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BRIEF REPORT

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# The anti-inflammatory cytokine IL-37 improves the NK cell-mediated anti-tumor response

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**ABSTRACT**

IL-37 is a member of the IL-1 superfamily exerting anti-inflammatory functions in a number of diseases. Extracellular IL-37 triggers the inhibitory receptor IL-1R8 that is known to regulate different NK cell pathways and functional activities including their anti-tumor effect. However, the effect of IL-37 on human NK cell functions is still to be unveiled. This study aimed to investigate the functional effect of IL-37 in human NK cells activated with IL-15. We found that IL-37 enhanced both NK cell cytotoxic activity against different tumor cell lines and cytokines production. These effects were associated with increased phosphorylation of ERK and NF-Kb. The improved NK cell activity was also strictly related to a time-dependent GSK3β-mediated degradation of IL-1R8. The enhanced activation profile of IL-37 treated NK cells possibly due to IL-1R8 degradation was confirmed by the results with IL-1R8-silenced NK cells. Lastly, in line with these data, through the analysis of the TNM plot database of a large group of patients, IL-37 mRNA expression was found to be significantly lower in colon and skin cancers than in normal tissues. Colon adenocarcinoma and neuroblastoma patients with higher IL-37 mRNA levels had significantly higher overall survival, suggesting that the presence of IL-37 might be considered an independent positive prognostic factor for this tumor. Our results provide novel information on the mechanisms regulating IL-1R8 function in human NK cells, highlighting the IL-37-IL-1R8 axis as a potential new target to improve the anti-tumor immune response.

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## Introduction

IL-37 is a cytokine belonging to the IL-1 family, which displays anti-inflammatory properties. Similar to other IL-1 family components, IL-37 is present as a precursor, which can be activated by caspase-1 cleavage into its mature forms.[1]

Five spliced variants have been identified including IL-37b, which is the most studied functional isoform.[2–4] IL-37 expression has been documented in natural killer (NK) cells, monocytes, skin keratinocytes, epithelial cells, and on tissues including lymph nodes, thymus, lung, colon, uterus, and bone marrow.[2]

Recently, it has been shown that IL-37 may suppress the progression of cervical cancer,[5] fibrosarcoma,[6] hepatocellular carcinoma,[7] lung cancer,[8,9] renal cell carcinoma,[10] and breast cancer.[11]

The effect of IL-37 in tumors has been associated with an increased recruitment of immune cells, including $CD57^+$ mature NK cells.[7] and dendritic cells (DC),[12] inhibition of the M2 polarization of tumor-associated macrophages,[13] and suppression of angiogenesis.[14] and of cancer cell migration.[15] Once produced by intratumoral regulatory T cells (Tregs), IL-37 has been reported to downregulate both the expression and function of the activating receptor TIM-3 on canonical NK cells thus, inhibiting their anti-tumor functions.[16]

IL-37 functions through intracellular and extracellular mechanisms. Intracellular IL-37 binds to cytoplasmic SMAD-3 and translocates into the nucleus where it suppresses the transcription of pro-inflammatory cytokine genes, including IL-6, IL-1α, IL-1β, and TNF–α. On the other hand, extracellular IL-37 forms a complex with IL18Rα and IL-1R8 on the cell surface and activates multiple signaling pathways.[2,3]

IL-1R8 is a highly conserved member of the IL-1 superfamily receptors displaying a single Ig domain, a Toll/interleukin-1 receptor (TIR) region, and a unique long tail differentiating this receptor from both IL-1 R and TIR domain-containing molecules.[17] Indeed, once IL-1 R or Toll-like receptor (TLR) ligands bind to their receptors, TIR domains dimerize, recruit TIR-containing adaptor proteins, such as MyD88, and activate downstream signaling molecules including tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated protein kinases (IRAKs). Subsequent activation of transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB, p65) and Mitogen-activated protein kinases (MAPKs), results in the triggering of molecules of innate immunity, potentiating inflammatory responses. In this context, IL-1R8 prevents the formation of the TIR-containing adaptor protein complex, thus negatively modulating the activation of such intracellular pathways.[17]

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Initial studies on the function of extracellular IL-37 showed that this molecule is able to interact with both IL-1R8 and IL-18 Rα. Of note, this binding was required for the transduction of the anti-inflammatory signal of IL-37.[18] Suppression of the immune response due to IL-1R8/IL-37 interaction has been shown to occur through inhibition of NF-kB activation and chemokine secretion.[19]

In our previous work, we evaluated the IL-1R8 impact on human NK cell functions demonstrating that it acts as a negative regulator of multiple cellular pathways including cytotoxic activity against different tumor cell targets.[20]

This study aimed to investigate the activity of exogenous IL-37 on human NK cells *via* IL-1R8. We demonstrated that the treatment of human NK cells with IL-15 in combination with IL-37 increases NK cell cytotoxicity and leads to the activation of different signaling pathways. Interestingly, in IL-37 (plus IL-15)-treated NK cells, we have shown the reduction of IL-1R8 protein levels, suggesting that IL-37 could upregulate NK cell function through a feedback loop controlling the IL-1R8 expression.

Characterization of the IL-37-IL-1R8 axis and its functional consequences might be considered a new strategy to promote NK cell antitumor functions.

