International Immunopharmacology 69 (2019) 136–142

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

IL-21-mediated expansion of Vγ9Vδ2 T cells is limited by the Tim-3 pathway

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ARTICLE INFO

Keywords:
IL-21
Tim-3
Vγ9Vδ2 T cell
Acute myeloid leukemia

ABSTRACT

Vγ9Vδ2 T cells are the main γδ T subset in the peripheral blood and lymphoid organs. Previous studies have shown that Vγ9Vδ2 T cells could expand in the presence of phosphoantigens and IL-2 and exert antitumor functions. However, their potency was limited because sustained proliferation could not be achieved, possibly due to exhaustion caused by prolonged antigenic stimulation. In this study, we examined the proliferative response of Vγ9Vδ2 T cells to IL-21, a cytokine previously shown to promote NK cell and CD8 T cell cytotoxicity. We found that IL-21 could significantly improve the proliferation of phosphoantigen-stimulated Vγ9Vδ2 T cells in a dose-dependent manner. However, in acute myeloid leukemia (AML) patients, the efficacy of IL-21 was significantly reduced. Vγ9Vδ2 T cells from AML patients exhibited lower expression of IL-21R, and required higher levels of IL-21 for expansion. IL-21-treated Vγ9Vδ2 T cells from AML patients presented lower increase in STAT1 phosphorylation than Vγ9Vδ2 T cells from healthy volunteers. Interestingly, AML Vγ9Vδ2 T cells presented significantly higher Tim-3 expression than healthy Vγ9Vδ2 T cells. IL-21 treatment further induced Tim-3 upregulation. Blocking Tim-3 increased the proliferation and the STAT phosphorylation in Vγ9Vδ2 T cells in response to IL-21. Together, these results demonstrated that IL-21 could significantly expand the Vγ9Vδ2 T cells, but its efficacy was limited since it also increased the expression of checkpoint molecule Tim-3.

1. Introduction

Vγ9Vδ2 T cells are the main γδ T subset in the peripheral blood and the lymphoid organs [1]. Previous studies have shown that Vγ9Vδ2 T cells are critical components of the anti-tumor immunosurveillance in many types of solid and hematopoietic cancers, including colorectal, lung, breast, ovary, prostate, and hepatocellular carcinomas, as well as lymphomas and myelomas [2]. Unlike the conventional αβ-T cells that recognize peptide ligands presented by the major histocompatibility complex (MHC), T cell receptors (TCRs) on Vγ9Vδ2 T cells recognize non-peptidic phosphorylated antigens that are not usually present in healthy cells, but can be upregulated under stress, such as during microbial infections or when undergoing malignant transformation [3–5]. Vγ9Vδ2 TCRs may also bind directly to upregulated self-antigens on transformed cells [6]. Depending on the environmental factors, Vγ9Vδ2 T cells can differentiate into Th1-like, Th2-like, Th17-like, Treg-like, and Tfh-like cells, and express the associated cytokines upon stimulation. In addition, Vγ9Vδ2 T cells can exert cytotoxic function by the expression of granzymes and perforin [7,8], and by the expression of CD16 through antibody-dependent cell-mediated cytotoxicity [9].

Because of the plasticity in Vγ9Vδ2 T cell differentiation, regulatory mechanisms are essential to the function of activated Vγ9Vδ2 T cells. IL-21 is a cytokine primarily expressed by Th cells and natural killer T (NKT) cells. Binding of IL-21 to IL-21 receptor (IL-21R) activates the kinases JAK1 and JAK3, which subsequently activate STAT1, STAT3, and STAT5 to varying degrees [10]. In Vγ9Vδ2 T cells, IL-21 induces limited proliferation, and potentiates Tfh-like B cell helper function with increased secretion of IL-4, IL-10, and CXCL13 [11,12]. γ9Vδ2 T cells expanded in the presence of both IL-2 and IL-21 present increased degranulation and expression of cytolytic molecules, together with increased expression of inhibitory markers [13,14].

Acute myeloid leukemia (AML) occurs when myeloid precursor cells in the bone marrow fail to mature into functional leukocytes, but instead continue to undergo abnormal proliferation [15]. Patients with

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https://doi.org/10.1016/j.intimp.2019.01.027
Received 20 September 2018; Received in revised form 10 January 2019; Accepted 17 January 2019
Available online 29 January 2019

AML are currently treated with a chemotherapy regimen that has changed little for many years. The complete remission rate is around 70% to 80% in patients under the age of 60 years, and is significantly lower in older patients, who also suffer significantly higher rate of relapse [16].

Vγ9Vδ2 T cells are considered a promising new candidate for AML treatment. Coculture of Vγ9Vδ2 T cells and AML cells induced effector functions in Vγ9Vδ2 T cells and resulted in elimination of AML cells [17]. However, numerous clinical trials of Vγ9Vδ2 T cell-based immunotherapy have shown that the full antitumor potential of Vγ9Vδ2 T cells was not realized, in part due to diminished proliferative responses to prolonged stimulation by phosphoantigens and IL-2 [18]. Here, we examine the proliferative response of Vγ9Vδ2 T cells from AML patients to exogenous IL-21.

2. Methods

2.1. Participants

Thirty AML patients exhibiting primary malignancy and thirty healthy volunteers were recruited after providing informed consent. The patients and healthy subjects were matched in age and sex. The demographic and clinical information of patients and controls are presented in Table 1. The patients were untreated before collection of peripheral blood samples. In addition, no study participant presented acute or chronic infections, inflammatory bowel diseases, cardiovascular diseases, obesity, or malignancy other than AML during recruitment. Mononuclear cells were collected using standard Ficoll density gradient separation method from peripheral blood, and cryopreserved in heat-inactivated fetal calf serum containing 10% DMSO (Sigma). Freezing was performed step-wise from −80 °C overnight to −150°t for long-term storage. Thawing was performed in 37 °C complete media supplemented with 1% DNase (Sigma). Thawed cells were rested overnight before use. All sample collection and experimental procedures were approved by the ethics committee of the First Affiliated Hospital of Xiamen University.

