

ARTICLE OPEN



Interleukin-15 enhanced the survival of human $\gamma\delta$ T cells by regulating the expression of Mcl-1 in neuroblastoma

Hui Wang^{1,2}, Xiaolin Wang^{1,2}, Wei Wang^{1,2}, Wenjia Chai^{1,2}, Wenqi Song³, Hui Zhang^{1,2}, Wenjun Mou^{1,2}, Mengmiao Pei^{1,2}, Yan Su⁴, Xiaoli Ma⁴ and Jingang Gui^{1,2}

© The Author(s) 2022

Neuroblastoma (NB) is the most common extracranial solid tumor and the treatment efficacy of high-risk NB is unsatisfactory. $\gamma\delta$ T-cell-based adoptive cell transfer is a promising approach for high-risk NB treatment. Our previous study has revealed that $\gamma\delta$ T cells in NB patients exhibit a poor proliferation activity and a decreased anti-tumor capacity in vitro. In the present study, we found that IL-15 could effectively enhance the proliferation of NB $\gamma\delta$ T cells, to a level that remains lower than healthy controls though. In addition, IL-15-fostered NB $\gamma\delta$ T cells robustly boosted cell survival against apoptosis induced by cytokines depletion. Our data revealed that Mcl-1 was a key anti-apoptotic protein in IL-15-fostered $\gamma\delta$ T cells during cytokine withdrawal and its expression was regulated via the activation of STAT5 and ERK. In addition, IL-2 and IL-15-fostered $\gamma\delta$ T cells harbored higher levels of tumoricidal capacity which is also beneficial for $\gamma\delta$ T-cell based immune therapy in NB. Understanding the survival control of $\gamma\delta$ T cells in a sub-optimal cytokine supportive microenvironment will expedite the clinical application of $\gamma\delta$ T cells for immunotherapy.

Cell Death Discovery (2022)8:139; <https://doi.org/10.1038/s41420-022-00942-5>

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor that accounts for 8% of childhood cancers [1]. At present, ~40–50% of NB patients are diagnosed with high-risk NB with an overall survival rate <50% owing to high recurrence and metastasis, which cannot be curtailed by traditional surgery and chemo/radiotherapy [2–4]. Novel strategies such as immunotherapy are eager to be developed for tackling the devastating NB in children.

Adoptive transfer of $\gamma\delta$ T cells holds great promise for immunotherapy [5], thanks to their strong anti-tumor activity, readily expansion, and independence of major histocompatibility complex molecules as opposed to $\alpha\beta$ T cells. It has been documented that in vitro expanded $\gamma\delta$ T cells showed clinically relevant cytotoxicity to NB cells [6]. However, the production of $\gamma\delta$ T cells stimulated with IL-2 in vivo was shown to concurrently promote the generation of regulatory T cells, potentially inhibiting immune surveillance for cancer cells [7]. Our previous study also demonstrated that in vitro expanded NB-derived $\gamma\delta$ T cells featured with a reduced proliferation capacity compared with expanded $\gamma\delta$ T cells from healthy controls (HC) [8]. Previous research revealed that IL-15 could promote cell proliferation, the anti-tumor function of $\gamma\delta$ T cells, and enhance the response of $\gamma\delta$ T cells to microbial pathogens [9]. Therefore, it is valuable to test whether IL-15 can rectify and promote subduced NB-derived $\gamma\delta$ T cells to regain better survivability with a reinforced proliferation potential competent for autogenic $\gamma\delta$ T adoptive transfer.

In the clinical setting, adoptive transfer therapy of $\gamma\delta$ T cells requires harvesting enough cells for engraftment, and sustaining cell survival and cytotoxicity lasting long enough for tumor eradication [10]. Although supraphysiological levels of cytokines were added in vitro culture to support $\gamma\delta$ T-cell expansion, cytokines with a level out of scope were not appropriate to administrate directly to patients due to potentially severe side effects [11]. In vitro expanded $\gamma\delta$ T cells prepared in a condition supplemented with high concentrations of IL-2 and IL-15 would abruptly dive into a survive niche with a physiological level of cytokines in the recipient host far below the culture ones upon adoptive transfer. The drastic environmental change in vivo could affect the survival and function of these well-behaved immunocompetent cells prepared in vitro. For instance, the proliferation of NK cells displayed a dose-dependent addiction to IL-15 and the sudden withdrawal of IL-15 increased the apoptosis with altered expression of Bcl-2 family proteins [12, 13]. The stronger survival and cytolytic activity were observed in IL-15-primed NK cells attributed to a STAT5-dependent upregulation of Bcl-2 [14]. Anti-apoptotic Bcl-2 family members including Bcl-2 and Mcl-1 express in different cells and play critical roles in regulating their life and death through the intrinsic death pathway [15]. Mcl-1, a short half-life anti-apoptotic protein, undergoes rapid upregulation and stabilization in response to cytokines or antigen signaling. IL-15, as a pro-survival cytokine, regulates the expression of Bcl-2 family members to maintain the survival of T cells [12]. However, it is

¹Laboratory of Tumor Immunology, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China. ²Key Laboratory of Major Diseases in Children, Ministry of Education, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China. ³Department of Clinical Laboratory Center, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China. ⁴Medical Oncology Department, Pediatric Oncology Center, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing Key Laboratory of Pediatric Hematology Oncology, Key Laboratory of Major Diseases in Children, Ministry of Education, Beijing 100045, China.

✉email: mxl1123@vip.sina.com; guijingang@bch.com.cn

Received: 25 October 2021 Revised: 20 February 2022 Accepted: 8 March 2022

Published online: 29 March 2022

unclear if the survival of $\gamma\delta$ T cells is regulated by IL-15 via the expression of Bcl-2 family proteins, and what is the destiny of expanded IL-15-primed $\gamma\delta$ T cells in a system devoid of cytokines.

In the present study, we aimed to enhance the survival of $\gamma\delta$ T cells for adoptive cell transfer therapy by optimizing the previous in vitro cell-expanding protocol and further explore the maintenance of $\gamma\delta$ T-cell survival in NB patients receiving immunotherapy. We found that IL-15 effectively promoted the proliferation of $\gamma\delta$ T cells and robustly increased apoptotic resistance when cytokines were suddenly depleted. We fathomed the underlined factors involved in the cell protection and found it was Mcl-1 but not Bcl-2 that promoted the cell longevity after IL-15 withdrawal, a process mimicking the abrupt change of cytokine levels when in vitro expanded cells are adoptively transferred to patients. In addition, the level of Mcl-1 was regulated by the activation of STAT5 and ERK in IL-15-fostered $\gamma\delta$ T cells. Notably, regardless of patient- or HC-derived preparation, IL-15-fostered $\gamma\delta$ T cells co-cultured with NB cell line exhibited an increased cytolytic activity. Results from our study suggested that in vitro expanding NB patient-derived $\gamma\delta$ T cells requires IL-15 nourishment to transform into apoptosis-resistant immunocompetent cells optimized for autologous adoptive cell transfer therapy.

RESULTS

IL-15 robustly promoted $\gamma\delta$ T-cell proliferation during in vitro culture

We previously have known that $\gamma\delta$ T cells in NB patients presented with a reduced proliferation capacity and diminished cytotoxicity against tumor cells [8]. Considering the crucial role of IL-15 in the maintenance of long-lasting, high-avidity T-cell responses [16], our speculation was that the insufficiency of IL-15 concentration in patients was possibly one of the adverse factors for the survival and function of $\gamma\delta$ T cells. By ELISA method, we found the serum concentration of IL-15 slightly decreased in NB patients (Fig. 1A). This decrease was subsequently verified by quantitative PCR (qPCR) taking templates out of from patient blood and tumor tissues (Fig. 1B). Conversely, CD215, the alpha subunit of IL-15 receptor (IL-15R α), was found to express a higher level in NB tissues as well as in NB-derived $\gamma\delta$ T cells (Fig. 1C, D). In an in vitro setting, the expression of IL-15R α on HC $\gamma\delta$ T cells was much higher than it was on the patient $\gamma\delta$ T cells in a 3-day expanding culture with IL-2 only. Nevertheless, the attrition of IL-15R α in patient $\gamma\delta$ T cells triggered by the addition of IL-15 in culture after cell expanding was at a slower rate in a 24 h observation time window suggesting dull signaling transduction (Fig. 1E).