## Materials and methods

### Human blood donors

Buffy coats were collected from volunteer blood donors (HD) after obtaining informed consent at the blood transfusion service of IRCCS Bambino Gesù Children's Hospital of Rome. The study was approved by the Ethical Committee (2058_OPBG_2020) and conducted according to the tenants of the declaration of Helsinki.

### Human cells isolation, electroporation, and culture

Human NK cells (≥95% pure) were isolated from HD PBMCs as described in.[20] NK cell purity was assessed by incubation with CD-56-PeCy7 and CD3-ECD (Beckman Coulter) antibodies. Cells were activated in the presence of IL-15 (20 ng/ml) (Miltenyi Biotech, Bergisch Gladbach, Germany) with or without IL-37 (100 ng/ml) (R&D Systems, Bio-Techne, Minneapolis, MI, USA) at 37°C for 18 h.

Electroporation with mock and IL-1R8 siRNA of freshly-isolated NK cells was performed as previously described.[20] Cells were cultured with IL-15 (2 ng/ml) (Miltenyi Biotech) at 37°C for 48 hours and then activated with IL-15 (20 ng/ml) in the presence or not of IL-37 (100 ng/ml) (R&D Systems).

For cytotoxicity assays, the following human cell lines were used: K562 (chronic myelogenous leukemia), IMR-32 (human neuroblastoma), HT-29, SW480, and COLO-205 (colorectal Cancer Cell). K562 and COLO-205 were cultured in RPMI 1640 (Euroclone, Milan, Italy) while IMR-32, HT-29, and SW480 in Dulbecco's Modified Eagle Medium high glucose (Euroclone). The following supplements were added to both culture media: 2 mM l-glutamine (Euroclone), 1% penicillin – streptomycin–neomycin mixture (Euroclone), and 10% heat-inactivated fetal bovine serum (Euroclone).

For GSK-3β inhibition experiments, NK cells were cultured with IL-15 or IL-15+ IL-37 with DMSO or TWS119 (20 μM, Merck) for 18 h and then further processed for western blot analysis.

### NK cell activation, cytotoxicity, and cytokine assessment

NK cell activation was evaluated after staining of IL-15 or IL-15 + IL-37-treated NK cells (CD56+ CD3-) with CD69 FITC (Miltenyi Biotec, Clone FN50) for 30 min at 4°C using Cytoflex S flow cytometer (Beckman Coulter). Dead cells were excluded as DAPI (Sigma-Aldrich) positive. Data analysis was performed using Kaluza software 2.1 (Beckman Coulter, Brea, California, USA).

Cytotoxicity was assessed by incubating previously activated NK cells (IL-1R8-silenced, mock, or resting NK cells) with different cell targets as previously described.[20] Briefly, NK cells were incubated at 37°C for 4 h with the tumor target cell lines previously stained with 5 μM Cell Tracker Green (CMFDA, Invitrogen, Thermo Fisher Scientific) at effector target ratios ranging from 5:1 to 0.25:1. Specific lysis was evaluated after adding propidium iodide to NK cell-tumor target cocultures. Flow cytometric analysis was performed by calculating dead target cells (Td, propidium iodide positive) cultured with effector cells – Td of target cells cultured without effector cells.

Release of IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) in cell supernatants, was measured, following NK cell activation and 4 h incubation with K562 using commercially available ELISA kits (DuoSet ELISA, R&D systems) and Milliplex Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel HCYTA-60K-PX38 (Millipore, Darmstadt, Germany) respectively, according to the manufacturer's instructions.

### Quantitative PCR (qRT-PCR)

mRNA isolation, reverse transcription to cDNA, and Quantitative PCR (qRT-PCR) were performed as previously described.[20]

### Western blotting (WB)

For WB analysis, NK cell lysates were processed as described by.[21] and stained with the following antibodies: α-IL-1R8 (Abcam, Cambridge, UK), α-P ERK (Cell Signaling Technologies, CST, Danvers, Massachusetts, USA), α- ERK (CST), α-PGSK3β (CST)- α-GSK3 (CST), α-P NF-kB (p65, CST), α-NF-kB (p65, CST) and α-Vinculin (Sigma-Aldrich, St. Louis, MO, USA).

### Immunofluorescence and confocal microscopy

Activated NK cells were incubated with PBS-bovine serum albumin (BSA) 5% goat serum 5% for 30 min at room temperature followed by staining with the α-IL-1R8 antibody overnight at 4°C 1:100 (Thermo-Fisher Scientific, Waltham, MA, USA/Abcam) in 1% PBS: BSA. After washes in PBS, cells were incubated with α-rabbit Alexa Fluor 555 1:500 (Thermo-Fisher Scientific) for 1 h at RT.

After washes with PBS, cells were resuspended in a fixation buffer for 30 min at 4°C. After rehydration in PBS, nuclei were stained with Hoechst 33,342 1:10,000 (Thermo-Fisher Scientific) for 5 min at room temperature. After washing, the cells were distributed dropwise onto positive-charged microscope slides (Super Frost Plus, Menzel-Glaser, Germany) and dried at 37°C. Slides were mounted with PBS: glycerol 1:1. Negative controls were performed using 1% PBS/BSA without the primary antibody, to verify specific staining.

