Original Research Article

Lactic acid inhibits the cytotoxicity of NK-92 cells via activating the lactate receptor GPR81

ABSTRACT

Aims: Lactate acid functions as not only an energy source but a signaling molecule through the lactate receptor GPR81 under physiological conditions. However, the pathological role of lactic acid in the tumor microenvironment remains unclear, particularly for immune cells.
Methodology: NK-92 cells were treated with L-lactic acid solutions at final concentrations of 10, 20, 30, and 40 mM, and its cell viability and cytotoxicity on A549 cells and A375 cells were evaluated by CCK8 assay and crystal violet assay, respectively. Furthermore, qPCR was used to assess the expression of GPR81 and cytotoxicity-related genes in NK-92 cells treated with antagonist and agonist. And their relationship between lactate/GPR81 pathway and cytotoxicity-related genes were analyzed by Pearson's correlation.
Results: The viability of NK-92 cells was inhibited by L-lactic acid with increasing concentration. Additionally, the cytotoxic activity against tumor cells of NK-92 cells treated with L-lactic acid decreased with increasing concentration. Moreover, qPCR results demonstrated that GPR81 can be activated by lactic acid or agonist (3,5-DHBA) and downregulate the expression cytotoxicity-related genes which included *FASLG* gene(Fas Ligand),*TNF-α* gene(Tumor necrosis factor-α), *INFG* gene (Interferon-γ), *RPF1* gene (Perforin 1), *GZMA* gene (Granzyme A), *GZMB* gene (Granzyme B), *GZMH* gene (Granzyme H), *GAMK* gene (Granzyme K) and *GZMM* gene (Granzyme M). And the expression of GPR81 returned to near-control level when treated with L-lactic acid in the presence of antagonist (3-OBA), the expression of cytotoxicity-related genes did as well. Pearson's correlation analysis of cytotoxicity-related genes with GPR81 revealed that their correlation coefficient seems negative.
Conclusion: lactic acid can activate the GPR81 to downregulate the expression of cytotoxicity-related genes, subsequently lower the cytotoxicity of NK-92 cells.

Keywords: lactate acid, RT-qPCR, cytotoxicity-related genes, correlation analysis

1. INTRODUCTION

"Accelerated glucose metabolism in tumor cells, the so-called "Warburg effect", can accumulate lactic acid even to high concentration of 40 mM in the extracellular tumor microenvironment (TME)" [1]. "It was highlighted that lactic acid, a glycolytic metabolite, is not a waste product of proliferating cancer cells but actually elicits a broad spectrum of effects that are critical for tumor progression and metastasis" [2]. "The production and export of lactate acid by rapidly proliferating tumor cells not only supports tumor to grow and to escape immune recognition [3], but also influences the function of immune cells, such as T cells [4], NK cells"[5]. "Additionally, acidification of the TME also limits the efficacy of cell-based immunotherapies" [6, 7].

“Playing an critical function as an energy source in various tissues, including brain [8], skeletal muscle [9], and cancer [10], lactate has been shown to function as a signaling molecule by binding to a specific receptor followed by signal transduction” [11]. “G protein-coupled receptor 81 (GPR81), also known as hydroxycarboxylic acid receptor 1 (HCAR1), has recently been identified as a lactate receptor” [12, 13]. “It was shown that L-lactate is a natural ligand and agonist of GPR81, along other monocarboxylates such as α-hydroxybutyrate, glycolate, α-hydroxyisobutyrate, and γ-hydroxybutyrate” [14]. “The receptor has been found in adipocytes [15], in the brain [16], in liver, skeletal muscle, and other human, mouse, and rat tissues” [17]. Recently, GPR81 also has been identified in immune cells [18-20]. It is required for inflammation protection and the downregulation of innate immunity in immune cells [18, 21, 22], as well as for cancer cell survival [23]. Through activating GPR81, tumor cell-derived lactic acid can promote tumor growth in multiple cancers [23, 24], and also block the presentation of tumor-specific antigens to other immune cells in DCs [25].

“Although previous studies have established the crucial role of metabolism in the function of immune effector cells, this approach to lactate/GPR81 association is still in its infancy and requires further investigation” [26, 27]. “Furthermore, with the advent of cell-based immunotherapies (NK cells, T cells), the importance of GPR81 in immune cells needs to be addressed appropriately. NK-92 cells, human natural killer cytotoxic cell line, are an off-the-shelf, cell-based immunotherapy currently in clinical trials for a variety of cancer types” [28]. Here, we studied the proliferation and cytotoxicity of NK-92 cells in the presence of lactic acid. In addition, RT-qPCR was performed to examine the expression and association between GPR81 and cytotoxicity-related genes of NK-92 cells treated with antagonist and agonist.