To further investigate the efficacy of IL-15 for fostering the proper proliferation and tumoricidal activity of in vitro expanded human $\gamma\delta$ T cells. We cultured the $\gamma\delta$ T cells from patients and healthy controls in the presence of stimulant PAM (pamidronate) supplemented with either IL-2, or IL-15, or both of them. Unstimulated $\gamma\delta$ T cells were not able to proliferate and were used as a negative control in this experiment (Fig. S1A). Following 4 days, 7 days, 9 days, and 14 days of culture, the percentage of $\gamma\delta$ T cells was determined by flow cytometry. Results showed a remarkable increase in the percentage of $\gamma\delta$ T cells along with the time of culture, but no differential percentages of $\gamma\delta$ T cells amongst the three culture conditions were observed in any of the time points we sampled (data not shown). To be noted, both NB- and HC-derived $\gamma\delta$ T cells achieved a much higher absolute cell number in the presence of IL-2 and IL-15 at the end of the 14-day expanding culture (Fig. 2B). Meanwhile, a greater fold change was also observed in IL-2 and IL-15 co-fostered $\gamma\delta$ T cells compared with IL-2 or IL-15 alone (Fig. 2C). Though IL-2 and IL-15 could robustly promote $\gamma\delta$ T-cell proliferation, NB-derived $\gamma\delta$ T cells exhibited a lower expansion capacity than HC with the IL-2 and IL-15 culture condition (Fig. 2B). On day 9, $\gamma\delta$ T cells from NB patients entered a fast-proliferating stage while it occurred as

early as the fourth day in the culture of HC-derived $\gamma\delta$ T cells. Furthermore, with IL-2 and IL-15 culture conditions, NB-derived $\gamma\delta$ T cells achieved $3.09 \pm 2.04 \times 10^6$ cells compared with $5.04 \pm 7.93 \times 10^6$ cells from HC-derived $\gamma\delta$ T cells at the end of 14-day culture. In other words, IL-15 together with IL-2 could effectively enhance the proliferation of NB-derived $\gamma\delta$ T cells, to a level remaining lower than that of HC (Fig. 2C).

IL-15-fostered $\gamma\delta$ T cells exhibited a stronger resistance to apoptosis after cytokine withdrawal

Under physiological conditions, IL-2 and IL-15 were at an extremely low level especially in NB patients (IL-2 0.44 ± 0.05 pg/ml in HC vs 0.27 ± 0.02 pg/ml in NB patients, $p < 0.05$; and IL-15 3.63 ± 0.6 pg/ml in HC vs 2.54 ± 0.39 pg/ml in NB patients, $p = 0.18$, respectively). To mimic physiological conditions, cytokines were withdrawn when $\gamma\delta$ T cells were successfully expanded, and cell apoptosis was measured by Annexin V expression at various time points within the additional 96 h incubation (Fig. 3A). As previously mentioned, cultures without stimulants could not proliferate and encountered massive apoptosis (~80% of Annexin V⁺ $\gamma\delta$ T cells), so did the cultures have stimulants but without any cytokine support. With PAM stimulation, no evident apoptosis was observed in Petri dishes supplemented with IL-2 or/and IL-15 on a 14-day cell-expanding culture (Fig. 3B). No matter they originated from healthy controls or patients, in the following 96 h culture without cytokines, $\gamma\delta$ T cells initialized apoptosis with an increment over culture time as detected by Annexin V staining (Fig. 3C–E). The divergence of cell survival protection in the cultures of different cytokine pre-conditioning became obvious at 72 h sampling time after cytokine withdrawal, while the difference was observed as early as 48 h in the culture of HC-derived $\gamma\delta$ T cells (Fig. 3C–E). Based on our data, the cell survival protection after cytokine withdrawal was closely relevant to IL-15, as $\gamma\delta$ T cells expanded from IL-15 or IL-15 plus IL-2 pre-conditioning perceptibly exhibited a decreased rate of cell death in a culture without further cytokine support (Fig. 3C–E). After 96 h cytokine-free culture time, ~60% of cells pre-conditioned only with IL-2 died ($66 \pm 5.08\%$ in HC and $60 \pm 4.57\%$ in NB patients). By contrast, only 30% of those cells pre-conditioned with IL-15 or IL-15 plus IL-2 underwent apoptosis ($30.5 \pm 4.97\%$ in HC and $24 \pm 5.64\%$ in NB patients) (Fig. 3D, E). To count the total viable cells in the Petri dishes after 96 h culture devoid of cytokine, we found starting from a cell seeding number 5×10^5 , cells pre-conditioned with IL-15 or IL-15 plus IL-2 outnumbered cells pre-conditioned only with IL-2. This trend was more obvious in the IL-15 plus IL-2 pre-conditioning group, suggesting a possible synergic effect from these two cytokines in cell protection during abrupt fluctuation of cytokine concentration (Fig. 3H).

Mitochondrial membrane potential (MMP) is a key indicator and initiator of cell apoptosis [17]. We used JC-1 dye to detect the loss of MMP 72 h post-cytokines withdrawal, at which massive apoptotic events occurred. As expected, JC-1 staining verified that $\gamma\delta$ T cells pre-conditioned with IL-2 showed a decreased aggregate to monomer ratio indicating that change in MPP was more obvious than in other cultures. Our JC-1 staining data revealed that IL-15 and IL-2 plus IL-15 fostered $\gamma\delta$ T cells exhibited a similar level of MPP change in cultures starting from both HC and NB Peripheral blood mononuclear cells (PBMCs) (Fig. 3F, G). To be noted, in any culture supplemented with IL-15 as pre-conditioning cytokine, a better survival rate was achieved in comparison with that supplemented with IL-2 only (Fig. 3C–H). These data revealed a more crucial role of IL-15 in protecting $\gamma\delta$ T cells from cell death during cytokines withdrawal. This underscores that IL-15 would be an indispensable factor for in vitro preparation of immunocompetent $\gamma\delta$ T cells with enduring survivability opt for immunotherapy, particularly for the autologous cell preparation where the fitness of starting cells is already an issue.