2.2. Flow cytometry

Mononuclear cells were thawed briefly in 37 °C water bath, and immediately washed in sterile cold PBS. The thawed cells were then rested in CO_2 incubator at 37 °C overnight. The next day, mononuclear cells were incubated with anti-human Vγ9, anti-human Vδ2 (both from BD Biosciences), anti-human CD3 and anti-human IL-21R (both from BioLegend) monoclonal antibodies, as well as Violet dead cell stain (Invitrogen), for 30 min at 4 °C. For P-STAT1 and P-STAT3 staining, surface stained cells were fixed and permeabilized using Fixation buffer and Perm buffer (both from BD), respectively, and then stained with anti-human pY701 for P-STAT1 and anti-human pY705 for P-STAT3 (both from BD). A Mouse IgG2a, κ isotype control (BD) was used for negative gating control. The cells were then washed and acquired in an LSR instrument (BD). Dead cells and debris were excluded from result analysis via Violet-negative gating and appropriate forward scatter vs. side scatter gating.

Table 1
Demographic and clinical information of AML patients and healthy controls.

	AML patient	Healthy control	*P*
N	30	30	
Age (y)	55.0 ± 6.1	54.6 ± 7.1	> 0.05
Sex (F/M)	11/19	11/19	> 0.05
Staging (N, %)			
M3	11, 37	N/A	
M4	8, 27	N/A	
M5	11, 37	N/A	

Values were given as mean ± standard deviation when applicable. Staging was based on French-American-British (FAB) Classification. N/A, not applicable.

2.3. Sorting and stimulation

NK cells and B cells were first depleted from whole blood mononuclear cells using mouse anti-human CD56 and CD19 antibodies coupled to an anti-mouse IgG column (Miltenyi Biotec). The remaining cells were then incubated with anti-human Vγ9 and anti-human Vδ2 for 30 min at 4 °C, washed, and then sorted in FACSAria (BD). For stimulation, Vγ9Vδ2 T cells were plated at 2×10^4 cells per 100 μL of complete culture medium, together with HMBPP (Sigma), IL-2 (Novartis), IL-21 (PeproTech), and/or anti-human Tim-3 clone 2E2 (BioLegend) at concentrations specified in the experiments. Irradiated mononuclear cells were used as feeder cells at 2 feeders per Vγ9Vδ2 T cell. Incubation was performed in a 37 °C CO_2 incubator for 72 h.

2.4. Proliferation assay

At the end of stimulation, 0.1 μCi/well of tritiated thymidine was added to Vγ9Vδ2 T cell for 12 h. The cells were then harvested and the level of thymidine incorporation was determined in a direct beta counter (Packard).

2.5. Statistical analysis

Mean ± standard deviation was provided for all datasets. Data normality was assessed using the D'Agostino-Pearson test. Comparisons of a single parameter between two groups were assessed using unpaired *t*-test with Welch's correction. Comparisons of multiple parameters between two groups were assessed using Two-way ANOVA followed by Dunnett's or Tukey's multiple comparisons. *P* smaller than 0.05 was required for statistical significance.

3. Results

3.1. Vγ9Vδ2 T cells from AML patients presented lower IL-21R expression directly ex vivo

To examine the IL-21 signaling pathway in Vγ9Vδ2 T cells, we first evaluated the expression of the IL-21R. Vγ9Vδ2 T cells were identified by anti-Vγ9 and anti-Vδ2 antibody binding in $CD3^+$ lymphocytes (Fig. 1A). The expression of surface IL-21R was then examined in Vγ9Vδ2 T cells by flow cytometry, and compared between healthy individuals and AML patients (Fig. 1B). The Vγ9Vδ2 T cells from AML patients presented significantly lower IL-21R expression than the Vγ9Vδ2 T cells from controls (Fig. 1C). In addition, we assessed IL21R expression in Vγ9Vδ2 T cells after stimulation with phosphoantigen HMBPP and IL-2. Stimulation significantly elevated the expression of IL-21R by Vγ9Vδ2 T cells (Fig. 1C).

It has been shown that Vγ9Vδ2 T cells in AML patients were characterized with increased frequencies of $CD45RA^-CD27^-$ effector memory cells, with reduction in IL-21R expression [17]. Using flow cytometry (Fig. 1D), we found that the frequencies of $CD45RA^-CD27^-$ effector memory cells directly ex vivo in our cohort of AML patients were significantly higher than that in healthy controls (Fig. 1E). However, after stimulation with phosphoantigen HMBPP and IL-2, the effector memory population was significantly reduced in both controls and patients, with no significant differences between the two (Fig. 1F). Indeed, effector memory cells from both controls and patients presented significantly lower IL-21R expression ($P < 0.001$ between Fig. 1C and Fig. 1F for both controls and patients). Stimulation significantly upregulated the expression of IL-21R in effector memory Vγ9Vδ2 T cells.