Confocal microscopy was performed on a Leica TCS-SP8X laser-scanning confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a tunable white light laser (WLL) source, 405 nm diode laser, 3 Internal Spectral Detector Channels (PMT) and 2 Internal Spectral Detector Channels (HyD) GaAsP. Sequential confocal images were acquired using an HC PL APO 63× oil-immersion objective (1.40 numerical aperture, NA, Leica Microsystems) with a 1024 × 1024 format, zoom factor 2×, scan speed 400 Hz.

Z-series images were obtained through the collection of serial confocal sections at 0.27 µm intervals and a factor zoom of 7.2 × .

The mean fluorescence intensity was measured by MetaMorph software (Molecular Devices). Tables of images were processed using Adobe Photoshop CS4 software (Adobe Systems Inc., San Jose, CA, USA).

### *Analysis of IL-37 gene expression in colon cancer patients from public repositories*

The violin plots in Figure 4a, showing IL-37 expression in colon, skin cancer, and healthy tissues were generated by interrogating the integrated database of gene chip data on TNMplot.com web tool.[22] Kaplan-Meier curves in Figure 4b, epresenting the overall survival test on colon cancer and neuroblastoma patients, were built by the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) on the colon adenocarcinoma dataset TCGA – 174 - agg4502a073 and Neuroblastoma – SEQC – 498 - custom – ag44kcwolf. The Kaplan Scan feature was used to divide the samples of a dataset into two groups based on the IL-37 gene expression, with a cutoff based on the highest p-value calculated with a log-rank test.

### Statistical analysis

Data were analyzed by GraphPad Prism V.6.0 software (La Jolla, California, USA).

### *Results and discussion*

Previous studies showed that IL-1R8 is highly expressed in NK cells.[20,23] and can negatively regulate their function.[20,23] Of note, in mice, IL-1R8 deficiency has been associated with enhanced NK cell maturation, increased numbers in peripheral blood (PB), and an activated phenotype. In line with these data, in humans, IL-1R8 silencing was shown to increase NK cell cytotoxicity and cytokine secretion.[20,23]

While in human NK cells, IL-1R8 has been well characterized, the actual function of its ligand, IL-37, remains unknown. Therefore, we investigated the effect of IL-1R8/IL-37 interaction on human NK cell function in the presence of IL-15, the main NK cell activating cytokine. Considering the negative effect of IL-37 on the immune response, we first evaluated whether IL-37 could impair the NK cell-mediated cytotoxicity. To this end, NK cells were cultured with IL-15 or IL-15 plus IL-37 for 18 h and then analyzed for their cytolytic activity against different targets. As shown in Figure 1a, upon treatment with IL-37, we found a significant increase in NK cell cytotoxic activity against different tumors including colon carcinoma cell lines (HT-29, SW480, and COLO-205), IMR-32 cells (Neuroblastoma, NB) and the leukemia cell line K562, compared to IL-15-treated NK cells. Among these targets, the SW480 cells were more sensitive to NK cell-mediated cytotoxic activity as compared to COLO-205 and IMR-32 (Figure 1a). In line with their enhanced killing activity, the combination of IL-15 plus IL-37 resulted in a significant increase in surface CD69 (Figure 1b) and higher production of IFN-γ and GM-CSF compared with NK cells cultured with IL-15 alone (Figure 1c,d). Thus, in the presence of IL-15, IL-37 upregulates NK cell functions. In contrast, no effects on NK cell-mediated cytotoxicity and cytokine production were detected upon stimulation at longer time points or in the presence of IL-37 alone (data not shown).

It has been shown that IL-1R8 engagement by IL-37 results in inhibition of phosphorylation of downstream signaling molecules, including MAPKs and NF-kB.[17] Thus, we investigated whether such phosphorylation events could occur following NK cell stimulation with IL-37. As shown in Figure 2a, in line with the improvement of cytotoxic activity, phosphorylation of both ERK1/2 and NF-kB p65 (at Thr202/Tyr204 and Ser536 residues, respectively) was increased in the samples treated with IL-15/IL-37 but not in controls (IL-15 alone). These data demonstrate that the treatment of human NK cells with IL-37, in combination with IL-15, for 18 h, leads to upregulation of signaling cascade molecules involved in NK cell effector function. Recent data have shown that IL-37/IL-1R8 interaction, in lung epithelial cells, promotes glycogen-synthase-kinase-3β (GSK3β) activation that, in turn, leads to IL-1R8 destabilization and degradation.[24] Thus, we investigated whether similar events could occur in IL-37/IL-15-stimulated NK cells. For this purpose, we examined the IL-1R8 membrane expression levels on NK cells stimulated either with IL-15 and IL-37 or with IL-15 alone by confocal microscopy. As reported in Figure 2b, the IL-1R8 fluorescence intensity decreased upon IL-37 treatment, indicating that, indeed, a reduction of IL-1R8 membrane localization also occurs in NK cells. In agreement with these data, we found that IL-1R8 protein levels were significantly reduced upon NK cell treatment with IL-37 plus IL-15 for 18 h, indicating that an IL-1R8 degradation may indeed occur (Figure 2c). Of note, no difference in IL-1R8 mRNA levels could be detected in both IL-15 and IL-15/IL-37 conditions. These data further confirm that the observed reduction of IL-1R8 protein was not due to the decrease of mRNA transcription but likely reflected protein degradation (Figure S1A).