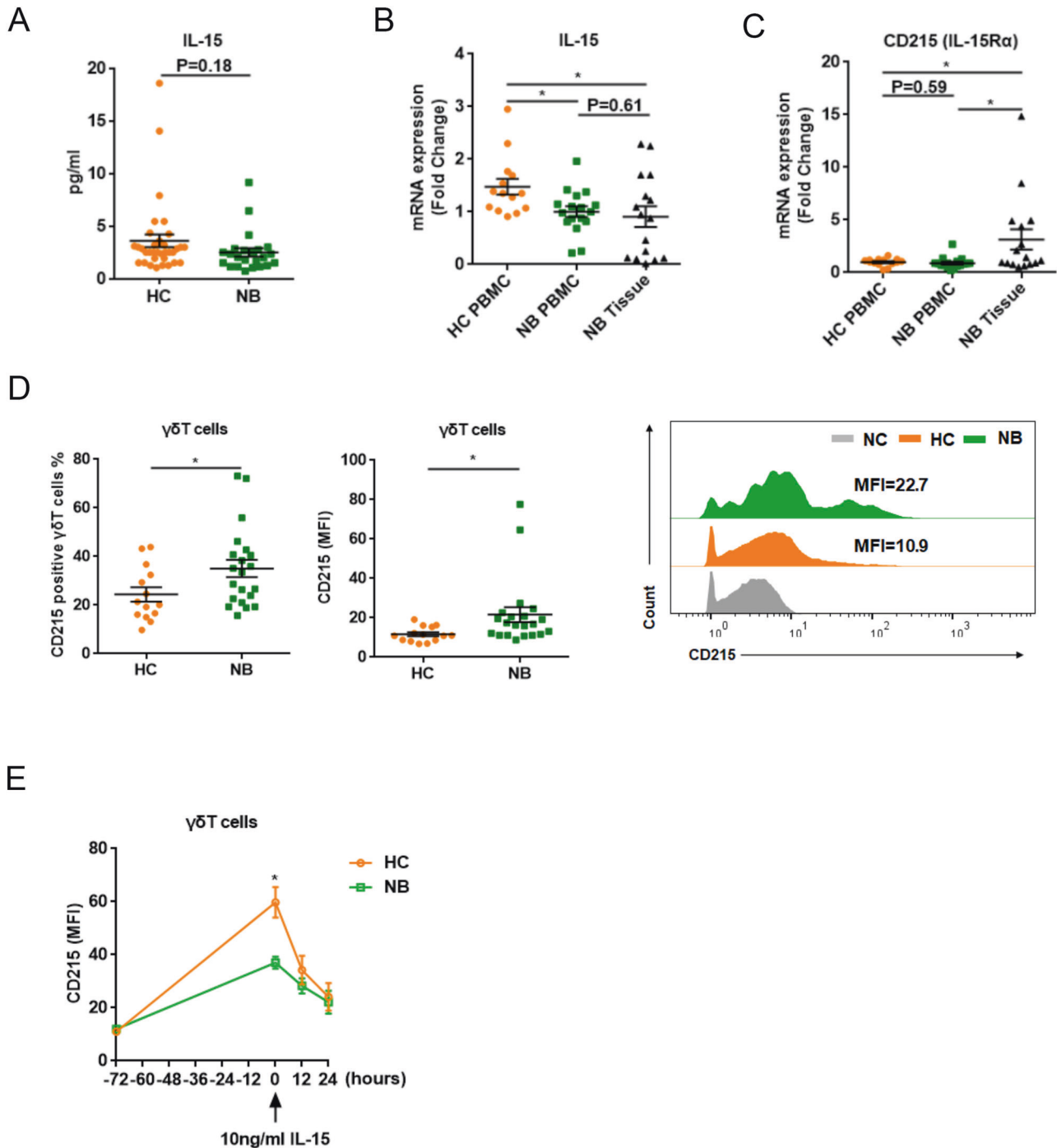


Fig. 1 Qualification of IL-15 and IL-15Ra in NB patients. **A** IL-15 levels in plasma were analyzed by ELISA (HC, $n = 34$; NB, $n = 23$). Data were mean \pm SEM and statistical analysis was performed using an unpaired t test. **B, C** The expression of IL-15 and CD215 (IL-15R α) were determined by qPCR (HC, $n = 14$; NB, $n = 33$, including 17 of NB PBMCs and 16 of NB tissues). Data were mean \pm SEM and statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$. **D** The expression of CD215 in $\gamma\delta$ T cells was analyzed by flow cytometry (HC, $n = 14$; NB, $n = 21$). Data were mean \pm SEM and statistical analysis was performed using an unpaired t test. *, $p < 0.05$. **E** The expression of CD215 in $\gamma\delta$ T cells was induced for 72 h and then IL-15 was added for another 24 h. The expression of CD215 was analyzed by flow cytometry at each time point. (HC, $n = 5$; NB, $n = 5$). Statistical analysis was performed using an unpaired t test. *, $p < 0.05$. NC negative control; HC healthy controls; NB neuroblastoma patients.

IL-15-fostered $\gamma\delta$ T cells possessed stronger survival capacity through retaining the expression of Mcl-1 but not Bcl-2

The homeostasis of T cells is strictly maintained by the up- and downregulation of Bcl-2 family members [18]. To clarify whether Bcl-2 family protein participates in the apoptosis resistance of IL-15-fostered $\gamma\delta$ T cells, we monitored the expression of Bcl-2 and

Mcl-1 in $\gamma\delta$ T cells in cultures devoid of pre-conditioning cytokines (Fig. 4A). At 72 h post cytokine removal, the protein expression of Mcl-1 but not Bcl-2 was largely retained in $\gamma\delta$ T cells from culture pre-conditioned with both IL-15 and IL-2, in contrast to the attrition of Mcl-1 expression observed in $\gamma\delta$ T cells in culture pre-conditioned with IL-2 only (Fig. 4B). The Mcl-1 expression was