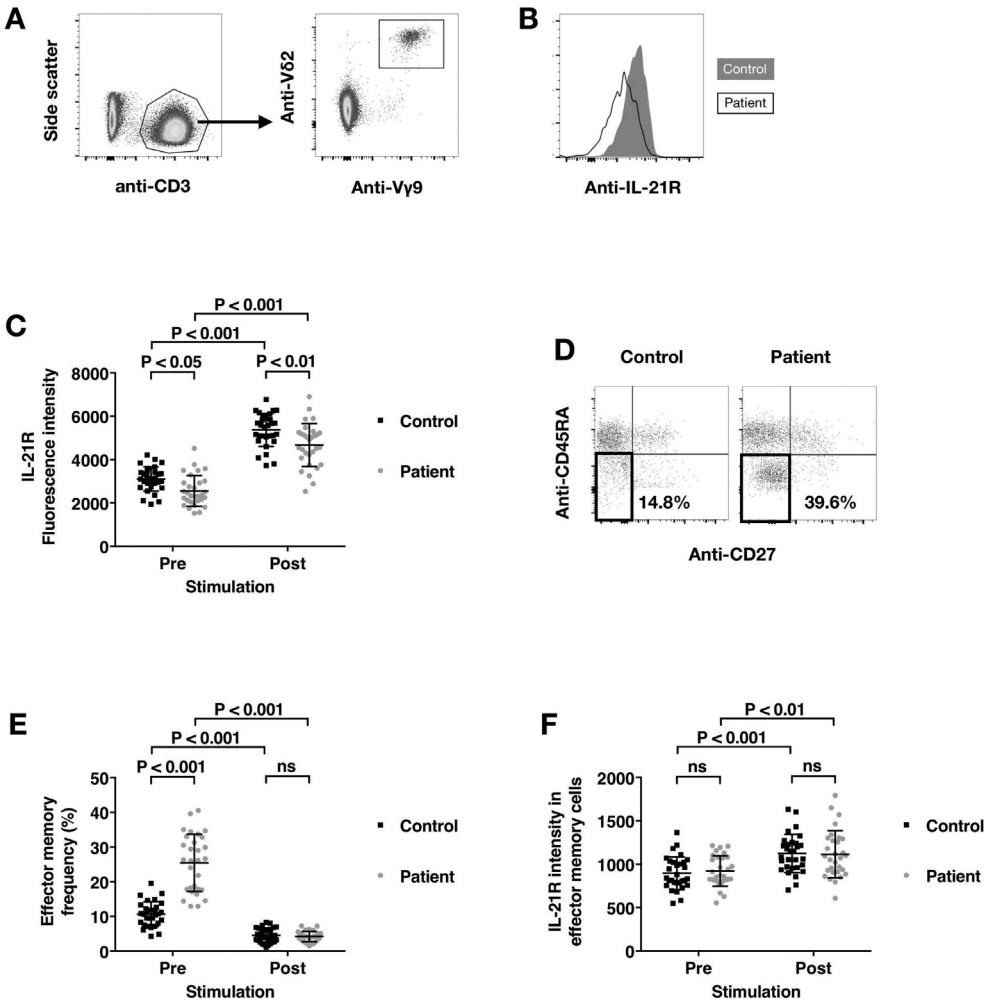

Fig. 1. Vγ9Vδ2 T cells in AML patients presented lower IL-21R expression.
A. Vγ9Vδ2 T cells were identified as Vγ9 and Vδ2 dual-positive cells in pre-gated $CD3^+$ lymphocytes. B. Expression of IL-21R in pre-gated Vγ9Vδ2 T cells from one representative healthy control (grey filled) and one representative AML patient (black unfilled). C. The IL-21R mean fluorescence intensity in Vγ9Vδ2 T cells from a cohort of 30 healthy subjects and 30 AML patients, pre- and post-stimulation with 20 ng/mL phosphoantigen HMBPP and 50 IU/mL IL-2. D. Expression of CD45RA vs. CD27 in Vγ9Vδ2 T cells from one representative healthy control and one representative AML patient. Numbers represent the frequency of $CD45RA^-CD27^-$ effector memory cells. E. The frequency of $CD45RA^-CD27^-$ effector memory cells in controls and patients, pre- and post-stimulation with 20 ng/mL phosphoantigen HMBPP and 50 IU/mL IL-2. F. The IL-21R mean fluorescence intensity by $CD45RA^-CD27^-$ effector memory cells pre- and post-stimulation. Two-way ANOVA followed by Tukey's multiple comparisons.

3.2. Expansion of Vγ9Vδ2 T cells upon IL-21 treatment was less potent in AML patients

To examine whether lower IL-21R expression in Vγ9Vδ2 T cells from AML patients was associated with lower response to IL-21-mediated stimulation, we examined the proliferation of isolated Vγ9Vδ2 T cells in response to a fixed concentration of phosphoantigen HMBPP and IL-2 and varying concentrations of IL-21 (Fig. 2A). The proliferation of Vγ9Vδ2 T cells was examined by pulsing the cells with tritiated thymidine and measuring thymidine incorporation. In both healthy subjects and AML patients, the addition of IL-21 significantly improved the proliferation of Vγ9Vδ2 T cells. In healthy subjects, a concentration of 10 ng/mL was sufficient to elicit a significant improvement, while in AML patients, concentrations of 30 ng/mL or above were required. At 0 concentration, no significant difference between healthy Vγ9Vδ2 T cells and patient Vγ9Vδ2 T cells was observed,