2. MATERIAL AND METHODS

2.1 CELL LINES AND REAGENTS

NK-92 cells, the lung cancer cell line A549 and human melanoma cell line A375 cells were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). NK-92 cells were cultured in MEM-α (Gibco) supplemented with 12.5% fetal calf serum (Sigma-Aldrich), 12.5% horse serum (Gibco). A549 and A375 cells were maintained in 10% Ham's F-12K medium and 10% DMEM, respectively.

L-lactic acid (L-LA), supplied by Aladdin Ltd(Shanghai, China), were dissolved in 10 mL of sterilized MEM-α medium to give a 0.1 M stock solution. Dosing solutions were prepared by serially diluting the stock solution with sterilized MEM-α medium. Final concentrations were set as 10 mM, 20mM, 30mM and 40 mM. 3,5-DHBA and 3-OBA, supplied by Haoyuan Ltd. (Shanghai, China), were dissolved in 10 mL of sterilized MEM-α medium to give a 1 mM solution. All other chemicals and solvents were of analytical or pharmaceutical grade.

2.2 CELL VIABILITY ASSAY

NK-92 cells were seeded in 96-well plates at a density of 2×10^5 cells/100μL. NK-92 cells were treated with L-lactic acid solutions at final concentrations of 10, 20, 30, and 40 mM. The control cells were not treated. After culturing for 12 hours, a total of 10 μL CCK8 solution (Beyotime, Ltd., China) was added into each well of a 96-well plate. Then, the plate was incubated at 37℃ in a 5% CO_2 environment for 2 hours. The absorbance at 450nm was measured using FlexA-200 microplate reader (Aosheng, Ltd., China).

2.3 CRYSTAL VIOLET ASSAY

The cytotoxicity of lactate-co-cultured NK-92 cells against tumor cells was assessed using a modified crystal violet assay which was described in a previous study [29]. The technique is based on the measurement of maintained adherence of cells which is the staining of attached cells with crystal violet dye binding to proteins and DNA. Briefly, A549 and A375 cells were seeded onto 96-well plates for their adherence at 1×10^5 cells/100μL in each well for 12 hours. Prior to co-culturing with NK-92 cells which were treated with 10, 20, 30 and 40mM L-lactic acid, the supernatant medium in the 96-well was carefully removed with a micropipette. 100 μL treated NK-92 cells at the concentration of 2×10^5 cells/mL were added to each well and incubated for 6 hours at 37℃ in a 5% CO_2 environment. A positive control and a blank control were set up, and all treatments were repeated three times. After 6 hours of incubation, the medium was carefully aspirated and each well cleaned twice with a gentle stream of PBS buffer. Subsequently, tap the plate on filter paper to remove any remaining liquid. Add 50 μL of 0.5% crystal violet staining solution (Sangon Ltd., China) into each well, and incubate for 20 minutes at room temperature. Each well was washed twice with PBS buffer, and the plate was allowed to air dry for 2 hours at room temperature without its lid. Absorbance (A) was detected at a wavelength of 590 nm with FlexA-200 microplate reader (Aosheng, Ltd., China). Results are expressed as cytotoxicity percentage of NK-92 cells using the formula: cytotoxicity percentage = [100% − ($A_{treatment}$− $A_{blank\ control}$) / ($A_{positive\ control}$− $A_{blank\ control}$) × 100%].