Different evidences support the involvement of GSK3β in the degradation of several proteins,[25,26] including IL-1R8.[24] GSK3β is a multifunctional protein whose phosphorylation at Tyr216 and Ser9 residues positively or negatively regulates its kinase activity, respectively.[27] Interestingly, it has been shown that IL-37 promotes GSK3β phosphorylation on Tyr216 residue inducing its activation and GSK3β-dependent IL-1R8

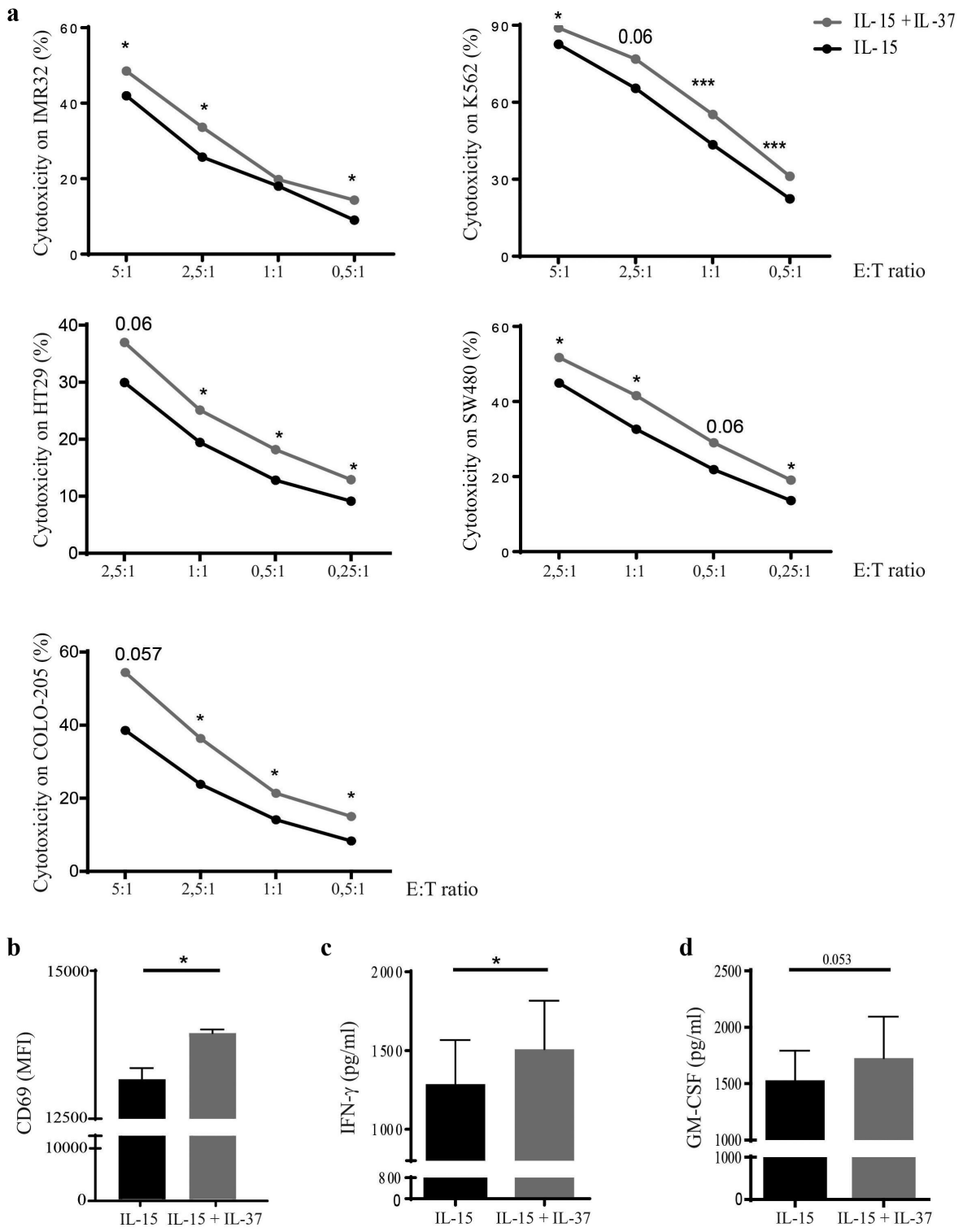


**Figure 1.** Effects of IL-37 on NK cell-effector functions. (a) Cytotoxic activity of IL-15- (black circles) or IL-15 plus IL-37-treated (gray circles) NK cells following co-culture with the indicated cancer cell lines at different effector (E): target (T) ratios. Data are plotted as percent of cytotoxicity of independent experiments performed on at least four HD. (b) CD69 expression of IL-15- (black column) or IL-15 plus IL-37-treated (gray column) NK cells for 18 h. Data are plotted as mean fluorescent intensity (MFI) of three independent experiments performed on live NK Cells (56+ DAPI -). (c-d) Interferon γ (IFN-γ) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) production of IL-15- (black column) or IL-15 plus IL-37-treated (gray column) NK cells following co-culture with K562 cells at 0.5:1 E:T ratios. Mean values (pg/ml) ± SEM are reported. Statistical analysis has been performed using paired t-test. *$p < 0.05$, ***$p < 0.005$.