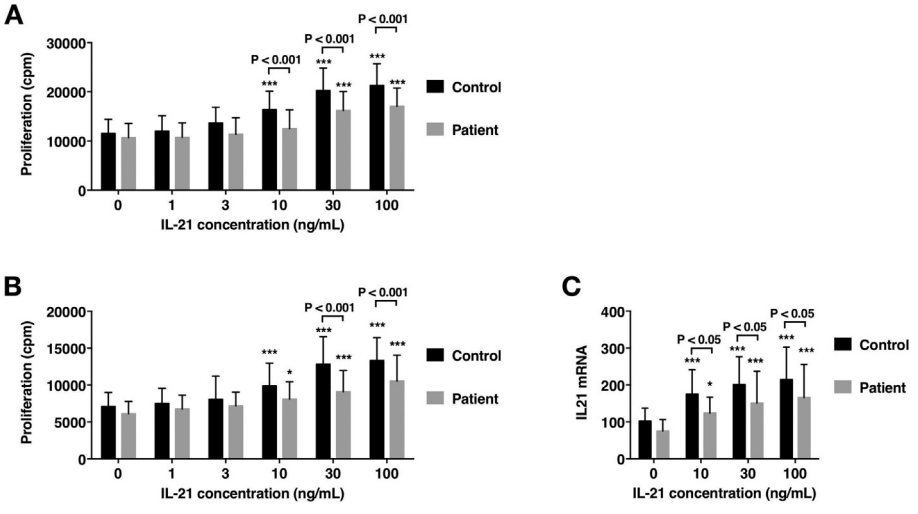

Fig. 2. IL-21 increased Vγ9Vδ2 T cell proliferation and IL-21 expression but was less potent in AML patients.
(A) Pre-isolated Vγ9Vδ2 T cells were stimulated with 20 ng/mL phosphoantigen HMBPP, 50 IU/mL IL-2, and various concentrations of IL-21. (B) PBMCs were stimulated with 20 ng/mL phosphoantigen HMBPP, 50 IU/mL IL-2, and various concentrations of IL-21. Vγ9Vδ2 T cells were then isolated and pulsed with tritiated thymidine for the measurement of proliferation. (C) Pre-isolated Vγ9Vδ2 T cells were stimulated with 20 ng/mL phosphoantigen HMBPP, 50 IU/mL IL-2, and various concentrations of IL-21. The expression of IL-21 mRNA was then examined. Two-way ANOVA followed by Dunnett's multiple comparisons. Asterisks indicate significant difference from the no IL-21 (concentration = 0) control. ***$P < 0.001$.

but from 10 ng/mL onward, the proliferation was significantly higher in healthy Vγ9Vδ2 T cells than in patient Vγ9Vδ2 T cells.

Instead of stimulating isolated Vγ9Vδ2 T cells, we performed another assay stimulating total PBMCs using the same condition as above. The Vγ9Vδ2 T cells were isolated after stimulation, and then pulsed with tritiated thymidine (Fig. 2B). Overall, the proliferation capacity of Vγ9Vδ2 T cells when stimulated with other PBMCs was significantly reduced than the proliferation capacity of Vγ9Vδ2 T cells when stimulated post-isolation ($P < 0.01$ or 0.001 for all comparisons between Fig. 2A and B). Nonetheless, no significant difference between healthy Vγ9Vδ2 T cells and patient Vγ9Vδ2 T cells was observed at 0 concentration, but from 30 ng/mL onward, the proliferation was significantly higher in healthy Vγ9Vδ2 T cells than in patient Vγ9Vδ2 T cells.

3.3. Expression of IL-21 by Vγ9Vδ2 T cells

Subsequently, we examined whether Vγ9Vδ2 T cells expressed endogenous IL-21. Little IL-21 expression from Vγ9Vδ2 T cells was observed under unstimulated condition in neither controls nor patients. Upon stimulation with HMBPP and IL-2, IL-21 mRNA could be detected in Vγ9Vδ2 T cells, without significant difference between healthy controls and AML patients (Fig. 2C). Interestingly, the addition of exogenous IL-21 significantly increased the endogenous IL-21 by Vγ9Vδ2 T cells, and the increase was higher in healthy controls than in AML patients.

3.4. IL-21 in patient Vγ9Vδ2 T cells presented reduced capacity to activate STAT1 and increased capacity to induce Tim-3

To explain the elevated requirement of IL-21 in patient Vγ9Vδ2 T cells, we examined the activation of downstream STATs upon IL-21 treatment. Vγ9Vδ2 T cells were stimulated in the absence or presence of IL-21. The level of phosphorylated STAT1 (P-STAT1) and P-STAT3 was then examined using intracellular flow cytometry (Fig. 3A and C). In both healthy subjects and AML patients, IL-21 elevated the level of P-STAT1 in Vγ9Vδ2 T cells; however, IL-21-mediated increase of STAT1 was smaller in AML patients than in healthy subjects (Fig. 3B). IL-21 also elevated the level of P-STAT3 in Vγ9Vδ2 T cells, without significant differences between healthy subjects and AML patients (Fig. 3D).

IL-21 was shown to induce Tim-3, a checkpoint molecule previously associated with immune suppression in cancer [19,20]. Hence, we examined the expression Tim-3 in Vγ9Vδ2 T cells in the absence or presence of IL-21 (Fig. 4A). In both healthy subjects and AML patients, IL-21 significantly increased the frequency of Tim-3 expressing Vγ9Vδ2 T cells (Fig. 4B). Also, the frequency of Tim-3 expressing Vγ9Vδ2 T cells was elevated in AML patients compared to that in healthy subjects, with or without IL-21. In AML patients, the IL-21R expression between Tim-3^+ and Tim-3^- Vγ9Vδ2 T cells were compared. Tim-3^+ Vγ9Vδ2 T cells presented significantly lower IL-21R expression than Tim-3^- Vγ9Vδ2 T cells (Fig. 4C).

3.5. Tim-3 blocking significantly increased Vγ9Vδ2 T cell proliferation, STAT1 phosphorylation and IL-21R expression

Since Tim-3 is an inhibitory receptor, we examined the effect of Tim-3 blocking on the IL-21 signal transduction pathway in Vγ9Vδ2 T cells from AML patients. As little as 3 ng/mL IL-21 could significantly enhance the proliferation of Vγ9Vδ2 T cells when Tim-3 blocking antibody was added in the cell culture, a result significantly improved from adding the isotype control to the anti-Tim-3 antibody (Fig. 5A). Blocking Tim-3 also significantly increased the STAT1 and STAT3 phosphorylation levels in response to IL-21 stimulation (Fig. 5B and C). Interestingly, blocking Tim-3 also significantly increased the expression of IL-21R in Vγ9Vδ2 T cells (Fig. 5D and E).

