (OCR) was measured and plotted as mean ± SEM from 10 different mPE tested on 4 different donors (2 independent experiments). Graphs show the (B) OCR values in basal condition, and (C) upon mitochondrial disruption. (D) Fold change of the MFI of the indicated markers evaluated by flow cytometry on HD-NK cells cultured with mPE in the presence or absence of IL15 for 72 hours (n = 9). (E) Flow cytometry histograms of the indicated markers evaluated on HD-NK cells cultured in the presence (purple histograms) or absence (blue histograms) of IL15 and treated with mPE for 72 hours. One representative experiments out of 9 performed. (F) Fold change of the MFI of the cytolytic granules in the presence or absence of IL15 and treated with mPE for 72 hours (n = 4). (G) Cytolytic activity of HD-NK cells cultured with mPE supplemented (purple line) or not (blue line) with IL15 (n = 4). Mean of ^{51}Cr release ± SEM using A549 as target cells. The Effector:Target (E:T) ratios are indicated. (H) Fold change (HD-NK cells cultured with mPE vs HD-NK cells culture with mPE supplemented with IL15) (n = 4) of degranulation (CD107a expression) and cytokine production (IFN-γ and TNF-α) in response to PMA and ionomicine stimuli (white bars) or to tumor target cells (NALM-18) (black bars) evaluated by flow cytometry (n = 4). (I) Overall survival of lung adenocarcinoma patients according to the level of IL-15 gene expression. High (red) and low (black) line indicate the level of gene expression using median cut-off modus. Dataset used for the analysis and *P* value is indicated (*$P < .05$).

that IL15 can rescue the mPE-induced metabolic dysfunction and induces an OCR profile similar to that of control cells (Figure 4B,C). In turn, the improved metabolic fitness, correlates with an increased expression of activating NK receptors (NKp44, NKp30, CD16, NKG2D, and DNAM-1) (Figure 4D,E and Figure S3A). Also cytolytic granules were present in higher amounts in NK cells cultured with mPE + IL15 in comparison with those cultured in mPE alone (Figure 4F). In addition, the presence of IL15, in agreement with the data above counteracted the inhibitory effect and induced an increase of cytotoxic activity, cytokine release, and degranulation (Figure 4G,H). Notably, also the viability and the proliferation rate were reverted by IL15 in mPE-NK cell cultures (Figure S3B-C). Interestingly, the proportion of $CD56^{bright}$ and $CD56^{dim}$ NK cells was not influenced by the presence of different stimuli (Figure S3D). Remarkably, the analysis of a dataset including 117 tumor samples of lung adenocarcinomas independent cohorts[16] confirms a better overall survival in the presence of high intratumoral IL15 expression (Figure 4I). In all, these data highlight the importance of IL15 as a tool to improve NK-based immunotherapy by reprogramming the NK metabolic functionality.

4 | DISCUSSION

Many studies provided evidence of the potent inhibitory effect of TME on immune cells.[4] Our present study indicates that mPE, a suitable and accessible TME surrogate, has a detrimental impact on NK cells affecting both their mitochondrial integrity and antitumor effector function. In particular, we highlight the importance of the metabolic interactions between TME and NK cells in shaping NK cell antitumor activity. We show that the mitochondrial dysfunction experienced by TME-exposed NK cells could impact on their bioenergetic profile, identifying metabolic disruption as one of the key mechanisms driving NK cell exhaustion. IL15 is known as a potent immunostimulating cytokine promoting both proliferation and cytolytic activity of NK cells.[17,18] However, limited information existed on its effect on the metabolic function of these cells. Our present data show that IL15-induced cytotoxic activity of NK cells[1] is paralleled by restored mitochondrial function, which, in turn, limits mPE-induced immunosuppression. These data offer a clue for therapeutic interventions in which IL15 could be exploited to reprogram NK cell metabolism and to increase the efficacy of NK-based tumor immunotherapy.

In conclusion, our data may be of clinical relevance as they reveal that defective mitochondrial metabolism may represent a common key driver of NK cell exhaustion in different tumors. This knowledge will help to design new NK-cell reprogramming strategies to increase their antitumor efficacy. This may allow the establishment of new NK cell-based immunotherapies which could be applied to a wider range of currently refractory pediatric and adult tumors.

AUTHOR CONTRIBUTIONS

The work reported in the article has been performed by the authors, unless clearly specified in the text. Designed experiments: Nicola Tumino, Paola Vacca, Teresa Manzo; performed the experiments: Silvia Tiberti, Carina B. Nava Lauson, Francesca Besi, Piera Filomena Fiore; analyzed the data: Silvia Tiberti, Carina B. Nava Lauson, Nicola Tumino, Paola Vacca, Stefania Martini; interpreted the results: Nicola Tumino, Paola Vacca, Teresa Manzo; wrote the article: Nicola Tumino, Paola Vacca, Teresa Manzo; provided samples from the patients: Francesca Scodamaglia; provided intellectual input and critically revised the article: Lorenzo Moretta, Maria Cristina Mingari.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding authors upon reasonable request.

ETHICS STATEMENT

Our study was approved by ASL3, Genova, Italy ethics board (N9-13, 2013), the Regione Liguria Ethics Board (Ethics Board ID: 4975, 2020) and by IRCCS Bambino Gesù Children's Hospital, Rome (ethics board prot. 899-2018). All patients gave written informed consent in accordance with the Declaration of Helsinki.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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