degradation.[24] Therefore, we investigated whether phosphorylation of the Tyr216 residue of GSK3β would take place in IL-37-stimulated NK cells. As shown in Figure 2d, an increase of GSK3β phosphorylation at residue Tyr 216 occurred upon IL-37 treatment, suggesting that the observed IL-1R8 degradation could be related to GSK3β activation. In order to determine the impact of GSK3β on IL-37-mediated effects, we incubated IL-15 or IL-15 plus IL-37-stimulated NK cells with TWS119, a specific inhibitor of GSK3β. As shown in Figure 2e, increased levels of IL-1R8 were observed in IL-15+IL-37-stimulated NK

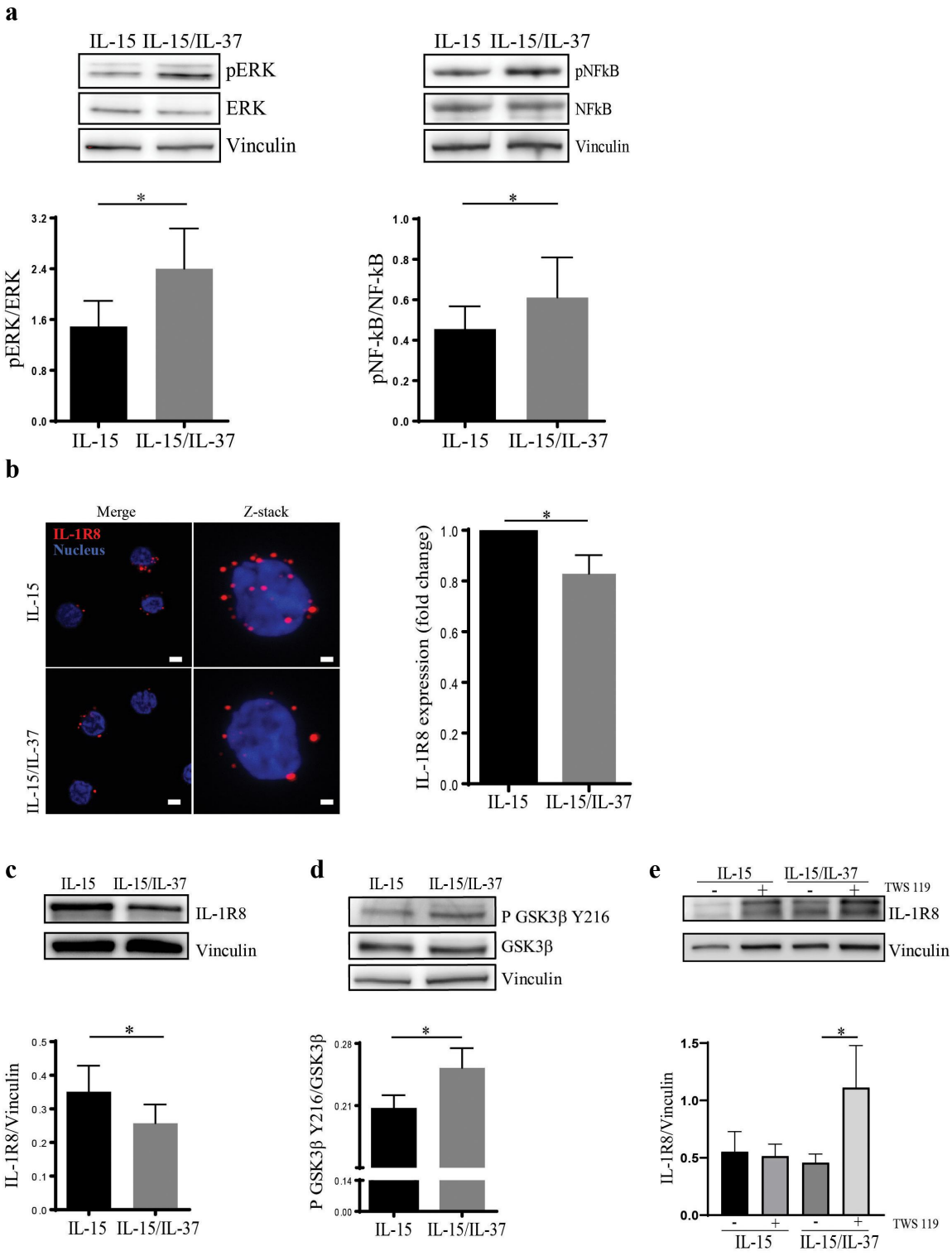


**Figure 2.** Effects of IL-37 on IL-1R8 expression and GSK3β phosphorylation. (a) Western Blot analysis and quantification of phosphorylated and total ERK (left panel) and NF-kB (p65, right panel) of IL-15- or IL-15 plus IL-37- treated NK cells for 18 h. Representative blots from one out of four independent experiments (upper panel). The results have been normalized over vinculin. Mean values ± SEM are reported. Statistical analysis has been performed using paired t-test and ratio paired t-test. *$p < 0.05$ (b) Confocal microscopy Imaging of IL-1R8 expression of IL-15- or IL-15 plus IL-37- treated NK cells for 18 h. A representative image is reported from seven independent experiments (left panel). Blue: nuclei (Hoechst 33,342); red: IL1R8. Scale bars 2.5 µm. IL-1R8 protein quantification is reported in the right panel. Mean values (fold changes) ± SEM are reported. Statistical analysis has been performed using ratio paired t-tests. *$p < 0.05$ (c) Western Blot analysis of IL-1R8 in IL-15- or IL-15 plus IL-37-treated NK cells for 18 h. A representative blot is reported from four independent experiments (upper panel). IL-1R8 protein quantification is reported in the lower panel. The results have been normalized over vinculin. Values are mean ± SEM. Statistical analysis has been performed using paired t-tests. *$p < 0.05$ (d) Western Blot analysis of phosphorylated and total GSK 3β in IL-15- or IL-15 plus IL-37- treated NK cells. A representative blot is reported from four independent experiments (upper panel). GSK3β protein quantification is reported in the lower panel. The results have been normalized over vinculin. Mean values (pg/ml) ± SEM are reported. Statistical analysis has been performed using paired t test. *$p < 0.05$ (e) Western Blot analysis of IL-1R8 in IL-15- or IL-15 plus IL-37-treated NK cells with TWS119 (20 µM) or DMSO for 18 h. IL-1R8 protein quantification is reported in the lower panel. The results have been normalized over vinculin. Values are mean ± SEM. Statistical analysis has been performed using ratio paired t-tests. *$p < 0.05$