2.4 RT-QPCR FOR GPR81 AND CYTOTOXICITY-RELATED GENES IN NK-92 CELLS TREATED WITH ANTAGONIST AND AGONIST

The relative expression of GPR81 and cytotoxicity-related genes (listed in Table 1), including *FASLG* involved in the death receptor pathway, TNF-α and IFNG involved in the cytokine pathway, RPF1, GZMA, GZMB, GZMH, GZMK, and GZMM involved in the perforin and granzyme pathway, was quantified to assess the role of lactic acid for GPR81. RT-qPCR was performed on two NK-92 groups treated with 20 mM L-lactic acid in the absence or presence of 1 mM GPR81 antagonist (3-hydroxybutyric acid, 3-OBA), as well as the GPR81 agonist group treated with 1 mM 3,5-dihydroxybenzoic acid (3,5-DHBA). Control group was treated with equal volume of MEM-α medium instead of 20 mM L-lactic acid. Four groups were cultured in 6-well plate for 12 hours. The cells were then transferred to 1.5-mL tubes, and the excess supernatant was removed by centrifugation (300 × g, 5 min). The cells were immediately frozen in liquid nitrogen for 5 minutes before being stored at −80℃. Total RNA was prepared promptly using RNA Extraction Kit (TakaRa MiniBEST Universal RNA Extraction Kit; Code No. 9767). RNAs were reverse-transcribed with cDNA Reverse Transcription Kits (PrimeScript™ RT reagent Kit with gDNA Eraser; Code No. RR047A) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR) was performed using Takara TB GREEN Premix Ex Taq II reagent (Takara Bio, Japan) on a Bio-Rad CFX 384 real-time PCR instrument. Reverse transcription PCR primer sequences for GPR81 and cytotoxicity-related genes were listed in Table 1. Primer design was performed with available sequences using the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and primer synthesis was performed by Sangon Ltd. (Shanghai, China). The PCR cycling conditions included a 30-second denaturation at 95℃ followed by 40 cycles of 5 seconds at 95℃, 31 seconds at 60℃. GPR81 and nine genes related with cytotoxicity of NK-92 were analyzed using CFX maestro version 4.1 (Bio-Rad, Hercules, CA, USA). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and presented as fold change [30]. The ΔCt value of each sample was calculated using GADPH as an endogenous control gene.

Table 1. Primer sequences for reverse transcription PCR

Symbol	Description	Primer sequence (5′-3′)	Amplicon size(bp)
GPR81	Hydroxycarboxylic acid receptor 1, HCAR1	F:AATTTGGCCGTGGCTGATTTC R:CCGTAAGGAACACGATGCTCC	157
FASLG	Fas Ligand	F:ATTTAACAGGCAAGTCCAACTCA R:GGCCACCCTTCTTATACTTCACT	99
TNF-α	Tumor Necrosis Factor-α	F:CCTCTCTCTAATCAGCCCTCTG R:GAGGACCTGGGAGTAGATGAG	220
IFNG	Interferon-γ	F:TCGGTAACTGACTTGAATGTCCA R:TCGCTTCCCTGTTTTAGCTGC	93
PRF1	Perforin 1	F:CCGTGTGGTGAGAACAGTGA R:TGGGAATACGAAGACAGCCC	93
GZMA	Granzyme A	F:TCTCTCTCAGTTGTCGTTTCTCT R:GCAGTCAACACCCAGTCTTTTG	170
GZMB	Granzyme B	F:CCCTGGGAAAACACTCACACA R:GCACAACTCAATGGTACTGTCG	110
GZMK	Granzyme K	F:CAAACAGCCGCAAAACTCAAT R:GCAGGGTGTCAGAAGGTCTT	139
GZMM	Granzyme M	F:GACGGGAAAGTGAAGCCCAG R:TCCAGAAGCGGCTGTTGTTA	196
GZMH	Granzyme H	F:CTGGCTGGGGTTATGTCTCAA R:GGCTACGTCCTTACACACGAG	203
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase	F:GGAGCGAGATCCCTCCAAAAT R:GGCTGTTGTCATACTTCTCATGG	197

2.5 STATISTICAL ANALYSIS

All the data were collected statically analyzed using DPS 7.05 statistical software [31]. Results are presented as mean ± standard deviation (SD). Comparison among different treatments was based on Fisher LSD to assess the difference between treatments at probability levels of 0.001, 0.05, and 0.01. Student's t-test was performed to statistically compare the gene expression of qRT-PCR results. Figures were plotted by GraphPad Prism 8.0.2 and Origin pro 2020b. The Pearson correlation coefficient was used to determine the association between *GPR81* and cytotoxicity-related genes with a significance level of 0.05.

3. RESULTS

3.1 EFFECTS OF L-LACTIC ACID ON THE VIABILITY OF NK-92 CELLS

Cell viability of NK-92 treated with L-lactic acid was shown in Figure 1. When the L-lactic acid concentration was 0, 10, 20, 30, 40 mM, the viability of NK-92 cells after treatment for 12 h were 97.9%, 79.3%, 14.3%, 6.7%, 4.8%. The viability of NK-92 cell exhibited a significant inhibition with increasing lactic acid concentration ($P<0.01$).