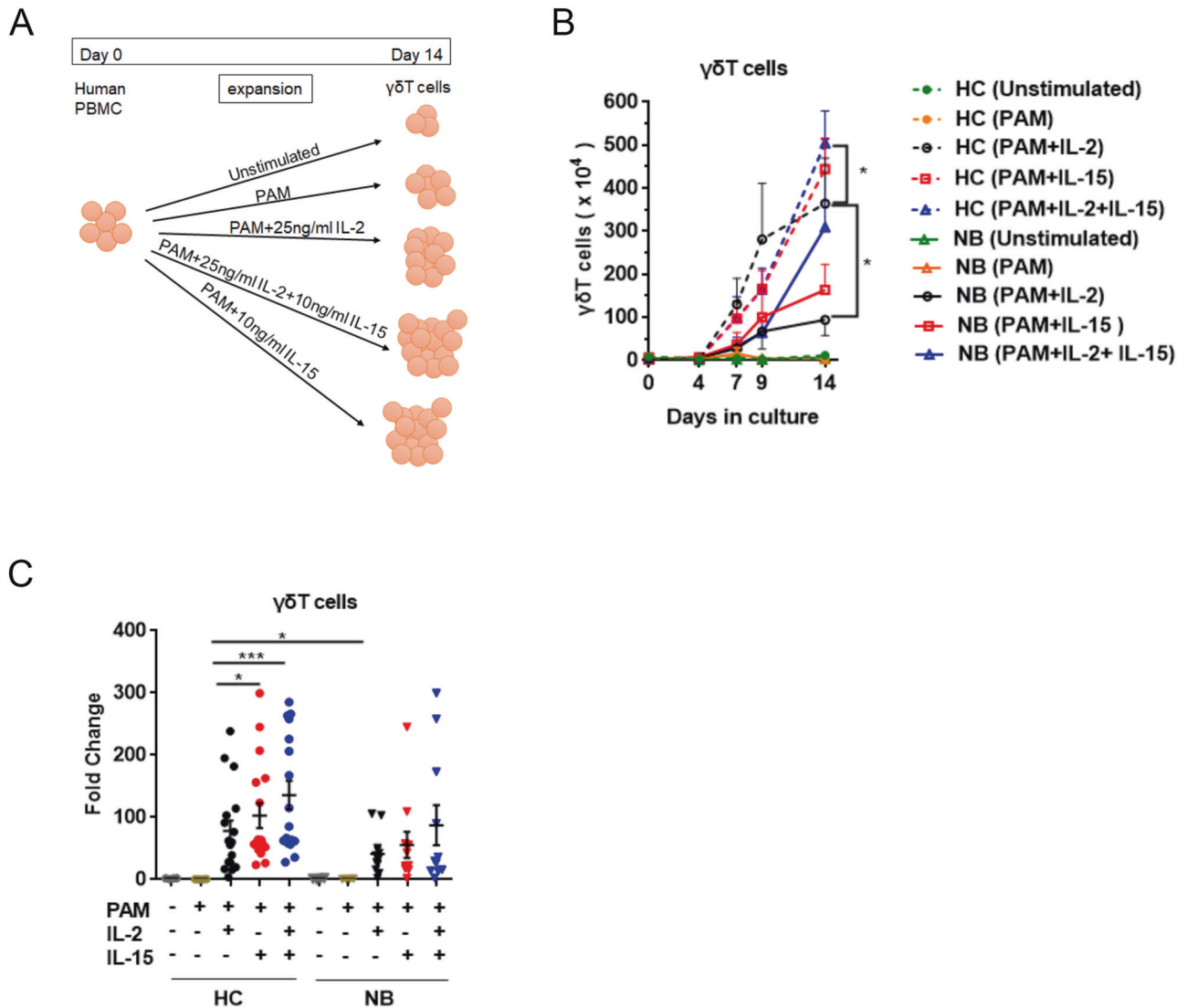


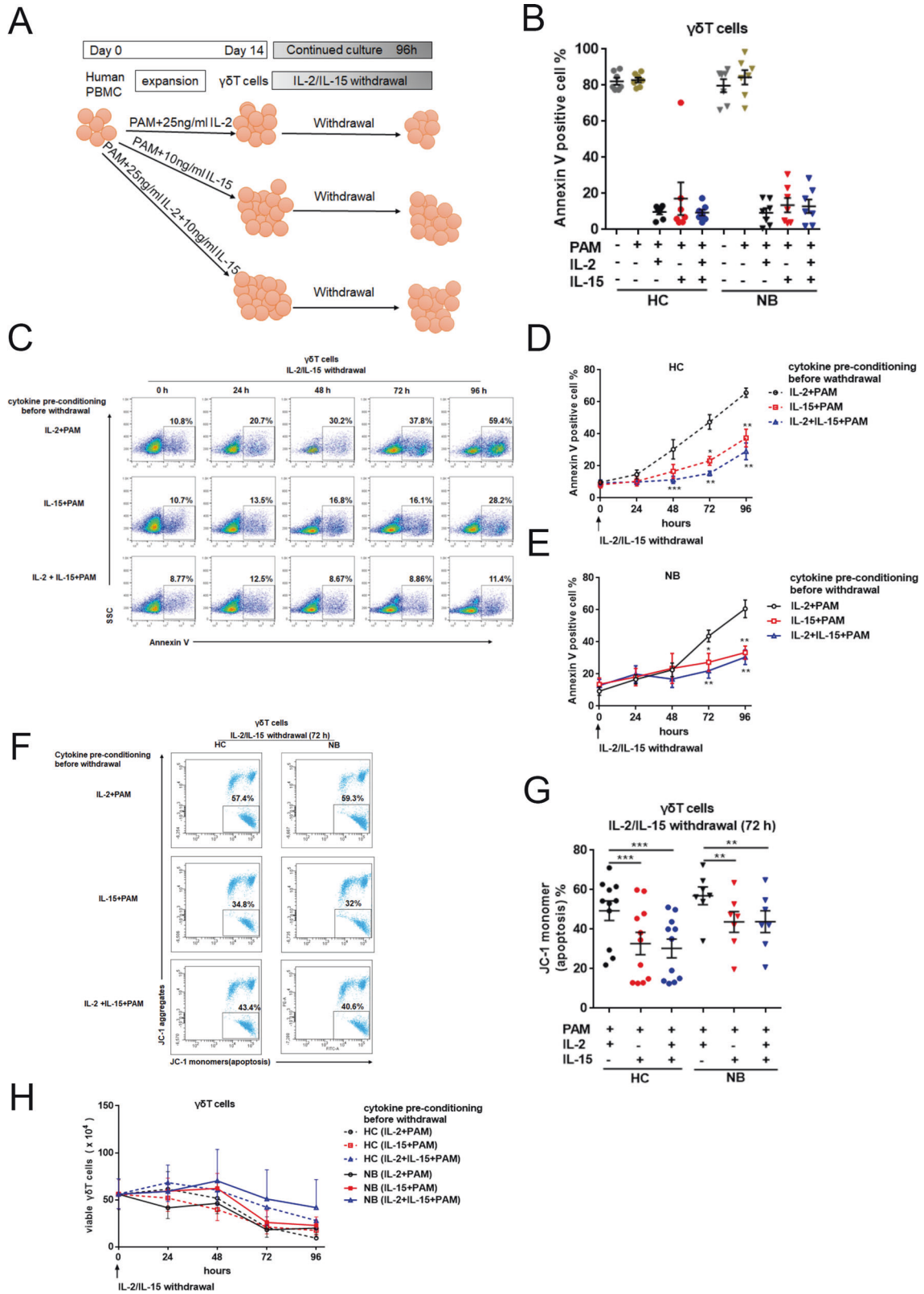
Fig. 2 Qualification of $\gamma\delta$ -cell expansion capacity in vitro culture conditions. $\gamma\delta$ T cells were stimulated with PAM supplemented with either IL-2 or IL-15 or both of them and expanded in vitro for 14 days. **A** Flowchart of the experimental setup to study in vitro $\gamma\delta$ T-cell expansion capacity with different culture conditions. **B** At culture days 0, 4, 7, 9, and 14, the absolute numbers of $\gamma\delta$ T cells from HC and NB were measured by flow cytometry (HC, $n = 7$; NB, $n = 7$). Data were mean \pm SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$. **C** fold change was calculated by dividing the number of $\gamma\delta$ T cells on day 14 by the numbers of $\gamma\delta$ T cells at the start (HC, $n = 17$; NB, $n = 11$). Data were mean \pm SEM from five independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$; ***, $p < 0.001$.

retained in cultures pre-conditioned only with IL-15 as well in comparison with those pre-conditioned with IL-2 only ($p = 0.06$), the expression level of Mcl-1 was in accordance with the apoptotic rate of $\gamma\delta$ T cells continued to culture in the medium without any cytokines. The IL-15 pre-conditioned cultures exhibited an apoptotic rate around $23.16 \pm 4.7\%$ in HC and $27.07 \pm 4.01\%$ in NB patients, while the cultures pre-conditioned only with IL-2 presented with a ratio of $47.46 \pm 4.54\%$ in HC and $43.66 \pm 3.61\%$ in NB patients. Taken together, these data suggested that IL-15 has a protective effect on the $\gamma\delta$ T-cell survivability through sustaining the Mcl-1 expression in an environment scarce of cytokines.

To further verify the dependence of Mcl-1 in resisting apoptosis of $\gamma\delta$ T cells deprived of cytokine support, AT-7519, a specific inhibitor of Mcl-1, but not Bcl-2, was added to cultures 48 h post cytokine removal [19]. As expected, the expression of Mcl-1 but not Bcl-2 was entirely inhibited by AT-7519 at the time point of 72 h post cytokine removal in comparison with cultures treated with DMSO as control (Fig. 4C). Accordingly, $\gamma\delta$ T cells in cultures

with AT-7519 indistinguishably underwent massive apoptosis in all culture groups regardless of what cytokines were added before the cytokine-free culture (Fig. 4D, E). These data indicated that the inhibition of Mcl-1 abolished the IL-15-mediated anti-apoptotic effect on in vitro expanded $\gamma\delta$ T cells.

STAT5 is well recognized as a common downstream pathway from cytokines receptors [20]. The expression of anti-apoptotic proteins Mcl-1 and Bim were upregulated by constitutively activated STAT5 [21]. According to our findings, to further investigate whether JAK/STAT pathway is involved in the Mcl-1 regulation in $\gamma\delta$ T cells receiving IL-15 nourishment, the phosphorylation of STAT5 was analyzed. After 72 h cytokine-free culture, it was perceivable that the phosphorylation of STAT5 in cultures pre-conditioned with IL-15 or IL-15 plus IL-2 was at a greater level than cells in cultures pre-conditioned only with IL-2 (Fig. 4F). Interestingly, the NB-derived $\gamma\delta$ T cells had a grossly higher level of phosphorylated STAT5 after 72 h cytokine removal in any pre-conditioned cultures. IL-15 is also known to stimulate MAPK/ERK



pathways in addition to the JAK/STAT pathway [20]. Our findings indicated that the MAPK/ERK pathway was activated as phosphorylation of ERK became higher in control-derived $\gamma\delta$ T cells from IL-15 pre-conditioned cultures, but unexpectedly not in respective patient-derived $\gamma\delta$ T cells cultures.

IL-15-fostered $\gamma\delta$ T cells increased the expression of activated phenotype and promoted the cytotoxic activity
Now that we knew IL-15 could enhance the viability of $\gamma\delta$ T cells, we were curious about their functionality. To assess this, we first performed the flow cytometry staining to measure the expression

Fig. 3 Qualification of $\gamma\delta$ T-cell apoptotic resistance after cytokine withdrawal. When $\gamma\delta$ T cells were cultured with IL-2, IL-15, and IL-2 plus IL-15 for 14 days, cytokines were removed and apoptosis was analyzed by measuring the level of Annexin V and 7-AAD by flow cytometry. **A** Flowchart of the experimental setup to study effects of IL-15 stimulation and withdrawal in $\gamma\delta$ T cells. **B** Annexin V⁺ $\gamma\delta$ T cells were analyzed at day 14 (HC, *n* = 7; NB, *n* = 7). Data were mean \pm SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. **C** Representative gating strategy for the flow-cytometric analysis. $\gamma\delta$ T cells fostered with different conditions were examined after cytokine withdrawal 0 h, 24 h, 48 h, 72 h, 96 h. **D, E** the proportion of Annexin V⁺ $\gamma\delta$ T cells were analyzed at each time point after cytokine withdrawal (HC, *n* = 7; NB, *n* = 7). Data were mean \pm SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001. **F** Representative gating strategy for the flow-cytometric analysis. Mitochondrial membrane potential was analyzed by flow cytometry following staining with JC-1 when cytokines were withdrawn for 72 h. JC-1 aggregates are representative of high mitochondrial membrane potential and JC-1 monomers are representative of low mitochondrial membrane potential (apoptosis). **G** JC-1 monomer percentage was analyzed (HC, *n* = 11; NB, *n* = 7). Data were mean \pm SEM from six independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. **, *p* < 0.01; ***, *p* < 0.001. **H** Annexin V⁻ 7-AAD⁻ $\gamma\delta$ T cells represented viable cells were assessed (HC, *n* = 7; NB, *n* = 7). Data were mean \pm SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons.

of activating markers on the $\gamma\delta$ T-cell surface. Expression of HLA-DR, CD56, and CD69 in the 14-day in vitro expanded control-derived $\gamma\delta$ T cells were remarkably upregulated in cultures supplemented with IL-15 related to those cultured with IL-2 only. In contrast to control-derived $\gamma\delta$ T cells, in vitro expanded patient-derived $\gamma\delta$ T cells were not much affected except that the expression of CD69 was boosted by the addition of IL-15 in cultures (Fig. 5B–D). That being said, the cytotoxicity of patient-derived $\gamma\delta$ T cells against target tumor cell SH-SY5Y was definitely enhanced in the presence of IL-15 only, compared to those cultured in IL-2 only. The IL-2 plus IL-15 culture, however, by unknown mechanism abolished the boost effect from IL-15. While the control-derived $\gamma\delta$ T cells cultured in IL-2 only have a better tumoricidal capability than patient-derived cells in the same culture condition, both IL-15 only and IL-15 plus IL-2 culture boosted the target cell killing ability in relation to culture with IL-2 only as baseline (Fig. 5E).

