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The potential markers of NK-92 associated to cytotoxicity against K562 cells

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ABSTRACT

Markers associated to NK cytolytic activity are in a great need to regulate NK cell immunotherapy products. We assume that biomarkers which response to cytolysis will change their transcription, expression or secretion. To find NK-92 indicator to cytolytic activity, we have evaluated the potential markers by quantifying the expression of well-known cytotoxicity functional molecules (cytokine IFN-γ, Granzyme B, perforin, CD69 and CD107a), and explored candidate markers by a sweeping transcription picture of NK-92 using a direct cytolysis model (incubation with K562). We found that IFN-γ secretion was highly correlated to cytotoxicity of NK-92, neither Granzyme B, perforin secretion, nor CD69, CD107a positive population were upregulated by K562 stimulation. RNAseq revealed 432 genes expression changed during cytolysis, several genes (BIRC3, CSF2, VCAM1 and TNFRSF9) mRNA expression were validated by real time RT-PCR under K562 being killed or protected from being killed conditions. Results suggested IFN-γ secretion, BIRC3 and TNFRSF9 transcription in NK-92 were responsive to K562 cytolysis. In a word, our results confirmed one marker and reveal an array of novel candidate markers associated with NK-92 cytotoxicity. Further studies are greatly needed to determine the roles these new makers play in NK-92 cytolysis process.

1. Introduction

Immuno-oncology is an emerging field that has revolutionized cancer treatment. Natural killer (NK) cells are highly cytotoxic anti-tumor and anti-infection immune effectors, and are promising candidates for cancer immunotherapy [1–9]. NK-92, a continuously growing human NK cell line [10], is an ideal model to study NK cell biology and is expected to bring new approaches for cancer immunotherapy. NK-92 or genetic modified NK-92 has been shown to be broadly and highly cytotoxic against a spectrum of malignant cells, and has been proved to be with potential protection efficacy in xenotransplantated immune-compromised mouse models among various types of human cancers [11–15]. The safety and partially efficacy have also been proved in several clinical trials [16–19].

Mechanismly, activated NK cells release perforin and granzyme or induce apoptosis to remove hostile cells. They also secrete a number of cytokines, such as IFN-γ to regulate innate and adaptive immunity [15]. Currently, many methods have been developed and used to evaluate NK activity such as cytokines secretion, activating receptors expression, cytotoxicity or target cells lysis or apoptosis inducing.

Accurately, timely and easily performed assays for detecting the NK cell immune responses have paramount importance in therapeutic cell products evaluation. Reliable biomarkers which highly correlated to cytotoxicity are ideal for establishment such efficacy assesment assays. Ideal cytotoxicity biomarkers of cytotoxic lymphocytes may change their expression when effectors execute killing functions. To search such biomarkers, on one hand, we quantitatively analyzed several cytolysis functional molecules expression during killing cancer cells. On the other hand, we explored the transcriptome of NK-92 to search candidate indicators to cytolysis. It is reported that increased expression of CD69 and CD107a on NK cells was parallel to cytotoxicity [20–22]. In our present study, we have detected the candidate molecules which were literaturely reported closely related to cytotoxic activity, including secretion of IFN-γ, granzyme, perforin, the surface expression of CD69 [22] and CD107a [20] on NK-92 during lysing K562, to seek specific indicators for assessment of NK-92 lytic ability [20,23,24]. Utilizing cell sorting and transcriptional profiling to identify cytotoxicity related transcripts and distinct biological pathways can unveil potential mechanisms by

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which NK cells execute direct cytolysis, which may inform how to better engage these cells for immunotherapy.

2. Material and methods

2.1. Cells and cell lines

NK-92 and K562 cells were confirmed by STR, and checked free of bacteria, fungi and mycoplasma. K562 cells were maintained in complete media consisting of RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). NK-92 cells were cultured in GH T551 H3 medium (Takara Biomedical Technology (Beijing) Co., Ltd) and 500 IU/mL IL-2 (Shandong Quangang Pharmaceutical Co., Ltd). All cells were cultured at 37 °C 5% CO_2.