cells upon treatment with TWS119 compared to control NK cells (IL-15+IL-37 DMSO).

These data indicate that, instead of promoting an inhibitory signal, IL-37 (in combination with IL-15) controls IL-1R8 degradation, through GSK3β activity downregulation, thus contributing to the upregulation of NK cell activation and the increase of anti-tumor activity.

In order to confirm that the increased NK cell activity upon IL-37 stimulation was mainly related to the modulation of IL-1R8 protein levels, a further analysis was performed by transfecting human NK cells with small interfering RNA (siRNA) specifically targeting IL-1R8 (Figure 3a). Indeed, consistent with the results reported in Figure 1a, IL-15 plus IL-37-stimulated mock (transfected with control siRNA) NK cells showed an increase in the

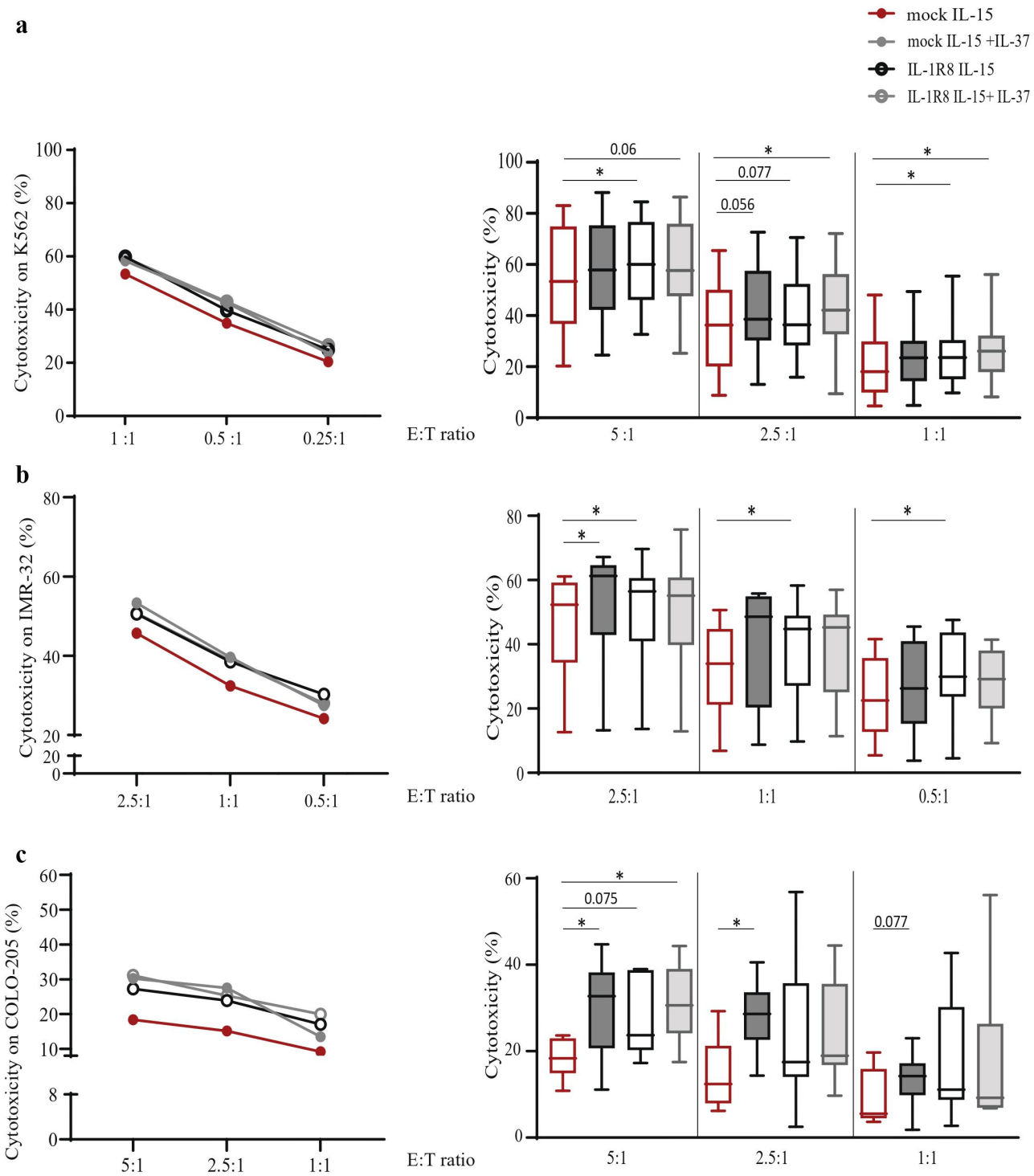


**Figure 3.** Effects of IL-37 on IL-1R8 silenced NK cells cytotoxicity. (a–c) Cytotoxic activity of IL-15- (black circles), IL-15 plus IL-37-treated (gray open circles) IL-1R8-silenced NK cells and IL-15- (red circles), IL-15 plus IL-37-treated (gray circles) mock NK cells following co-culture with K562 (a), IMR-32 (b) and COLO-205 (c) cells at different effector (E): target (T) ratios. Data are plotted as a percentage of cytotoxicity of independent experiments performed on at least four HD. Statistical analysis has been performed using paired t-test. *$p < 0.05$

killing of K562 (Figure 3a), IMR-32 (Figure 3b) and COLO-205 (Figure 3c) as compared to IL-15- treated mock NK cells. In agreement with our previous study,[20] IL-1R8-silenced NK cells were more cytotoxic as compared to mock NK cells. A similar increase in cytotoxicity was reported in IL-1R8-silenced NK cells upon stimulation (IL-15 plus IL-37) compared to IL-15-treated mock transfected NK cells whereas no differences were detected in IL-1R8-silenced NK cells regardless of cytokine stimulation. These data demonstrate that IL-37-stimulated and IL-1R8-silenced NK cells, both display enhanced cytotoxicity toward different cell lines further confirming that blocking the IL-1R8 inhibitory axis, through its degradation, improved NK cell effector functions.

In an attempt to provide additional information of potential clinical interest, we further verified IL-37 gene expression in colon adenocarcinoma and skin cancer from 1450 to 253 patients, respectively, by using a TNMplot database.

Indeed, in agreement with Yan X et al.,[28] IL-37 gene expression was significantly ($p = 4.39E{-}03$) lower in colon carcinoma than in healthy tissue (Figure 4a). A similar, although much striking decrease in IL-37 gene expression ($p = 7E{-}53$) was reported in skin cancer patients compared to skin samples of healthy individuals (Figure 4a). IL-37 reduction in cancer tissues compared to healthy ones is in line with the positive role played by IL-37 in promoting NK cell-dependent anti-tumor response. Furthermore, the Kaplan–Meier analysis using public databases also demonstrated that colon adenocarcinoma and neuroblastoma patients with high IL-37 gene expression have higher overall survival (OS) than those with low IL-37