DISCUSSION

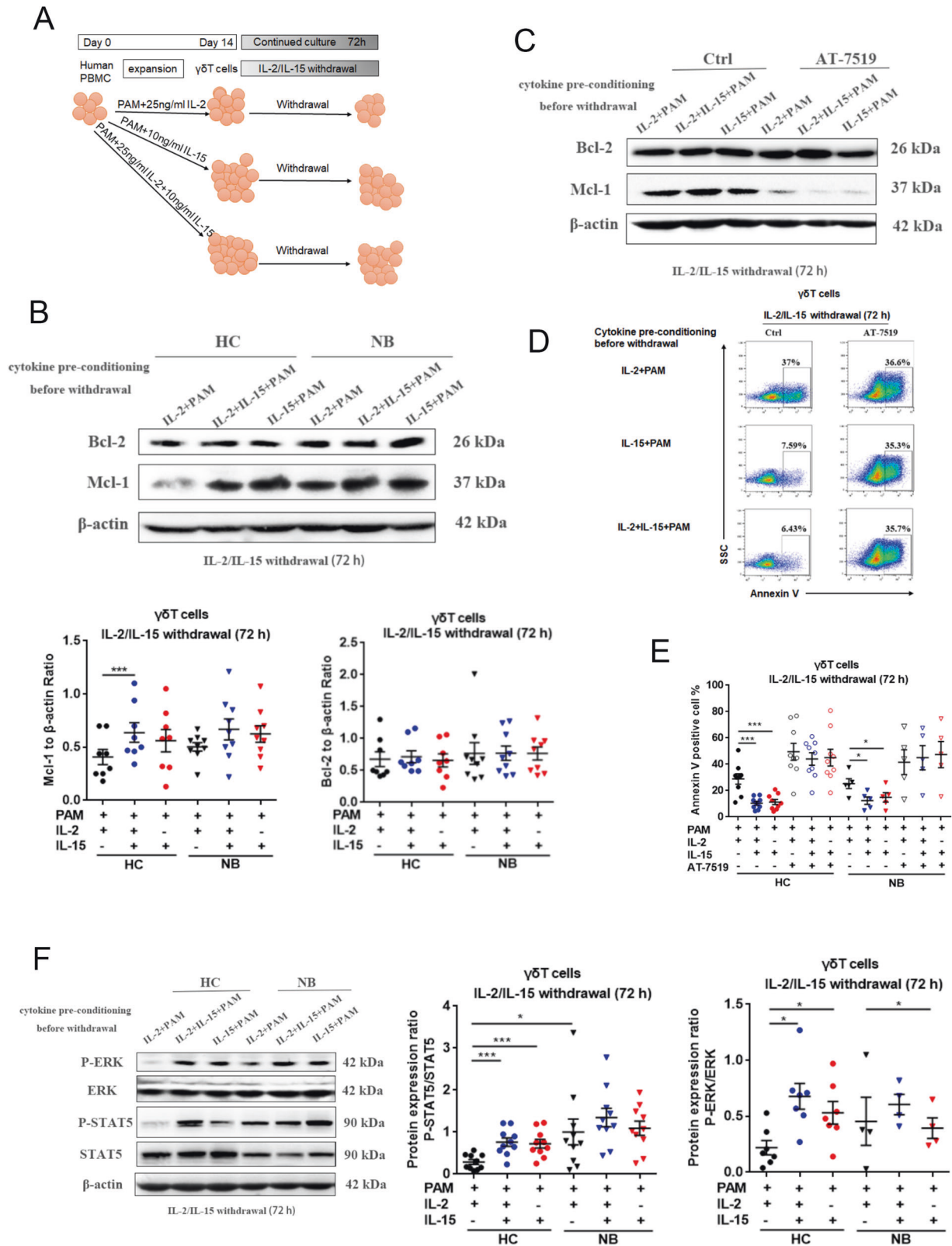
$\gamma\delta$ T-cell immunotherapy is a promising treatment for NB patients which requires a large number of $\gamma\delta$ T cells preparation [22, 23]. Numerous studies have focused on expanding $\gamma\delta$ T cells using phosphoantigens (such as PAM) in the presence of IL-2 as a co-stimulator [11, 24]. As expected, the unstimulated $\gamma\delta$ T cells encountered massive apoptosis after a 14-day culture. While IL-15 shares many functions with IL-2, it was proved to promote the homeostatic expansion of mouse $\gamma\delta$ T cells in vivo and to inhibit the growth of $\alpha\beta$ T cells which expedite the expansion of $\gamma\delta$ T cells [25, 26]. This is crucial for tumor immunotherapy since the infiltration of $\gamma\delta$ T cells in most tumors is inefficient in comparison to $\alpha\beta$ T cells. The former, however, in most cases is functionally more tumoricidal. In our study, we successfully produced more $\gamma\delta$ T cells in vitro by adding IL-15 in the culture for both HC-derived and NB-derived PBMCs. In the end-stage of cell expansion, neither cultures with different cytokine supplements nor cultures from different cell origins of seeding (HC vs NB) were found different in terms of cell apoptosis. This implied that IL-15 could enhance the proliferation of $\gamma\delta$ T cells during in vitro expansion by PAM. That being said, even with the IL-15 supplement, the total number of $\gamma\delta$ T cells generated from culture seeded with patient-derived PBMCs was not able to reach the level of those started from HC-derived PBMCs. One of the reasons for curtailed maximum expansion in patient-derived $\gamma\delta$ T cells could be the dull responsiveness of IL-15R α to IL-15 resulting from the long-term deficiency in IL-15 within the tumor microenvironment. Our data indicated that in NB patients, the expression of IL-15R α in $\gamma\delta$ T cells was significantly higher than that from HC. This might be a salvage compensation to the sub-physiological level of IL-15 in the patient with a chronic tumor burden. The internalization of IL-15/IL-15R α on NK cells modulated cellular response and IL-15R α shedding

following IL-15 stimulant was crucial to T-cell proliferation [27, 28]. Unexpectedly, reduction of IL-15R α with a slower rate was observed in patient $\gamma\delta$ T cells triggered by the addition of IL-15, suggesting a sluggish IL-15/IL-15R α complex internalization or shedding. We speculated that it was the desensitized IL-15/IL-15R α signaling that led to a sub-optimized in vitro expansion of patient $\gamma\delta$ T cells. Whether and, if any, how the IL-15/IL-15R α signaling pathway was attenuated requires further investigation.

It has been evidenced that absence of IL-15 in ~30% of patients with metastatic colon cancer was associated with high disease relapse and mortality [29]. In an in vitro experiment, IL-15-fostered NK cells maintained a lower level of apoptosis and a higher level of cytotoxicity after cytokine withdrawal compared with IL-2-fostered cells [20]. In our study, by mimicking the in vivo tumor microenvironment, we depleted the IL-15 after the 14-day cell expansion and found the patient-derived $\gamma\delta$ T cells possessed a much stronger anti-apoptotic ability in the following 96 h culture. We boldly believed that $\gamma\delta$ T cells in NB patients bore a tolerance to the extremely low level of IL-15 within the tumor microenvironment.