2.2. Chemicals and reagents

Monensin, PMA and Ionomycin were purchased from Sigma-Aldrich. The DELFIA® BATDA Reagent was from PerkinElmer (Waltham, MA, USA). The multi-cytokines detection kit was purchased from Merk Millipore (HCD8MAG-15-03). Mouse anti human monoclonal antibodies specific for Alexa Fluor 647 CD56, Percp-Cy5.5 CD69, PE-Cy™5 CD107a, CD54 were BD products, anti CD102 is Thermo Fisher (14-1029-82). Primers were designed using Vector NTI Advance 9.0 softwares (InforMax, Frederick, USA) and synthesized in Sangon (Shanghai, China).

2.3. BATDA method to quantify cytolysis

After the incubation, the K562 cells (1×10^6 cells/mL) were labeled with 5 μL BATDA reagent for 10 min at 37 °C. Labeled cells were washed three times with RPMI 1640 containing 10% FBS and incubated with NK-92 at various effector: target ratio (E:T = 5:1, 10:1, 20:1, 40:1). Set up background, spontaneous release and maximum release groups. After co-incubation of the effector and target cells for 1 h at 37 °C in a humidified 5% CO2 incubator, the cells were mixed, centrifuged and 20 μL of the supernatant was mixed with 200 μL europium solution and incubated for 15 min at room temperature while shaking in a flat-bottom 96-well plate. The determination of specific lysis was carried out by time-resolved fluorometry. The percentage of cell lysis was calculated as (experimental release-spontaneous release)/(maximal release-spontaneous release) × 100%.

In antibody protection tests, K562 cells were blocked for 1 h at room temperature with 5 μg/mL anti CD54 and CD102 monoclonal antibody before incubation with NK-92, followed by BATDA labeling and calculated cell lysis percentage.

2.4. Cytokine production and de-granulation

Cytokine production was analyzed by ELISA. De-granulation was analyzed with flow cytometry. After incubation, K562 and NK-92 cells were collected and re-suspended with RPMI 1640 containing 10% FBS at an E:T ratio of 10:1. K562 absent group was set as background control. NK-92 cells (10^5 per well) were seeded in 96-well plates alone, as a control, or together with 10^4 K562 cells and cultivated for different time. The supernatant was collected every 15min incubation and stored at −80 °C until use. The concentration of IFN-γ, Granzyme B and perforin were detected according to the manufacture's instruction by Luminex (R&D Systems) and analyzed with MagPix software. Meanwhile, target cell killing was monitored with BATDA method at an E:T ratio of 10:1, every 30 min in 2 h.

2.5. CD69 and CD107a surface marker expression

NK-92 cells were incubated with or without K562 cells. To detect CD69, NK-92 and K562 incubated at an E:T ratio of 10:1. For analysis of CD107a, NK-92 and K562 incubated 5:1, 10:1, 20:1 and 40:1. The emission/excitation wavelengths (Alexa Fluor 647 CD56, Percp-Cy5.5 CD69, PE-Cy™5 CD107a) were set according to the manufacturer's specifications of wavelength combinations. After incubation, effector cells were isolated with anti-CD56 antibody and stained with anti-CD69 antibody or anti-CD107a antibody separately. CD69 or CD107a positive cells were detected using flow cytometry. For detection of CD107a, 2 or 8 μM monensin was added to inhibit endocytosis [16], 2.5 μg/mL PMA, 0.5 μg/mL ionomycin were added to obtain maximum release [25]. All samples and data analysis were performed with FACS Calibur (BD, Franklin Lakes, NJ, USA) and Flowjo software.