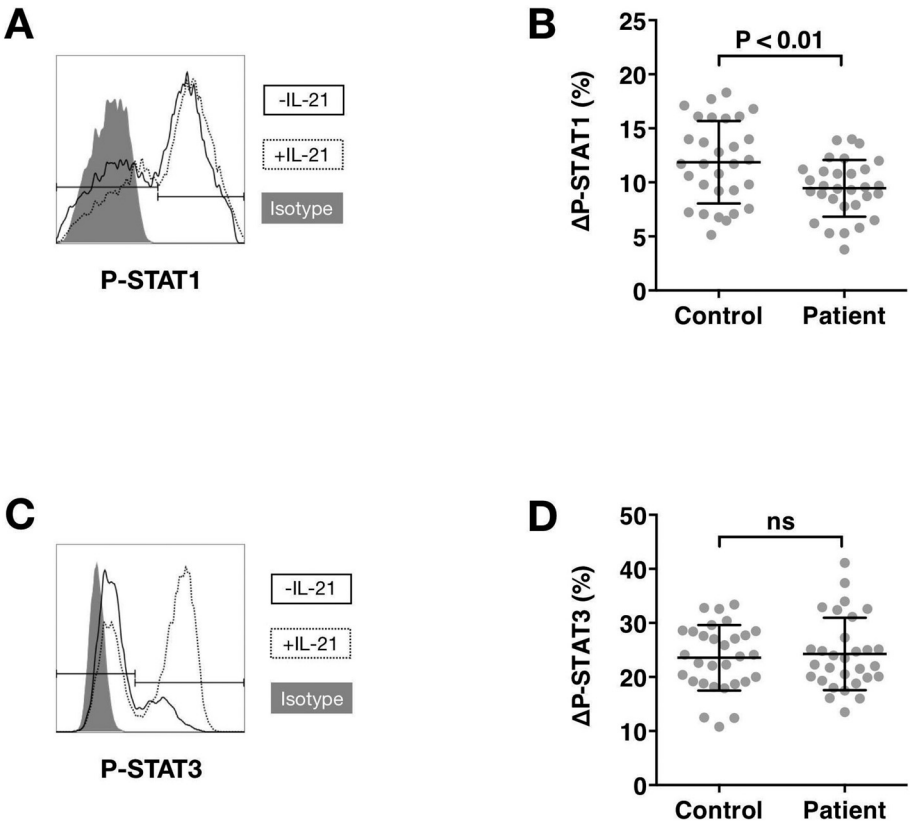

Fig. 3. Phosphorylation of STAT1 and STAT3 following IL-21 stimulation.
Vγ9Vδ2 T cells were stimulated with 20 ng/mL phosphoantigen HMBPP and 50 IU/mL IL-2 in the absence of IL-21 or in the presence of 100 ng/mL IL-21. A. Representative staining of STAT1 phosphorylation in Vγ9Vδ2 T cells without IL-21 (black solid) and with IL-21 (black dotted), overlaid with isotype control (grey filled). B. Increase in P-STAT1 with exogenous IL-21 in Vγ9Vδ2 T cells from 30 healthy subjects and 30 AML patients. C. Representative staining of STAT3 phosphorylation in Vγ9Vδ2 T cells without IL-21 (black solid) and with IL-21 (black dotted), overlaid with isotype control (grey filled). D. Increase in P-STAT3 with exogenous IL-21 in Vγ9Vδ2 T cells from 30 healthy subjects and 30 AML patients. B and D. Unpaired *t*-test with Welch's correction. ns, not significant.

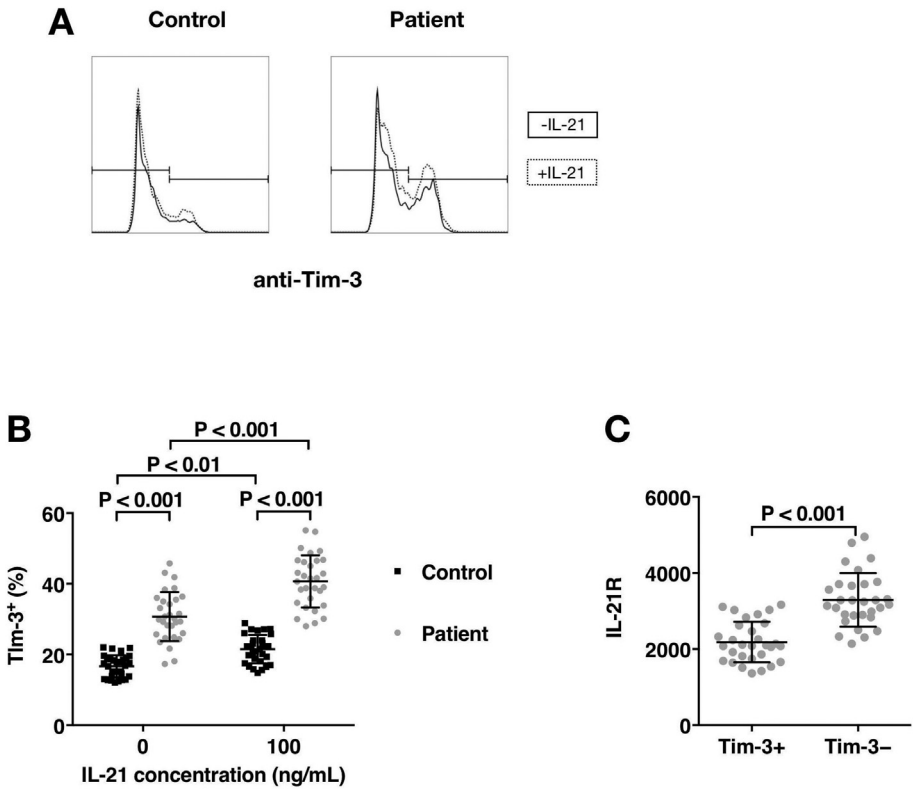

Fig. 4. Tim-3 expression in stimulated Vγ9Vδ2 T cells in the absence or presence of IL-21.