IL-15 regulated the apoptosis of $\gamma\delta$ T cells mainly through the intrinsic apoptosis pathway, a pathway similarly delayed human neutrophil apoptosis by Mcl-1 [30]. Our data clarified that IL-15 protected $\gamma\delta$ T cells from scarce of cytokines through sustaining the Mcl-1 expression, and the effect could be reversed by Mcl-1 inhibition with a CDK inhibitor (AT-7519). It has been reported that IL-15 failed to increase the level of Mcl-1 mRNA but changed its proteasomal degradation. Furthermore, IL-15 regulated Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells [21, 31]. As we discovered, the phosphorylation of STAT5 and ERK were dramatically changed in IL-15-fostered $\gamma\delta$ T cells. It is very likely that the anti-apoptotic effect in $\gamma\delta$ T cells conferred by IL-15 was through retaining the Mcl-1 via JAK/STAT5 or MAPK/ERK pathways.

Our results showed that the activation markers CD56, CD69, and HLA-DR were significantly upregulated in IL-15-expanded $\gamma\delta$ T cells on day-14 culture. This indicated that IL-15-fostered $\gamma\delta$ T cells exhibited an increased cytotoxic capacity, as confirmed by the promoted killing of NB cell line SH-SY5Y in our research. These results were consistent with the previous study that IL-15^{-/-} knockout mice only produced less amounts of IFN- γ upon stimulation and showed remarkably attenuated cytotoxicity against target cells as compared to wild-type mice [32]. Interestingly, in our study, the NB $\gamma\delta$ T cells supposedly having dull responsiveness to IL-15 signaling was still capable of killing target tumor cells, in a level similar to that from HC $\gamma\delta$ T cells. It reinforced our confidence in the usage of IL-15 pre-conditioned patient-derived $\gamma\delta$ T cells in an autologous adoptive transfer setting for tumor eradication. Furthermore, many hurdles, such as $\gamma\delta$ T-cell exhaustion, restricted trafficking, and limited tumor infiltration should be considered before clinical application.



MATERIALS AND METHODS

Sample collection

A total of 63 patients (mean age 4.2 ± 0.5 years) with NB at intermediate (13 boys and 7 girls) and high (25 boys and 18 girls) risk stages, and 58 healthy children (30 boys, 28 girls; mean age 3.9 ± 0.4 years) under regular physical examination were recruited from the Beijing Children's Hospital Affiliated to

the Capital Medical University. The study was approved by the Medical Ethics Committee of Beijing Children's Hospital, the Capital Medical University. Written consent for research purposes of sample use was read and signed by all participants or their biological parents or legal guardians. Tumor samples from 16 NB patients were collected for this study. Peripheral blood samples from healthy children and NB patients were collected in BD Vacutainer™

Fig. 4 Mechanism of the enhanced survivability of $\gamma\delta$ T cells under IL-15 culture condition. When $\gamma\delta$ T cells were cultured for 14 days, cytokines were withdrawn for 72 h and $\gamma\delta$ T cells were collected and analyzed. IL-2-fostered $\gamma\delta$ T cells as a control culture condition. **A** Flowchart of the experimental setup to study potential the mechanism of IL-15 to enhance the survivability of $\gamma\delta$ T cells. **B** Western blotting was performed to analyze the expression of Mcl-1 and Bcl-2 (HC, $n = 8$; NB, $n = 9$). Data were mean \pm SEM from five independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. ***, $p < 0.001$. **C, D** $\gamma\delta$ T cells were treated with AT-7519 for 24 h and representative results were shown for the Western blotting and the flow-cytometric analysis. **E** Annexin V⁺ $\gamma\delta$ T cells were analyzed when $\gamma\delta$ T cells were treated with AT-7519 for 24 h (HC, $n = 11$; NB, $n = 5$). Data were mean \pm SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$; ***, $p < 0.001$. **F** Western blotting was performed to analyze the expression of STAT5 and ERK and the phosphorylation levels of STAT5 and ERK. The protein band intensity was quantified and analyzed by ImageJ (HC, $n = 8$; NB, $n = 9$). Data were mean \pm SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$; ***, $p < 0.001$.

plastic blood collection tubes with ethylenediaminetetraacetic acid (EDTA)-K2 as an anticoagulant during the onset of the disease. Serum samples were collected in tubes without anticoagulant by centrifugation at $600 \times g$ for 5 min and the aqueous phase was taken.

PBMC isolation

PBMCs were isolated from EDTA anticoagulated blood with Ficoll-Hypaque solution by density gradient centrifugation. After centrifuged at $1000 \times g$ for 20 min at room temperature (RT), the interphase layer was carefully transferred into a 15 ml falcon tube with 10 ml PBS. PBMCs were obtained with another centrifugation at $600 \times g$ for 5 min and resuspended in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (fetal bovine serum, Gibco, Invitrogen, Carlsbad, CA, USA).

NB cell line cultures

The NB cell line SH-SY5Y (CRL-2266) was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS at 37 °C in 5% CO₂.

$\gamma\delta$ T-cell expansion

In order to induce activation of $\gamma\delta$ T cells, PBMCs were incubated with 10 μ g/ml PAM (Sigma-Aldrich, St Louis, USA) in 10% FBS RPMI-1640 plus 25 ng/ml recombinant human IL-2 (PeproTech, Rehovot, Israel) and/or 10 ng/ml recombinant human IL-15 (PeproTech, Rehovot, Israel) at 37 °C humidified cell incubator with 5% CO₂. Half of the culture medium was replaced by a fresh medium and recombinant cytokines were added every 3 days. The purity of $\gamma\delta$ T cells was examined on days 0, 4, 7, 9, and 14 of culture by flow cytometry analysis. Then cell counting was determined by Precision Count Beads™ (BioLegend, San Diego, CA, USA). Only those cells under a 14-day culture presented with a ratio >90% $\gamma\delta$ -TCR and CD3-positive cells were considered as a successful expansion and pure enough for further experiments.

For some cultures, $\gamma\delta$ T cells were pre-treated with AT-7519 (Selleck Chemicals, Houston, TX, USA) for 24 h before further culture in a regular culture medium devoid of cytokines. Cells were then harvested for corresponding assays.

Apoptosis analysis by Annexin V surface exposure

To determine the viability of in vitro expanded $\gamma\delta$ T cells in different cytokine-conditioned culture medium, $\gamma\delta$ T cells were collected on culture day 14 and resuspended in RPMI-1640 plus 10% FBS in 48-well plates for further culture in the absence of cytokines. Cells were then collected for apoptosis analysis 0 h, 24 h, 48 h, 72 h, 96 h post-IL-2 and/or IL-15 withdrawal. In brief, $\gamma\delta$ T cells were washed with PBS and resuspended in cell staining buffer (PBS + 3% FBS), followed by Fluorescein isothiocyanate-TCR $\gamma\delta$ antibody (BioLegend, San Diego, California, USA) staining at RT for 20 min in dark. Cells were then subjected to a washing protocol with Annexin V-binding buffer (0.1 M Tris (PH 7.4), 1.5 M NaCl, 25 mM CaCl₂) followed with APC- Annexin V (BioLegend, San Diego, California, USA) staining for 15 min at RT in dark. 7-ADD (BioLegend, San Diego, California, USA) was added to the cell suspension and mixed gently before flow cytometry analysis. Cell events were acquired with a BD FACSCalibur flow cytometer. Data were analyzed with Flowjo software.

MMP assay

The JC-1 detection assay was performed to measure the MMP of cultured $\gamma\delta$ T cells after IL-2 and/or IL-15 withdrawal as described above. In brief, cultured $\gamma\delta$ T cells in the absence of cytokines for 72 h were collected and

stained with APC- TCR $\gamma\delta$, the cells were then washed twice with PBS and resuspended with 1 ml of pre-warmed medium containing 2.5 μ g/ml JC-1 following incubation at 37 °C in dark for 30 min. The JC-1 fluorescence shift by flow cytometry was used to evaluate the cell MMP change.