2.6. RNAseq by deep sequencing

Total RNA from CD56 beads sorted NK-92 (no target cell incubation as control) and NK-92 incubation with K562 at an E/T ratio of 10:1 for 0 h, 1 h, 2 h, and 4 h with three replications was isolated using Trizol for the construction of a RNA-seq library and sequencing. The construction of RNA-seq library was performed using the KAPA Stranded mRNA-Seq Kit (Illumina® platform) (product codes KK8420 and KK8421, Boston, Massachusetts, United States) following the manufacturer's instructions. Briefly, mRNA was extracted and purified from total RNA, then fragmented and primed for cDNA synthesis. Double-stranded cDNAs were synthesized and then purified with 1.8 × Agencourt AMPure XP beads (Beckman Coulter, Beverly, USA) followed by the end 2nd Strand Synthesis. After A-Tailing, Illumina adapter oligonucleotides were ligated to cDNA fragments, and the 1X SPRI® clean-up was performed. Suitable cDNA fragments were selected as templates for PCR amplification using the KAPA Library Amplification Primer Mix and KAPA HiFi Hot Start Ready Mix. Products were purified with the AMPure XP bead system and quantified using a Bioanalyzer (Agilent high sensitivity chip). Finally, RNA-seq libraries were sequenced using an Illumina HiSeq at Beijing Microread Genetics Co., Ltd (Beijing, China). Raw data were processed with Fastp using recommended parameters. The filtered reads were mapped to Hg19 by hista2. The bam files were processed with samtools. Feature Counts was used to calculate gene expression. A list of differential expression genes (DEGs) was identified using the R packages" EdgeR". P-value of 0.05 and $|\log2(\text{foldchange})|>1$ were set as the threshold for significantly differential expression by default. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes were implemented with KOBAS3.0. GO classification was done by the R packages "TopGO". To further validate the RNAseq results, we selected 4 DEGs of our interests and conditional biomarkers to evaluate their expression in NK-92 using qRT-PCR. The gene-specific primers for these genes are listed in Table 1.

Table 1
Real time PCR primers.

primer	sequence
BIRC3 forward primer	5′-TGTTGGGAATCTGGAGATGA-3′
BIRC3 reverse primer	5′-CGGATGAACTCCTGTCCTTT-3′
CSF2 forward primer	5′-ACTCAGGATGGTCATCTTGGAG-3′
CSF2 reverse primer	5′-GCCATGCCTGTATCAGGGTC-3′
VCAM1 forward primer	5′-GGATTTTCGGAGCAGGAAAG-3′
VCAM1 reverse primer	5′-CAAAGGCAGAGTACGCAAACA-3′
CD69 forward primer	5′-CAAGTTCCTGTCCTGTGTGCTG-3′
CD69 reverse primer	5′-GCCCACTGATAAGGCAATGAG-3′
CD107a forward primer	5′-TCAAGGGCTGGCACTTTTTT-3′
CD107a reverse primer	5′-CCGAATGTCAACAGAAGACCAAA -3′
IFNγ forward primer	5-TGTAGCGGATAATGGAACTCTTTTC-3
IFNγ reverse primer	5-AATTTGGCTCTGCATTATTTTTCTG-3
TNFRSF9 forward primer	5′-ACTGGTGCCATTTCAGGAACAA-3′
TNFRSF9 reverse primer	5′-ACACCATGTGTCCAAAGCCAAG-3′
β-actin forward primer	5′-GGACTTCGAGCAAGAGATGG-3′
β-actin reverse primer	5′-AGCACTGTGTTGGCGTACAG-3′

2.7. Real-time RT-PCR

After the incubation with K562 at E:T 10:1 for 0 h, 1 h, 2 h and 4 h as described in RNAseq sequencing, NK-92 cells were separated using CD56 MicroBeads and total RNA was isolated using the miRNeasy Mini Kit with QIAzol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Concentration of total RNA was determined with NanoQuant instrument, 1 μg total RNA was reversely transcribed into cDNAs following the Super Script III First-Strand kit's protocols (Invitrogen, Carlsbad, CA, USA). The reverse transcription condition was 65 °C for 5 min, 50 °C for 50 min, 85 °C for 5 min. Real-time PCR was performed with SYBR premix (Takara Biomedical Technology (Beijing) Co., Ltd). PCR program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min (signal acquisition). The real-time PCR were performed with an ABI ?R?*R* 7500 Fast Real-Time PCR System (Applied Biosystems) with gene specific primers (Table 1). The comparative threshold cycle (CT) method was used with β-actin as an internal control. The mRNA expression levels of target genes were calculated by $2^{-\triangle\triangle Ct}$ method and normalized to non-stimulation NK-92 group (0 h).

3. Results

3.1. The secretion of IFN-γ is highly correlated with the NK-92 cell cytotoxicity

The secretory kinetics of IFN-γ, perforin, and granzyme were described by detecting their concentration every 15 min after incubating NK-92 with K562 cells, the E:T ratio is 10:1, using K562 absent group as control (Fig. 1). This experiment has been done twice independently with triplications. The concentration of perforin and granzyme only showed slightly increasing after 75 min incubation, Granzyme B slowly dropped to initial level (Fig. 1A and B). The concentration of IFN-γ released by NK-92 showed an exponential growth after 75 min incubation with K562 (Fig. 1C), its concentration has not yet reached a platform stage within 2 h. Correlation analysis between cytolytic activity (BATDA method) and IFN-γ secretion demonstrated that IFN-γ secretion is highly correlated with cytolytic activity of NK-92, the correlation coefficient is as high as 0.9678 (Fig. 1D).