4. Discussion

It has been shown that depending on the types of environmental factors during stimulation, Vγ9Vδ2 T cells can exhibit a variety of functional subtypes, some of which are implicated in antitumor immune responses [2]. IL-21 is an attractive candidate for cancer immunotherapies due to its ability to enhance cytotoxicity and promote cell proliferation [21–23]. This study demonstrated that IL-21 could significantly expand the Vγ9Vδ2 T cells and enhance their endogenous IL-21 expression in vitro. However, the efficacy of IL-21 was limited, as IL-21 also increased the expression of checkpoint molecule Tim-3. The reduction in the efficacy of IL-21 was especially pronounced in AML patients, who presented lower IL-21R and higher Tim-3 expression. Blocking Tim-3 could significantly improve the efficacy of IL-21. Interestingly, blocking Tim-3 also significantly increased the expression of IL-21R.

Interestingly, isolated Vγ9Vδ2 T cells alone presented significantly more potent proliferation than Vγ9Vδ2 T cells in the presence of other PBMCs, possibly due to the existence of regulatory cells, such as Treg cells, Tr1 cells, and inhibitory antigen-presenting cells, in the unfractionated PBMCs. In the future, the regulatory mechanisms that suppress Vγ9Vδ2 T cell expansion should be investigated. In addition, Tim-3 blocking only induced a small upregulation of proliferation, STAT1 phosphorylation, and IL-21R expression in the Vγ9Vδ2 T cells from AML patients. Co-expression of Tim-3 and other inhibitory molecules, such as LAG-3, PD-1, and TIGIT, has been shown in conventional VαVβ T cells [24]. It is possible that these Tim-3$^+$ Vγ9Vδ2 T cells expressed those inhibitory molecules as well. Antagonization of Tim-3 with additional receptors may be required for the optimal response from Vγ9Vδ2 T cells. Whether IL-21 regulates the expression of these additional inhibitory molecules should be examined in future studies. Results from this study will improve the design of future Vγ9Vδ2 T cell-based clinical trials. The current trials involve a Vγ9Vδ2 T cell expansion step, either in vivo using an administration of pamidronate, zoledronate, and low-dose IL-2, or ex vivo using phosphoantigens and a combination of cytokines followed by adoptive transfer into the recipient [18,25]. One common limitation is the progressive loss of proliferative capacity, previously attributed to the induction of exhaustion by continuous stimulation by phosphoantigens and IL-2. Here, we showed that IL-21-mediated upregulation of Tim-3 could be limiting Vγ9Vδ2 T cell proliferation as well. Hence, an anti-Tim-3 antibody may be incorporated in the expansion step, both to remove Tim-3-mediated inhibition and to improve the responsiveness toward IL-21.

This study has many limitations that need to be addressed in future investigations. First, the characteristics of Vγ9Vδ2 T cell before and after IL-21 stimulation were not examined. IL-21 could potentiate the cytotoxicity of NK cells and CD8 T cells [26,27]. Hence, it should be addressed in future studies whether IL-21-treated Vγ9Vδ2 T cells could eliminate AML cells. Second, the molecular signaling events linking Tim-3 and IL-21R expression and STAT phosphorylation should be investigated. Inhibitors to STAT phosphorylation should also be used to examine the involvement of STATs in Tim-3-mediated inhibitory pathways. In addition, the efficiency of IL-21-treated Tim-3-blocked Vγ9Vδ2 T cells should be examined in animal models. Whether IL-21- and anti-Tim-3-treated Vγ9Vδ2 T cells could be tolerated by AML patients and mediate AML remission in vivo should be investigated.

Vγ9Vδ2 T cells were stimulated with 20 ng/mL HMBPP and 50 IU/mL IL-2 in the absence or presence of 100 ng/mL IL-21. A. Gating of Tim-3 + Vγ9Vδ2 T cells in the absence (solid) or presence (dotted) of IL-21, in one representative healthy control and one representative AML patient. B. The frequency of Tim-3 + Vγ9Vδ2 T cells in 30 healthy subjects and 30 AML patients, without or with IL-21. Two-way ANOVA followed by Tukey's multiple comparisons. C. The IL-21R mean fluorescence intensity of Tim-3 + and Tim-3 − Vγ9Vδ2 T cells in 30 AML patients. Unpaired t-test with Welch's correction.

Conflict of interest

None.