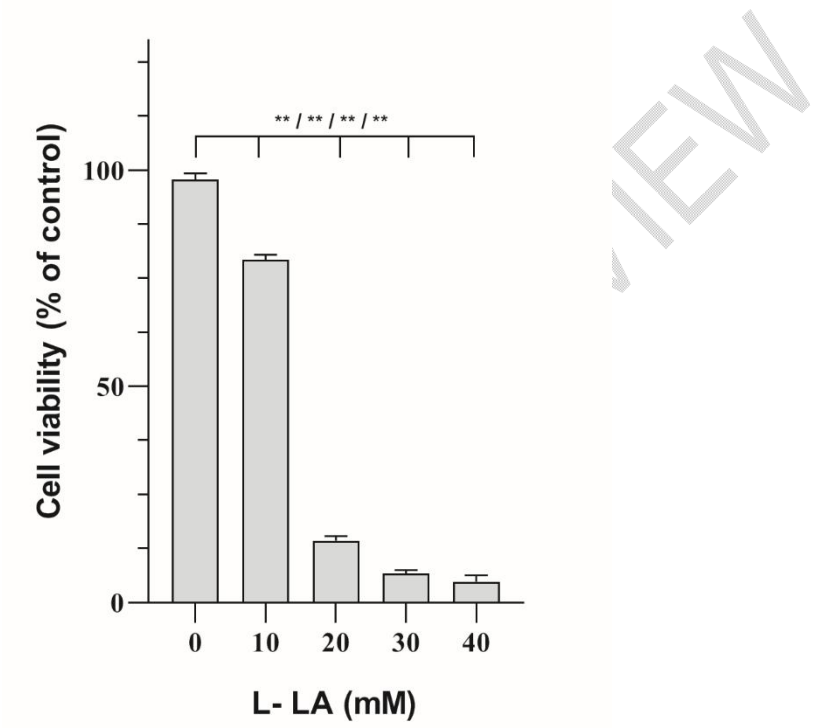

Fig. 1. Inhibitory effect of L-lactic acid on the viability of NK-92 cells.

*L-LA represents L-Lactic acid. Each treatment has three duplicates. Differences compared to control group were considered significant at ** p< 0.01.*

3.2 NK-92 CYTOTOXICITY ON A549 CELLS AND A375 CELLS IN THE PRESENCE OF DIFFERENT CONCENTRATION OF L-LACTIC ACID

The cytotoxicity of NK-92 on A549 cells and A375 cells evaluated by crystal violet assay were shown in Figure 2-A and 2-B. While the concentrations of L-lactic acid were 0, 10, 20, 30, 40mM, the average cytotoxicity of NK-92 cells on A549 was 91.9%, 77.5%, 59.0%, 53.6%, 38.7%, respectively. Similarly, treated with a series concentrations of L-lactic acid with 0 (CK), 10, 20, 30 and 40mM, the average cytotoxicity of NK-92 cells on A375 decreased from 70.7%, 63.8%, 57.3%, 48.3%, to 33.0%. The cytotoxicity results revealed that L-lactic acid could significantly suppress the cytotoxic impact of NK-92 cells on A549 and A375 ($P<0.01$), and the inhibition rates increased with the concentration of L-lactic acid.

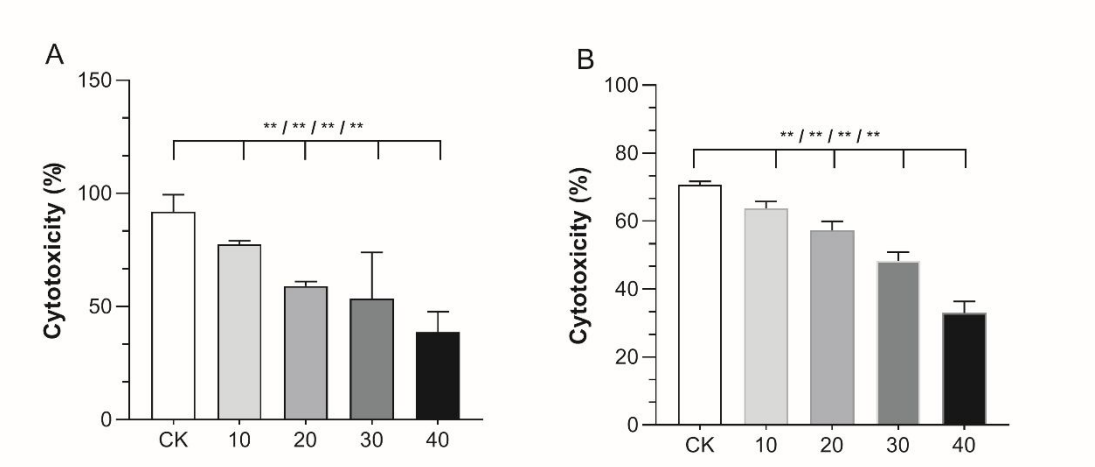

Fig. 2. Cytotoxicity of NK-92 treated with different concentrations of L-lactic acid on A549(A) and A375(B) cells.