3.2. The CD69 and CD107a surface expression is not correlated with the NK-92 cell cytotoxicity

Stimulation of NK-92 with K562 could not up-regulate the percentage of CD69 and CD107a expressing cells compared to unstimulated control. As shown in Fig. 2A, more than 90% of NK-92 population is CD69 positive, no matter K562 is present or not, increasing incubation time from 1 h to 4 h with K562 has no effect on CD69 cell positive population.

Studies report that CD107a is a marker for de-granulation of NK, and its expression is a sensitive marker for the cytotoxic activity. In this study, the expression of CD107a was monitored during NK-92 lysed K562 cell at E:T ratios 5, 10, 20, 40. Monensin was added to avoid endocytosis. To determine the appropriate Monensin addition time point, post 0, 1, 2 h incubation NK-92 with K562, Monensin was added, then detected the CD107a expressing cells. Results showed that Monensin was added at the initiation stage of cytolysis (0 h), the percentage of CD107a expressing was the highest, post 1 h cytolysis added Monensin, CD107a positive cells was the lowest (Fig. 2B–a). So it is better to add monensin at the initiation stage of cytolysis. The CD107a positive cell count slight increase at higher E:T ratio. We also evaluated the effect of Monensin concentration on CD107a expression, results proved that 8 μM Monensin was better than 2 μM added at the cytolysis initiation (Fig. 2B–b). Then we evaluated the correlation between expression of CD107a and NK-92 cytotoxicity by measuring the surface expression of CD107a at different K562 duration 1 h, 2 h, 3 h, 4 h. There was no significant variation in CD107a expression at different duration time, CD107a expression cells count were no increasing (Fig. 2B and c) and CD107a positive population was <30% (Fig. 2B–d).