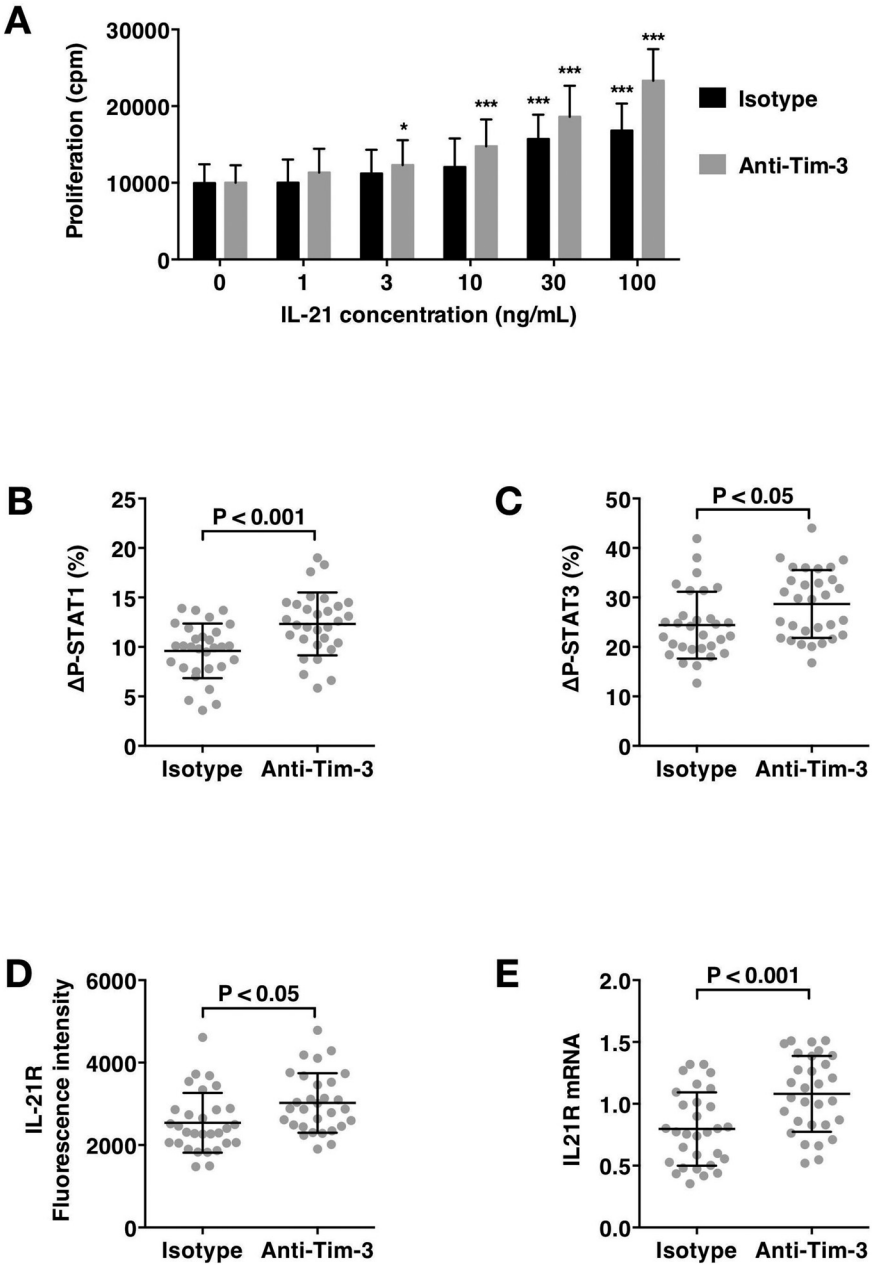

Fig. 5. The effects of Tim-3 blocking on Vγ9Vδ2 T cell proliferation and STAT phosphorylation.
A. Vγ9Vδ2 T cells from AML patients were stimulated with 20 ng/mL HMBPP, 50 IU/mL IL-2, and varying concentrations of IL-21, in the presence of 10 μg/mL anti-Tim-3 antibody or isotype control. Two-way ANOVA followed by Dunnett's multiple comparisons. Asterisks indicate significant difference from the no IL-21 (concentration = 0) control. $*P < 0.05$. $***P < 0.001$. B to E. Vγ9Vδ2 T cells from AML patients were stimulated with 20 ng/mL HMBPP, 50 IU/mL IL-2, and 100 ng/mL IL-21, in the presence of 10 μg/mL anti-Tim-3 antibody or isotype control. B. Increase in P-STAT1 with anti-Tim-3 or isotype control. C. Increase in P-STAT3 with anti-Tim-3 or isotype control. D. IL-21R mean fluorescence intensity with anti-Tim-3 or isotype control. E. IL21R mRNA expression with anti-Tim-3 or isotype control. Unpaired *t*-test with Welch's correction.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81600131, No. 81770126, and No. 81800163), and Fujian Provincial Natural Science Foundation (No. 2017J01354, and No. 2017J01360, and No. 2018D0015).

References

[1] Y. Chien, C. Meyer, M. Bonneville, γδ T cells: first line of defense and beyond, Annu. Rev. Immunol. (2014), https://doi.org/10.1146/annurev-immunol-032713-120216.
[2] Z. Xiang, W. Tu, Dual face of Vγ9Vdδ2-T cells in tumor immunology: anti- versus pro-tumoral activities, Front. Immunol. (2017), https://doi.org/10.3389/fimmu.2017.01041.
[3] B. Altincicek, J. Moll, N. Campos, G. Foerster, E. Beck, J.F. Hoeffler, et al., Cutting edge: human gamma delta T cells are activated by intermediates of the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis, J. Immunol. (2001), https://doi.org/10.4049/jimmunol.166.6.3655.
[4] Z.W. Chen, Multifunctional immune responses of HMBPP-specific Vγ2Vδ2 T cells in M. tuberculosis and other infections, Cell. Mol. Immunol. (2013), https://doi.org/10.1038/cmi.2012.46.
[5] H.-J. Gober, M. Kistowska, L. Angman, P. Jenö, L. Mori, G. De Libero, Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells, J. Exp. Med. (2003), https://doi.org/10.1084/jem.20021500.
[6] D.V. Correia, A. Lopes, B. Silva-Santos, Tumor cell recognition by γδ T lymphocytes: T-cell receptor vs. NK-cell receptors, Oncoimmunology (2013), https://doi.org/10.4161/onci.22892.
[7] P. Wrobel, H. Shojaei, B. Schittek, F. Gieseler, B. Wollenberg, H. Kalthoff, et al., Lysis of a broad range of epithelial tumour cells by human γδ T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition, Scand. J. Immunol. (2007), https://doi.org/10.1111/j.1365-3083.2007.01963.x.
[8] M. Von Lilienfeld-Toal, J. Nattermann, G. Feldmann, E. Sievers, S. Frank, J. Strehl, et al., Activated γδ T cells express the natural cytotoxicity receptor natural killer p44 and show cytotoxic activity against myeloma cells, Clin. Exp. Immunol. (2006), https://doi.org/10.1111/j.1365-2249.2006.03078.x.
[9] M.S. Braza, B. Klein, G. Fiol, J.F. Rossi, γδT-cell killing of primary follicular lymphoma cells is dramatically potentiated by GA101, a type II glycoengineered anti-CD20 monoclonal antibody, Haematologica (2011), https://doi.org/10.3324/haematol.2010.029520.
[10] M.R. Davis, Z. Zhu, D.M. Hansen, Q. Bai, Y. Fang, The role of IL-21 in immunity and cancer, Cancer Lett. 358 (2015) 107–114, https://doi.org/10.1016/j.canlet.2014.12.047.
[11] N. Caccamo, M. Todaro, M.P. Manna, G. Sireci, G. Stassi, F. Dieli, IL-21 regulates the differentiation of a human γδ T cell subset equipped with B cell helper activity,