*Each treatment has three duplicates. Differences compared to control group were considered significant at ** p< 0.01.*

3.3 RT-QPCR FOR THE EXPRESSION OF GPR81 IN NK-92 CELLS

GPR81 expression in NK-92 cells treated with antagonist and agonist were demonstrated in Figure 3. The relative expression of lactate receptors GPR81 of NK-92 cells treated with 20 mM L-lactic acid was approximately 37 times higher than that of the control (0 mM L-lactic acid). However, while NK-92 cells were treated with 20 mM L-lactic acid in the presence of GPR81 antagonist (1 mM 3-OBA), it dropped dramatically, and had no significant difference with the control. In addition, when the NK-92 cells were treated with agonist (1 mM 3,5-DHBA), the relative expression reached to approximately 171 times higher than that of the control. Surprisingly, the relative expression of GPR81 in NK-92 cells treated with 20 mM sodium L-lactate exhibited no significant increase compared to that of NK-92 cells treated with 20 mM L-lactic acid in the presence of GPR81 antagonist. It was therefore proposed that L-lactic acid could have an immunosuppressive impact via the lactate receptor (GPR81).

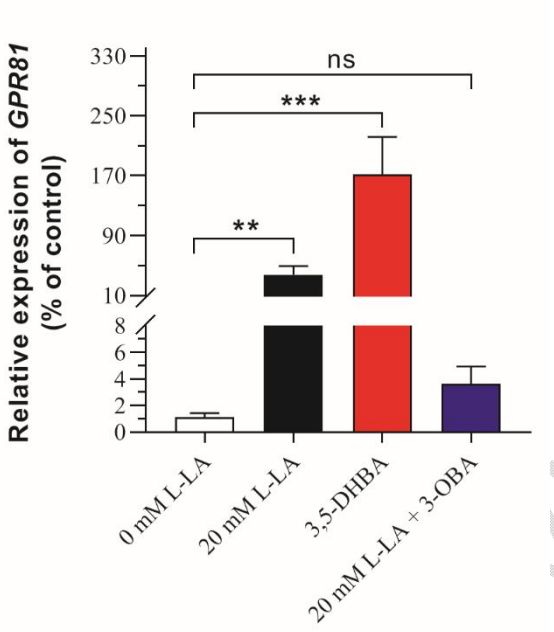

Fig. 3. Relative expression of GPR81 in NK-92 cells treated with L-lactic acid, GPR81antagonist and GPR81agonist.

*ns means p≥0.05, ** means p<0.01, and *** means p<0.001..*

3.4 RT-QPCR FOR THE EXPRESSION OF CYTOTOXICITY-RELATED GENE IN NK-92 CELLS

Statistically, the relative expression of FASLG in NK-92 cells treated with L-lactic acid or GPR81-specific agonist had no significant difference with the control (P>0.05) (Fig. 4-A). This indicated that activating or inhibiting GPR81 had no effect on the cytotoxicity of NK-92 cells via the death receptor pathway. On the contrary, the relative expression of TNF-α and IFNG treated with L-lactic acid or GPR81-specific agonist was significantly reduced compared with the control (P<0.01) (Fig. 4-B, C). Nevertheless, their expression level returned to near-control with no significant difference while they were treated with L-lactic acid in the presence of GPR81-specific antagonist. Therefore, it indicated that L-lactic acid conducted signal transduction to cells through GPR81, affected the gene expression of TNF and IFNG, thereby inhibiting the anti-tumor ability of NK-92 cells. In addition, the relative expression of cytotoxic-related genes of NK-92 cells, *RPF1*, *GZMA, GZMB, GZMH, GZMK* and *GZMM*, which are involved in perforin and granzyme pathways, were showed in Figure 4-D, E, F, G, H, I, respectively. Except for the *GZMM*, all of the aforementioned genes were significantly down-regulated after treatment with L-lactic acid or GPR81 agonists (3,5-DHBA), and returned to near-control expression level with no significant difference when treated with L-lactic acid in the presence of GPR81 antagonist(3-OBA). Notably, GZMM expression was downregulated in GPR81 antagonist treatment but not in L-lactic acid treatment (Fig. 4-I), indicating that its expression may only be regulated by the lactate receptor GPR81. These data suggested that lactic acid induced viability and cytotoxic function in NK-92 cells, resulting in decreased expression of perforin and granzyme A, B, H, and K genes.