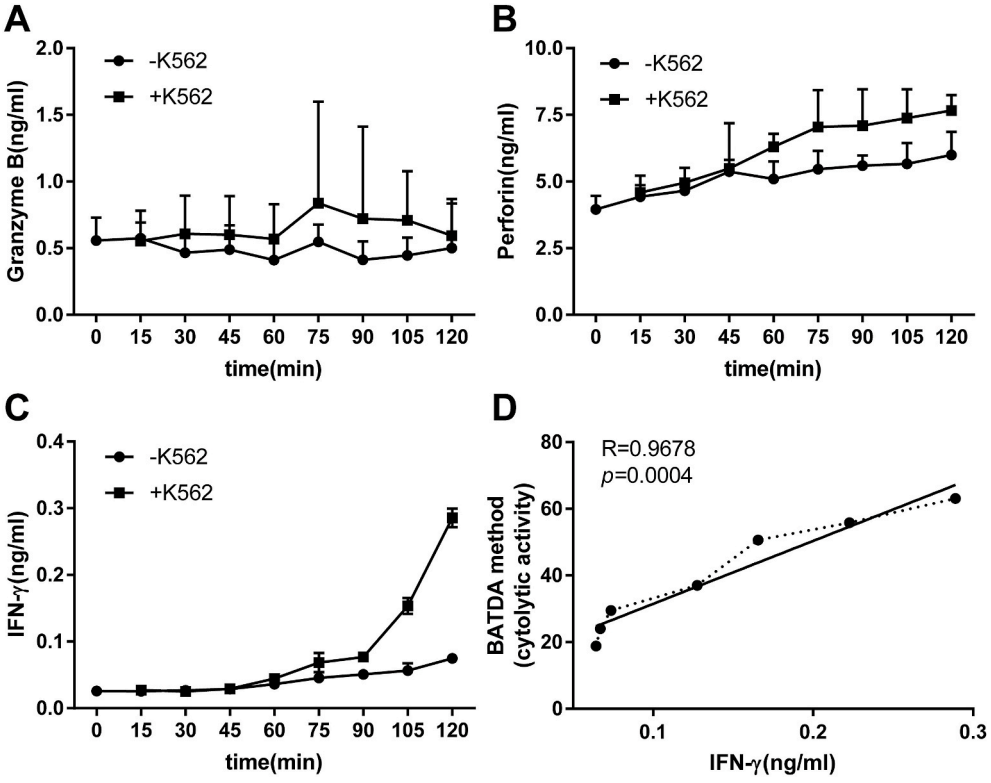


Fig. 1. Secretory kinetics of Granzyme B (A), perforin (B) and IFN-γ (C) during incubating NK-92 with K562 measured with ELISA. D. Correlation analysis between cytolytic activity (BATDA method) and concentration of IFN-γ secreting from NK-92 which has been stimulated with K562. Meanwhile, target cell killing was monitored with BATDA method at an E:T ratio of 10:1, every 30 min in 2 h during incubation. The dots are BATDA and IFN-γ concentration data, solid lines represented the correlation analysis between cytolytic activity (BATDA method) and IFN-γ secretion. R, correlation coefficient is 0.9678.

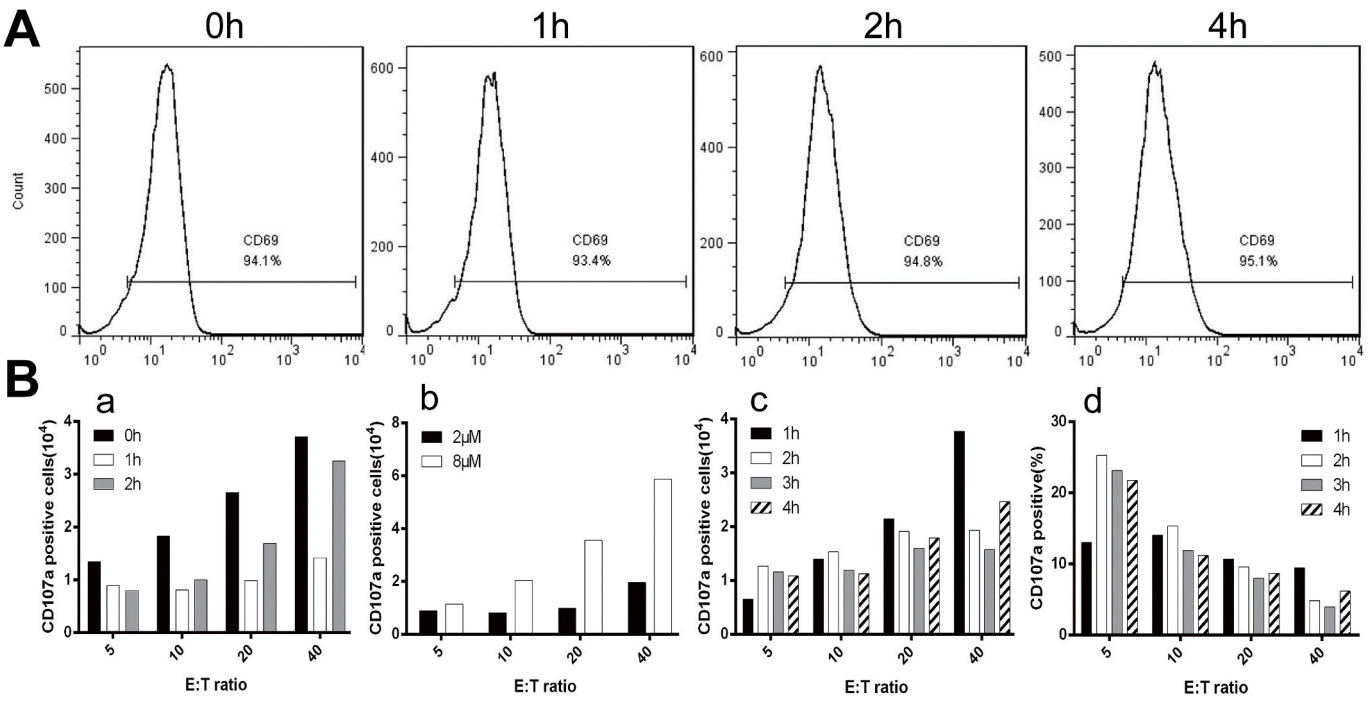


Fig. 2. CD69 and CD107a positive population of NK-92 during NK-92 cytolysis process detected by flow cytometry. A. CD69 expression on NK-92 incubated with K562 for 0 h, 1 h, 2 h and 4 h at E:T ratio 10:1. B-a. 2 μM monensin added post 0, 1, 2 h incubation at E:T ratio 5, 10, 20, 40. B-b. 2 μM and 8 μM monensin were added separately when the cytolysis initiation. B-c. Cell counts with CD107a surface expression of NK-92 at different K562 incubation time 1 h, 2 h, 3 h, 4 h at E:T ratio 5, 10, 20, 40. B-d. CD107a positive population of NK-92 at different K562 incubation time 1 h, 2 h, 3 h, 4 h at E:T ratio 5, 10, 20, 40. Result from one of three independent experiments is represented.