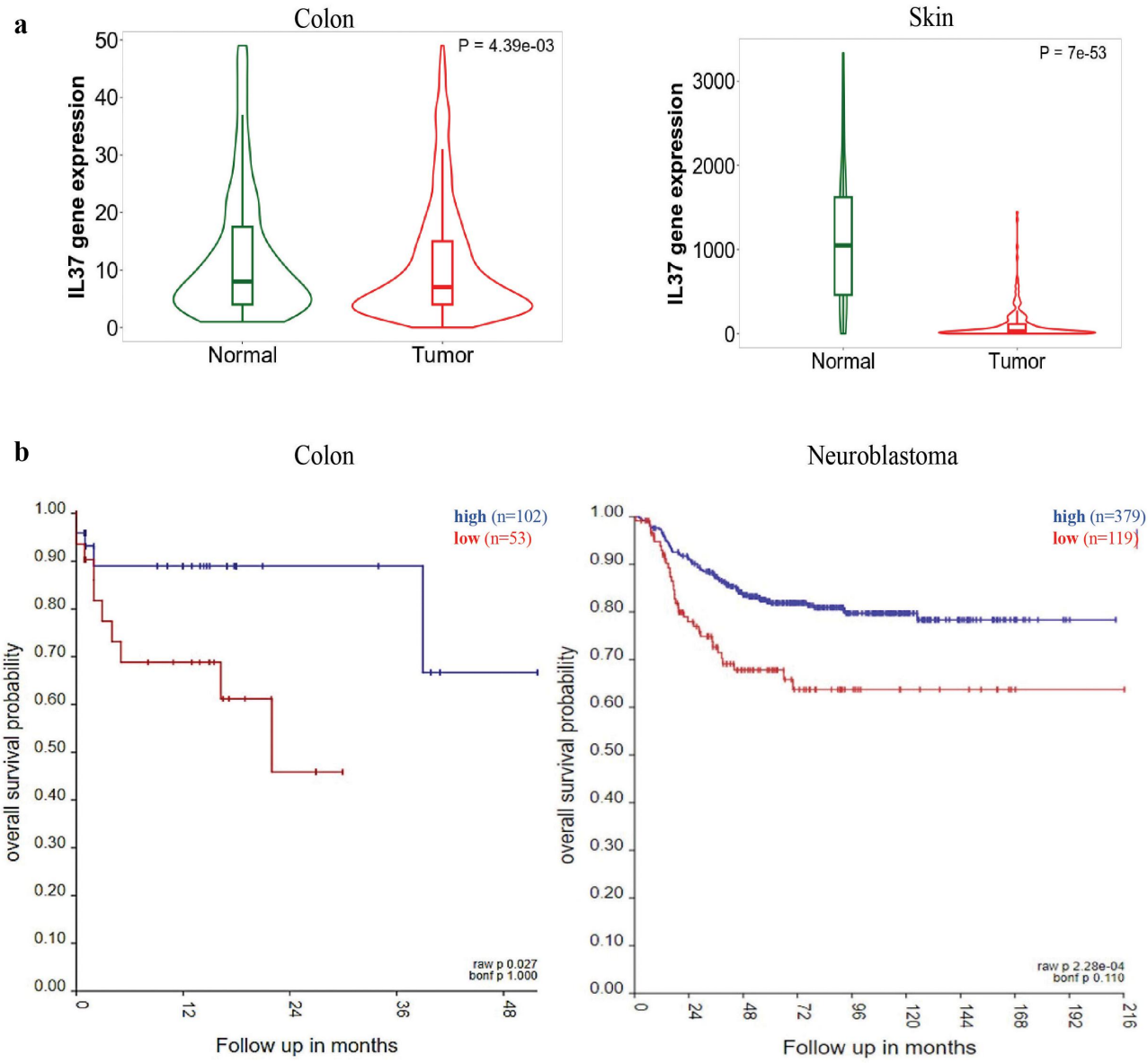


**Figure 4.** IL-37 gene expression on cancer patients and overall survival. (a) Differential gene expression analysis of IL-37 gene in colon cancer ($n = 1450$) and in healthy tissues ($n = 377$) (left panel) and in skin cancer ($n = 253$) and in healthy tissues ($n = 174$) (right panel) from gene chip public databases. Analyses were performed by the TNM plot web tool[22] Statistical analyses have been performed using the Mann-Whitney U test. $p = 4.39e{-}03$ (colon) and $p = 7E{-}53$ (skin) (b) Kaplan–Meier curve of overall survival (OS) in colon adenocarcinoma (left panel) and neuroblastoma (right panel) patients categorized on IL-37 gene expression. The analysis was generated by R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) using the dataset TCGA – 174 – custom – agg4502a073 ($n = 155$) ($n = 155$; high IL-37 $n = 102$; low IL-37 $n = 53$ raw p-value = 0.027) and tumor neuroblastoma – SEQC - 498 - custom – ag44kcwolf ($n = 498$; raw p-value = $2.28 \times 10^{-4}$).

gene expression (Figure 4b). These data suggest that, in these cancer types, the enhanced IL-37 gene expression might be considered a positive prognostic factor, associated with increased NK cells anti-tumor function.

Taken together, our data describe a new mechanism through which IL-37 regulates human NK cell function. Indeed, we have shown that stimulation of NK cells with IL-37 for 18 h, in the presence of IL-15, enhances both NK cell cytolytic function and release of IFN-γ and GM-CSF. However, longer exposure to IL-37 did not result in any improvement in NK cell activities (not shown).

Furthermore, in comparison with IL-15-treated NK cells, the additional stimulation with IL-37 promotes the activation of both NK cell MAPK and NF-KB signaling pathways. To better investigate the involvement of the IL-37/IL-1R8 axis, we evaluated the IL-1R8 expression following NK cell stimulation with IL-37 plus IL-15. We demonstrated that a reduction of IL-1R8 membrane localization occurs in IL-37-stimulated NK cells. In addition, we detected a reduction of IL-1R8 total protein levels only in IL-15- plus IL-37-stimulated samples suggesting that IL-37 