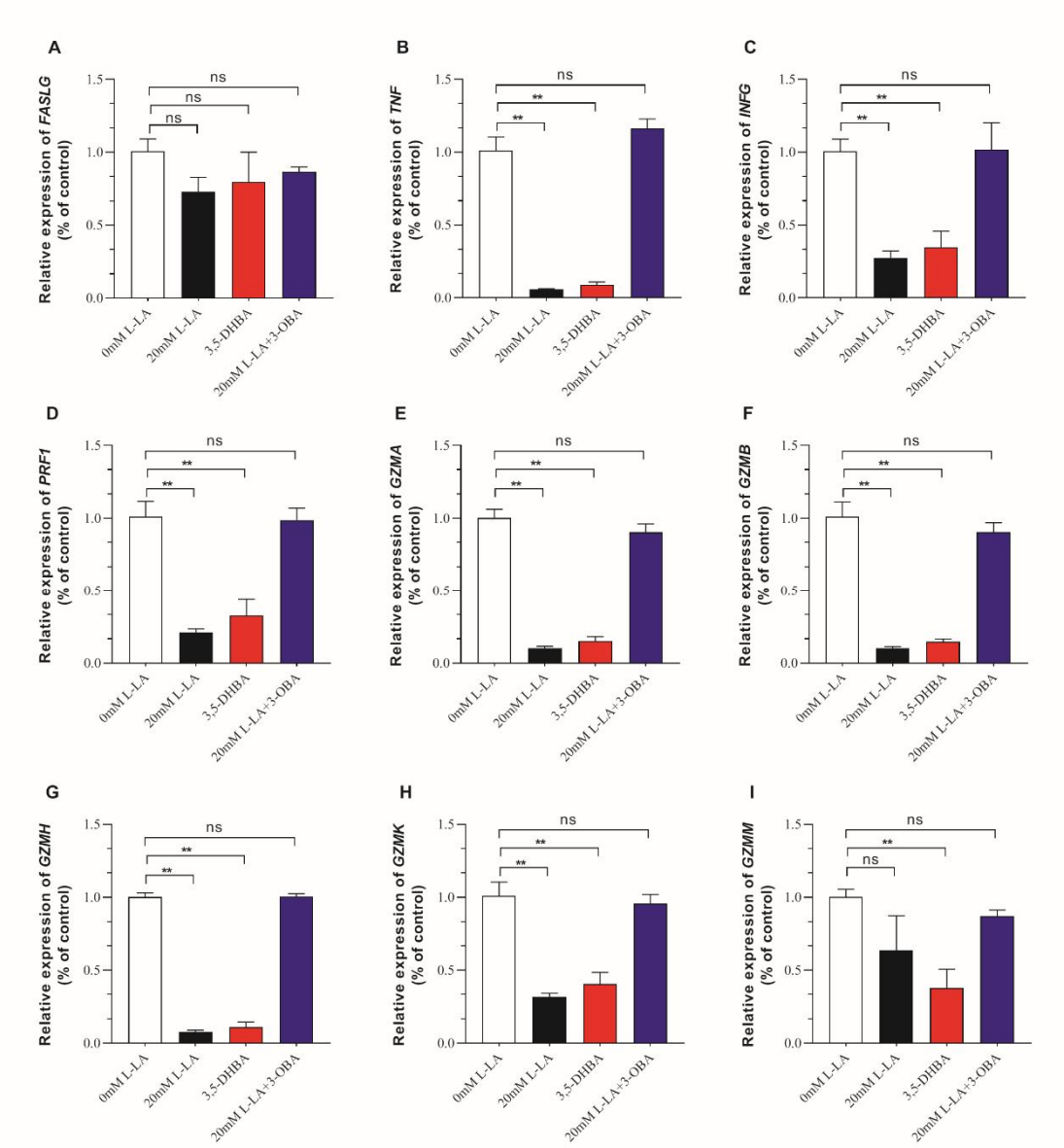

Fig. 4. Relative expression of cytotoxicity-related genes in NK-92 cells treated with antagonist and agonist.