PLoS One (2012), https://doi.org/10.1371/journal.pone.0041940.
[12] R.R. Bansal, C.R. Mackay, B. Moser, M. Eberl, IL-21 enhances the potential of human γδ T cells to provide B-cell help, Eur. J. Immunol. (2012), https://doi.org/10.1002/eji.201142017.
[13] A. Thedrez, C. Harly, A. Morice, S. Salot, M. Bonneville, E. Scotet, IL-21-mediated potentiation of antitumor cytolytic and proinflammatory responses of human V{gamma}9V{delta}2 T cells for adoptive immunotherapy, J. Immunol. (2009), https://doi.org/10.4049/jimmunol.0803068.
[14] D.C. Deniger, S.N. Maiti, T. Mi, K.C. Switzer, V. Ramachandran, L.V. Hurton, et al., Activating and propagating polyclonal gamma delta T cells with broad specificity for malignancies, Clin. Cancer Res. (2014), https://doi.org/10.1158/1078-0432.CCR-13-3451.
[15] M. Alcalay, A. Orleth, C. Sebastiani, N. Meani, F. Chiaradonna, C. Casciari, et al., Common themes in the pathogenesis of acute myeloid leukemia, Oncogene (2001), https://doi.org/10.1038/sj.onc.1204642.
[16] H. Bower, T.M.L. Andersson, M. Bjorkholm, P.W. Dickman, P.C. Lambert, A.R. Derolf, Continued improvement in survival of acute myeloid leukemia patients: an application of the loss in expectation of life, Blood Cancer J. (2016), https://doi.org/10.1038/bcj.2016.3.
[17] J. Gertner-dardenne, R. Castellano, E. Mamessier, S. Garbit, E. Kochbati, A. Etienne, et al., Human Vγ9Vδ2 T cells specifically recognize and kill acute myeloid leukemic blasts, J. Immunol. (2012), https://doi.org/10.4049/jimmunol.1103710.
[18] D.C. Deniger, J.S. Moyes, L.J.N. Cooper, Clinical applications of gamma delta T cells with multivalent immunity, Front. Immunol. (2014), https://doi.org/10.3389/fimmu.2014.00636.
[19] G. Han, G. Chen, B. Shen, Y. Li, Tim-3: an activation marker and activation limiter of innate immune cells, Front. Immunol. (2013) 449, https://doi.org/10.3389/fimmu.2013.00449 [Internet].
[20] A.C. Anderson, Tim-3: an emerging target in the cancer immunotherapy landscape, Cancer Immunol. Res. 2 (2014) 393–398, https://doi.org/10.1158/2326-6066.CIR-14-0039.
[21] R. Zeng, R. Spolski, S.E. Finkelstein, S. Oh, P.E. Kovanen, C.S. Hinrichs, et al., Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function, J. Exp. Med. 201 (2005) 139–148, https://doi.org/10.1084/jem.20041057.
[22] S.J. Santegoets, A.W. Turksma, D.J. Powell Jr., E. Hooijberg, T.D. de Gruijl, IL-21 in cancer immunotherapy: at the right place at the right time, Oncoimmunology 2 (2013) e24522, , https://doi.org/10.4161/onci.24522.
[23] H. Seo, I. Jeon, B.S. Kim, M. Park, E.A. Bae, B. Song, et al., IL-21-mediated reversal of NK cell exhaustion facilitates anti-tumour immunity in MHC class I-deficient tumours, Nat. Commun. 8 (2017), https://doi.org/10.1038/ncomms15776.
[24] A.C. Anderson, N. Joller, V.K. Kuchroo, Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation, Immunity 44 (2016) 989–1004, https://doi.org/10.1016/j.immuni.2016.05.001.
[25] B. Silva-Santos, K. Serre, H. Norell, γδ T cells in cancer, Nat. Rev. Immunol. (2015), https://doi.org/10.1038/nri3904.
[26] J. Parrish-Novak, S.R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J.A. Gross, et al., Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function, Nature (2000), https://doi.org/10.1038/35040504.
[27] A.P.R. Sutherland, N. Joller, M. Michaud, S.M. Liu, V.K. Kuchroo, M.J. Grusby, IL-21 promotes CD8+ CTL activity via the transcription factor T-bet, J. Immunol. (2013), https://doi.org/10.4049/jimmunol.1201730.