Western blotting

Cultured $\gamma\delta$ T cells in vitro after cytokine removal were collected by centrifugation at indicated time point within a 72-h culture. The cell pellet was lysed by RIPA buffer with protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and 1% PhosSTOP (Roche, Basel, Switzerland) with three times freeze (−80 °C) and thaw RT cycles. The lysate was centrifuged at 12000 rpm for 5 min at 4 °C and the supernatant was harvested as a total protein sample. Protein concentration was determined by a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) following the manufacturer's instructions. The protein was separated by 12% SDS-PAGE before being transferred to a methanol pre-soaked PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20 detergent (TBST)-buffer (50 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 0.1% Tween 20) for 2 h at RT followed by an overnight incubation at 4 °C with primary antibodies: anti-Mcl-1 antibody (cat. no. D2616) from Santa Cruz Biotechnology; anti-Bcl-2 antibody (cat. no. GR3232704-8) from Abcam; anti-ERK1/2 antibody (cat. no. 21), anti-P-ERK antibody (cat. no. 18), anti-STAT5 antibody (cat. no. 1), anti-P-STAT5 antibody (cat. no. 9) and anti- β -actin antibody from Cell Signaling Technology. The signal was detected with an ECL detection reagent after 1 h incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Signals on immunoblots were quantified using ImageJ software.

Cytotoxicity assays

To measure the tumoricidal capacity of in vitro expanded $\gamma\delta$ T cells in different cytokine-conditioned culture mediums, cytotoxicity assays were done with a CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. NB cell line SH-SY5Y was used as target cell. In brief, a total of 2×10^5 $\gamma\delta$ T cells were seeded in a round-bottom 96-well culture plate as the spontaneous LDH release control for effector cells; a total of 2×10^4 SH-SY5Y cells were seeded as the spontaneous LDH release control and equal numbers were also seeded as the maximal LDH release for target cells; effect cells were incubated with target cells at the ratio of the effector: target with 10:1 as the experimental well. After 5 h incubation at 37 °C, the lysis solution was added to the target cell control to determine maximal LDH release. Then a total of 50 μ l/well lysis supernatant was transferred to a flat-bottom 96-well plate with 50 μ l/well-reconstituted substrate mix. Following incubation in dark for 30 min, 50 μ l stop solution was added and absorbance was measured at 490 nm by TriStar² LB 942 Multimode Reader (Berthold, Germany). The cytotoxicity of effector $\gamma\delta$ T cells to target cells was calculated.

Flow cytometry

For surface staining, the PBMCs and cultured $\gamma\delta$ T cells were stained with the indicated antibodies (Anti-human CD3, CD25, CD215, TCR $\gamma\delta$, CD56, CD69, HLA-DR) at RT for 20 min in dark. For cytotoxic molecules detection, $\gamma\delta$ T cells were treated with 50 ng/ml PMA, 1 ng/ml ionomycin, and GolgiStop protein transport inhibitor (BD Biosciences, San Jose, CA, USA) for 5 h. Intracellular staining with antibodies (Anti-human IFN- γ , Granzyme B, perforin) was carried out with a Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. All antibodies were from BioLegend (San Diego, CA, USA) and titration of best concentration was determined before experiments. Cell

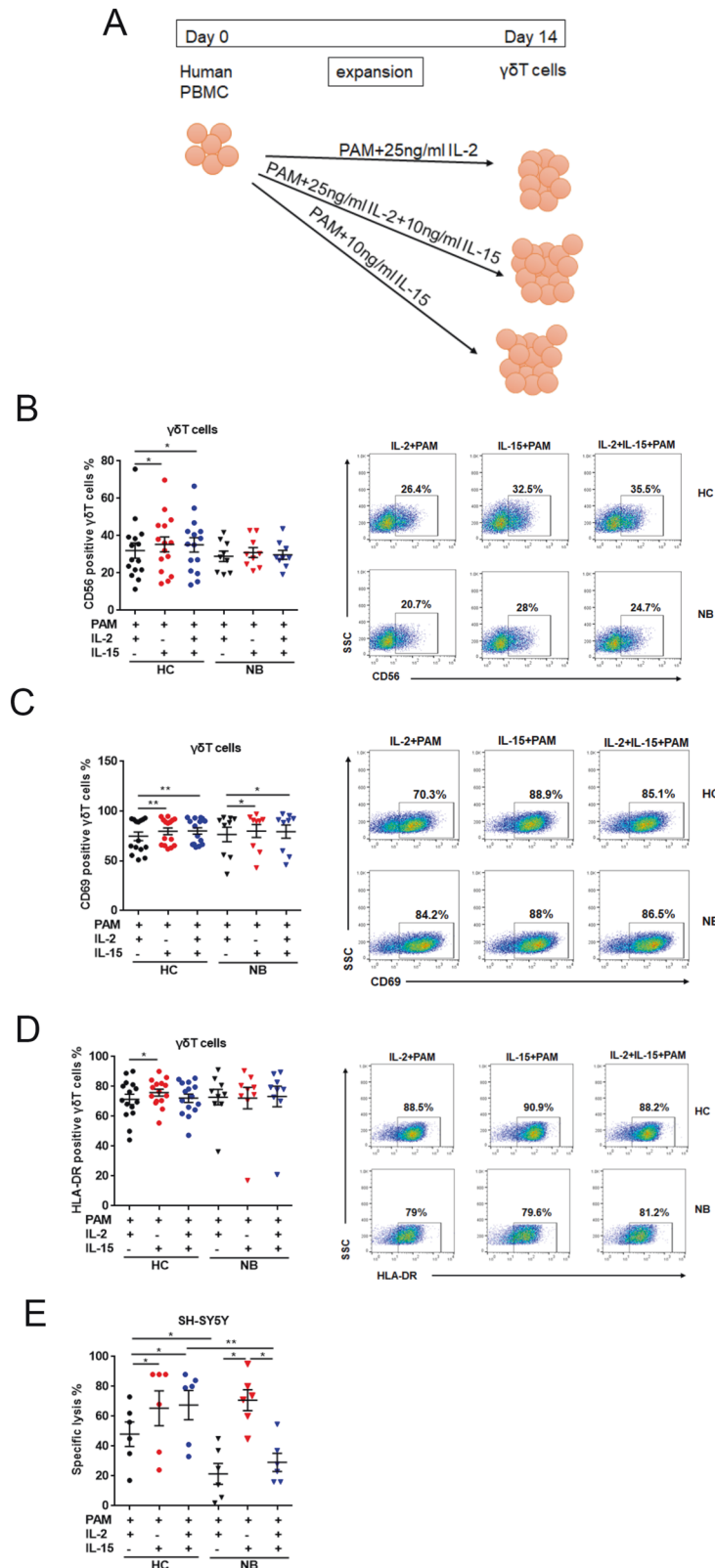


Fig. 5 Qualification of $\gamma\delta$ T-cell activated phenotype and cytolytic activity. **A** Flowchart of the experimental setup to study activated phenotype and cytolytic activity of IL-15-fostered $\gamma\delta$ T cells. **B–D** The expression of activated markers including CD69, CD56, and HLA-DR in expanded $\gamma\delta$ T cells was measured by flow cytometry at day 14 (HC, $n = 15$; NB, $n = 9$). Data were mean \pm SEM from three independent experiments. Statistical analysis was performed using an unpaired t test. *, $p < 0.05$; **, $p < 0.01$. **E** The cytotoxic assays were performed and expanded $\gamma\delta$ T cells were cultured with NB cell line SH-SY5Y at the ratio of 1:10 (HC, $n = 6$; NB, $n = 6$). Data were mean \pm SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons. *, $p < 0.05$; **, $p < 0.01$.

events were acquired using a BD LSRFortessa X20 flow cytometer, and the collected data were analyzed using FlowJo v10.

RT-PCR

Total RNA from PBMCs and NB tissues was purified using the Direct-zol RNA Miniprep (ZYMO research, Orange, CA, USA), and the concentration was determined by a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reverse transcription was performed according to standard protocols using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). qPCR was performed using SYBR Green PCR Master Mix (TIANGEN, Beijing, China) and the fluorescence was read by a QuantStudio 6 flex real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as the endogenous housekeeping gene control.

PCR primer sequences:

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	GGAGCCAAAAGGGTCATCACTC	GAGGGGGCCATCCACAGTCTTCT
IL-15	GGCCCAAAGCACCTAACCTA	AGGAAGCCCTGCCTGAAAC
IL-15R α	TGGCTATCTCCAGTCCACTGT	CATGGCTTCCATTCAACGCTGG

Measurement of IL-15

Plasma concentration of IL-15 was quantified with the ELISA kit (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. Briefly, 100 μ l serum of each sample or standard was added to the wells pre-coated with capture antibody. Then 50 μ l Biotin-conjugated detecting antibody was added and incubated at RT for 2 h. The plate was then washed and 100 μ l streptavidin-HRP added to all wells for 1 h incubation at RT. Following with plate wash, 100 μ l substrate was added and incubated at RT for 30 min in dark. The reaction was stopped with stop solution and colorimetric signals were collected by an Elisa reader (Berthold, Germany) at OD 450.