*The letters A, B, C, D, E, F, G, H, I represent the relative expression of FASLG gene(Fas Ligand),TNF-α gene(Tumor necrosis factor-α), INFG gene (Interferon-γ), RPF1 gene (Perforin 1), GZMA gene (Granzyme A), GZMB gene (Granzyme B), GZMH gene (Granzyme H), GAMK gene (Granzyme K) and GZMM gene (Granzyme M). ns means p≥0.05, whereas ** means p<0.01.*

3.5 PEARSON'S CORRELATION ANALYSIS OF CYTOTOXICITY-RELATED GENES WITHIN THE LACTATE/GPR81 PATHWAY

The expression of *GPR81* was demonstrated to have a significant negative correlation with the expression of other cytotoxicity-related genes using a heatmap (Fig. 5). Obviously, higher expression of *GPR81* induced by lactate significantly led to lower expression of cytotoxicity-related genes. *GPR81* and *FASLG* have the lowest correlation coefficient of any cytotoxicity-related genes, with a correlation coefficient of -0.47. *GPR81* had the weakest

association with *GZMM* of any cytotoxicity-related genes, with a correlation coefficient of -0.93.

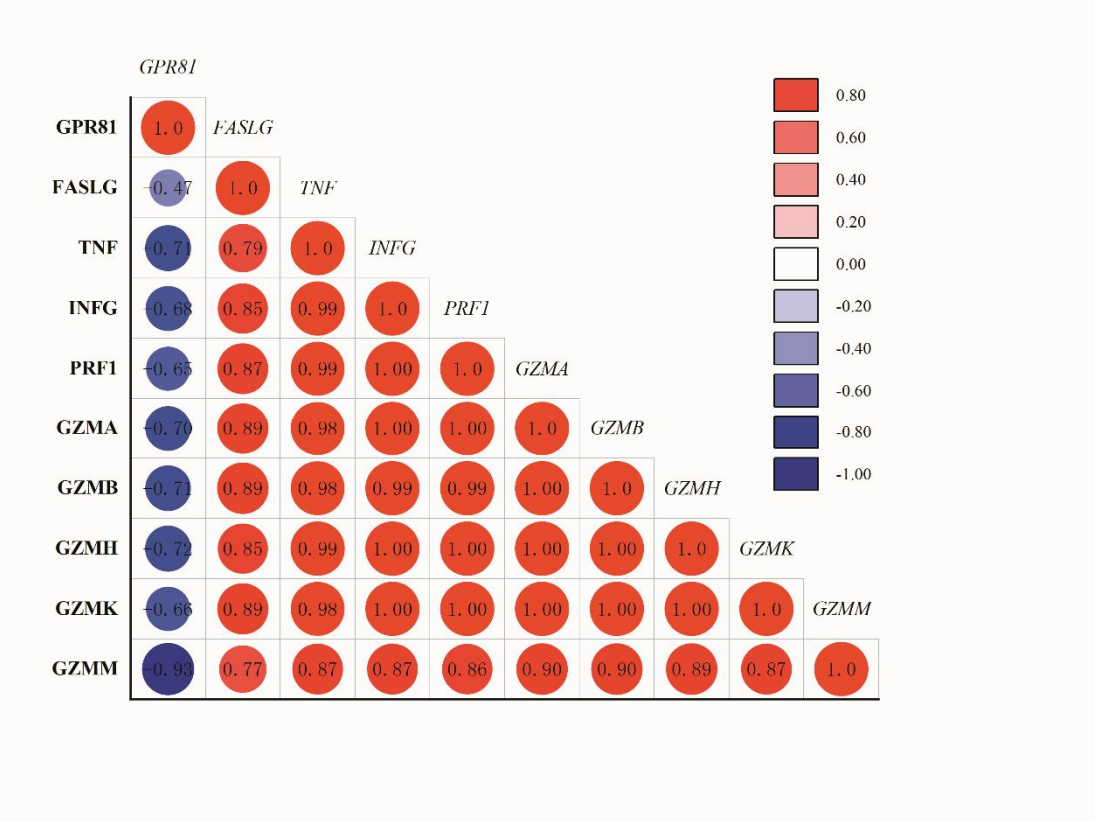

Fig. 5. Pearson's correlation matrix between *GPR81* and cytotoxicity-related genes in NK-92 cells.