treatment could modulate IL-1R8 expression. Importantly, IL-37-mediated regulation on IL-1R8 expression likely does not occur at the transcriptional level but rather at the protein level. In agreement with studies in lung epithelial cells, it is consequent to GSK3β phosphorylation which leads to IL-1R8 reduction. Indeed, inhibition of GSK3β restored IL-1R8 protein levels. IL-1R8/IL-37 pathway might, thus, function as a negative-feedback mechanism by which NK cells control their activities by downregulating inhibitory pathways.

In order to acquire further evidence of IL-1R8 in IL-37-mediated effects, IL-1R8-silenced NK cells were further stimulated with IL-37 (and IL-15). This treatment did not induce any further increase in cytotoxicity as compared to IL-1R8-silenced NK cells stimulated with IL-15 only. This may indicate that the addition of IL-37 in already IL-1R8-silenced NK cells cannot induce any additional downregulation of IL-1R8. Importantly, IL-37 gene expression was lower in colon carcinoma and skin cancer than in normal tissues.

Data on IL-37 gene expression would suggest to evaluate its expression profiles in other adult and pediatric cancers. While this study gives new insights into the IL-37/IL-1R8 signaling pathway in human NK cells, in the presence of IL-15, a cytokine present in the tumor microenvironment, it will be relevant to analyze the effect of other cytokines.

Despite further studies are required to better elucidate the precise mechanism(s) regulating the IL-37/IL-1R8 pathway, our data provide a rationale for IL-37 targeting in order to improve NK cell effector function in both adult and pediatric tumors.

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## Authors' contributions

Designed experiments NL, FRM EM; Performed the experiments NL, FRM, AP, VD, CA, TI; Analyzed the data NL, FRM, NL, AP, CA, VD; TI; Interpreted the results EM, NL, FRM, AP; Wrote and edited the manuscript NL, FRM, EM; Provided intellectual input and critically revised the manuscript LM, PV, EM. All authors read and approved the final manuscript.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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