Statistical analysis

The student's *t* test (paired or unpaired) was used for statistical analysis between two groups. Multiple group comparisons were performed by one-way analysis of variance (ANOVA) with the appropriate post hoc test. Data were represented as mean \pm standard error of the mean. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), and statistical significance was denoted as *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

DATA AND MATERIALS AVAILABILITY

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES

- Liu KX, Joshi S. "Re-educating" tumor associated macrophages as a novel immunotherapy strategy for neuroblastoma. *Front Immunol.* 2020;11:1947.
- Zhao Q, Liu Y, Zhang Y, Meng L, Wei J, Wang B, et al. Role and toxicity of radiation therapy in neuroblastoma patients: a literature review. *Crit Rev Oncol Hematol.* 2020;149:102924.
- Park JA, Cheung NV. Targets and antibody formats for immunotherapy of neuroblastoma. *J Clin Oncol.* 2020;38:1836–48.
- Pastor ER, Mousa SA. Current management of neuroblastoma and future direction. *Crit Rev Oncol Hematol.* 2019;138:38–43.
- Vantourout P, Hayday A. Six-of-the-best: unique contributions of $\gamma\delta$ T cells to immunology. *Nat Rev Immunol* 2013;13:88–100.
- Fisher JP, Yan M, Heuwerkerk J, Carter L, Abolhassani A, Frosch J, et al. Neuroblastoma killing properties of Vdelta2 and Vdelta2-negative gammadeltaT cells following expansion by artificial antigen-presenting cells. *Clin Cancer Res.* 2014;20:5720–32.
- Liu R, Zhou Q, La Cava A, Campagnolo DI, Van Kaer L, Shi F-D. Expansion of regulatory T cells via IL-2/anti-IL-2 mAb complexes suppresses experimental myasthenia. *Eur J Immunol* 2010;40:1577–89.
- Wang X, Mou W, Han W, Xi Y, Chen X, Zhang H, et al. Diminished cytolytic activity of gammadelta T cells with reduced DNAM-1 expression in neuroblastoma patients. *Clin Immunol.* 2019;203:63–71.
- Van Acker HH, Anguille S, Willemen Y, Van den Bergh JM, Berneman ZN, Lion E, et al. Interleukin-15 enhances the proliferation, stimulatory phenotype, and antitumor effector functions of human gamma delta T cells. *J Hematol Oncol.* 2016;9:101.

- Rozenbaum M, Meir A, Aharoni Y, Itzhaki O, Schachter J, Bank I, et al. Gammadelta CAR-T cells show CAR-directed and independent activity against leukemia. *Front Immunol.* 2020;11:1347.
- Fisher J, Anderson J. Engineering approaches in human gamma delta T cells for cancer immunotherapy. *Front Immunol.* 2018;9:1409.
- Huntington ND, Puthalakath H, Gunn P, Naik E, Michalak EM, Smyth MJ, et al. Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat Immunol.* 2007;8:856–63.
- Jacobs B, Pfefferle A, Clement D, Berg-Larsen A, Saetersmoen ML, Lorenz S, et al. Induction of the BIM short splice variant sensitizes proliferating NK cells to IL-15 withdrawal. *J Immunol* 2019;202:736–46.
- Mao Y, van Hoef V, Zhang X, Wennerberg E, Lorent J, Witt K, et al. IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. *Blood.* 2016;128:1475–89.
- Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science.* 1998;281:1322–6.
- Brincks EL, Woodland DL. Novel roles for IL-15 in T cell survival. *F1000 Biol Rep.* 2010;2:67.
- Bock FJ, Tait SWG. Mitochondria as multifaceted regulators of cell death. *Nat Rev Mol Cell Biol.* 2020;21:85–100.
- Opferman JT. Apoptosis in the development of the immune system. *Cell Death Differ.* 2008;15:234–42.
- Squires MS, Cooke L, Lock V, Qi W, Lewis EJ, Thompson NT, et al. AT7519, a cyclin-dependent kinase inhibitor, exerts its effects by transcriptional inhibition in leukemia cell lines and patient samples. *Mol Cancer Ther.* 2010;9:920–8.
- Felices M, Lenvik TR, Ankarlo DE, Foley B, Curtisinger J, Luo X, et al. Functional NK cell repertoires are maintained through IL-2Ralpha and Fas ligand. *J Immunol* 2014;192:3889–97.
- Shenoy AR, Kirschnek S, Hacker G. IL-15 regulates Bcl-2 family members Bim and Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells. *Eur J Immunol* 2014;44:2500–7.
- Pressey JG, Adams J, Harkins L, Kelly D, You Z, Lamb LS Jr. In vivo expansion and activation of gammadelta T cells as immunotherapy for refractory neuroblastoma: a phase 1 study. *Medicine.* 2016;95:e4909.
- Puan K-J, Jin C, Wang H, Sarikonda G, Raker AM, Lee HK, et al. Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. *Int Immunol.* 2007;19:657–73.
- Wang R-N, Wen Q, He W-T, Yang J-H, Zhou C-Y, Xiong W-J, et al. Optimized protocols for $\gamma\delta$ T cell expansion and lentiviral transduction. *Mol Med Rep.* 2019;19:1471–80.
- Nguyen R, Moustaki A, Norrie JL, Brown S, Akers WJ, Shirinifard A, et al. Interleukin-15 enhances anti-GD2 antibody-mediated cytotoxicity in an orthotopic PDX model of neuroblastoma. *Clin Cancer Res.* 2019;25:7554–64.
- Baccala R, Witherden D, Gonzalez-Quintal R, Dummer W, Surh CD, Havran WL, et al. Gamma delta T cell homeostasis is controlled by IL-7 and IL-15 together with subset-specific factors. *J Immunol* 2005;174:4606–12.
- Zhao YM, French AR. Mechanistic model of natural killer cell proliferative response to IL-15 receptor stimulation. *PLoS Comput Biol.* 2013;9:e1003222.
- Tamzalit F, Barbioux I, Plet A, Heim J, Nedellec S, Morisseau S, et al. IL-15R α complex shedding following trans-presentation is essential for the survival of IL-15 responding NK and T cells. *Proc Natl Acad Sci USA.* 2014;111:8565–70.
- Fiore PF, Di Matteo S, Tumino N, Mariotti FR, Pietra G, Ottonello S, et al. Interleukin-15 and cancer: some solved and many unsolved questions. *J Immunother.* 2020;8:e001428.
- Bouchard A, Ratthe C, Girard D. Interleukin-15 delays human neutrophil apoptosis by intracellular events and not via extracellular factors: role of Mcl-1 and decreased activity of caspase-3 and caspase-8. *J Leukoc Biol.* 2004;75:893–900.
- Senichkin VV, Streletskaia AY, Gorbunova AS, Zhivotovskiy B, Kopeina GS. Saga of Mcl-1: regulation from transcription to degradation. *Cell Death Differ.* 2020;27:405–19.
- Nakazato K, Yamada H, Yajima T, Kagimoto Y, Kuwano H, Yoshikai Y. Enforced expression of Bcl-2 partially restores cell numbers but not functions of TCRgammadelta intestinal intraepithelial T lymphocytes in IL-15-deficient mice. *J Immunol* 2007;178:757–64.

ACKNOWLEDGEMENTS

We would like to thank all participants in this study.

AUTHOR CONTRIBUTIONS

H.W., J.G.M., and X.W. conceived the conceptual designs. H.W., M.P. performed the experiments. H.W., J.G., and X.M. analyzed the data. H.W., J.G. wrote the manuscript. W.W., W.C. and W.M. were involved in the methodology and investigation. H.Z., W.S., and Y.S. provided the patients' clinical information and blood samples.

FUNDING

This work was supported by the National Natural Science Foundation of China (no. 81772668, no. 82002637, and no. 81802491).

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Written consent to participate as a patient or healthy control was obtained from the parents/guardians of the minors included in this study (minors are considered anyone under the age of 16). The Institutional Review Board of Beijing Children's Hospital approved all research on human subjects.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41420-022-00942-5>.

Correspondence and requests for materials should be addressed to Xiaoli Ma or Jingang Gui.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2022