Correlations are displayed in blue (negative) and red (positive); color intensity and circle size are proportional to the correlation coefficient.

4. DISCUSSION

Concentration of lactic acid in the acidic TME can be as high as 40 mM [1].In the present study, lactic acid was found to lower viability and downregulate cytotoxicity of NK-92cells with rising concentrations (Fig. 1, 2). It is consistent with previous report in NK cells, which attribute to inhibit NK cell viability and survival via intracellular acidification by lactic acid [32]. As we know, lactic acid contains two components: lactate and H^+. And lactic acid's immunosuppressive effects has been reported to reverse completely by buffering lactic acid-containing medium to a neutral pH of 7.4 [33]. In order to identify the role of GPR81 in lactic acid treatment, the expression of GPR81 were compared between 20 mM L-lactic acid in the presence of GPR81 antagonist (1 mM 3-OBA) and the control (0 mM L-lactic acid) (Fig. 3). The results were of no significant difference, suggesting L-lactate acid was a natural ligand of GPR81 of NK-92 cell.

"GPR81 expression varies depending on the cell type and tissue microenvironment" [34]. "GPR81 is expressed at a relatively high level in adipocytes and at lower levels in brain, intestine, kidney and many other tissues" [18]. Actually, the NK-92 cells in the present study expressed low levels of GPR81, and its expression was upregulated by the lactate or

GPR81 agonist (Fig. 3). This was consistent with the recent study that GPR81expression of DCs and macrophages is regulated by the tissue microenvironment [25].

Figure 2 and Figure 3 are suggested that high concentrations of lactic acid up-regulate the expression of GPR81 to inhibit the proliferation and cytotoxicity of NK-92 cells. The cytotoxicity of NK-92 cells against tumor cells was well known to involve in a number of cytokines (such as IFN-γ, TNF-α), lysed granules (such as perforin, granzyme) and Fas-FasL pathway [35]. These cytotoxicity-related genes were therefore investigated for their association with the lactate/GPR81 pathway in this study.

The lactate/GPR81 pathway may have distinct functions on cells. Lactate/GPR81-induced immune tolerance is protective for mice against experimental colitis via regulating intestinal homeostasis [18]. "In cervical cancer, the activated GPR81 can enhance the expression levels of DNA repair proteins for improving the efficiency of DNA repair" [24]. "Blocking the GPR81 activation will induces the production of the pro-angiogenic mediator amphiregulin (AREG) and thereby the angiogenic effect in breast cancer" [36]. However, in the case of NK-92 cells, the GPR81 may have strong relationship with the cytotoxicity-related genes due to the activating GPR81 can downregulate the expression of cytotoxicity-related genes, while preventing GPR81 activation with an antagonist can restore their expression (Fig. 4).

Lactic acid can inhibit interferon (IFN)- *γ* production and the cytotoxic activity of NK-92cells, as well as in other NK cells [37]. The convincing reason seems to be that pathophysiological concentrations of lactic acid prevented upregulation of nuclear factor of activated T cells (NFAT) in NK cells, resulting in diminished IFN-γ and IL-2 production.37

According to the correlation analysis GPR81 and cytotoxicity-related genes (Fig. 5), the cytotoxicity of NK-92 cells appears to be strongly associated with the lactate/GPR81 pathway although the underlying molecular mechanisms remain largely unknown. Additionally, the previous study that lactate can result immune escape through binding to its receptor GPR81 or independent of GPR81 in lung cancer cells [3], imply to the downregulation of cytotoxicity might be co-activated results combined with GPR81 and monocarboxylate transporters 1/4 (MCT1/4). To date, the strategy targeted on lactate-related receptors, such as MCT1/4, almost exclusively by blocking its secretion from tumor cells, shows encouraging results in some cancers [38]. Therefore, the extent to which GPR81 signaling regulates the expression of MCT1 and MCT4 in NK-92 remains to be determined in the further study.

5. CONCLUSION

L-Lactic acid decreased the viability, and downregulated the cytotoxicity of NK-92 cells. And RT-qPCR for the lactate/GPR81 pathway-related genes suggested that the lactate receptor GPR81 was an inhibitor in reducing the cytotoxicity of NK-92 cells after they were activated by lactic acid. The findings may provide a new insight for the relationship between lactate/GPR81 pathway and other cell functions in immune cells.

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