3.3. NK-92 transcription signatures during K562 cytolysis indicated by transcriptome analysis

To screen candidate indicators for cytotoxicity activity of NK-92, we study the transcriptome through defining dynamic, unique and common transcription signatures of NK-92 during K562 cytolysis process (K562 incubation was regarded as a NK-92 direct cell cytolysis model). We incubated NK-92 with K562 target cells for 1 h, 2 h, 4 h at E:T 10:1, then isolated NK-92 for RNAseq, used no target cell as control. Key to such analysis was the use of biologically replicated datasets to control for reproducibility, specificity, stringent statistical criteria for data analysis ($p < 0.05$, log2|FC|>1). Processed RNAseq data, identified differently expressed genes (DEGs) showed in Fig. 3 (and Table S1.). We thus identified a set of high-confidence indicators (top 20 DEGs) and generated a transcription heatmap during NK-92 cytolysing K562 (Fig. 3D, Table S2.).

First, we detected common sets of DEGs that were up-regulated or down-regulated during NK-92 killing K562 (Fig. 3). There were total 2392 transcripts were differentially expressed in K562 stimulating NK-92 cells compared to un-stimulated cells during 4 h incubation (Fig. 3 A and B). Among these genes, 1134 genes were up-regulated, 1258 genes were down-regulated. 432 common genes changed expression level during K562 incubation from 1 h to 4 h (Table S2.).

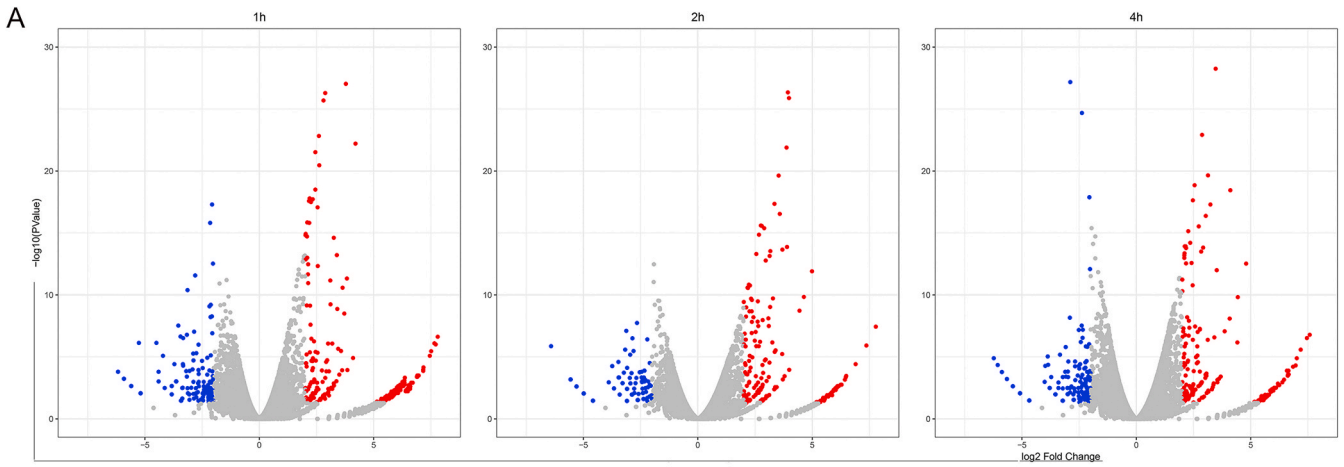
GO terms enrichment analysis of these 432 DEGs (Fig. 3C) suggested that 'cytokine activity' was the most significant enriched function. There were 18 molecules enriched in this part, these molecules include 5 cytokines (such as IL-4, IL-10, IL-16, IL-18, CSF2), 7 kinds of chemokine (XCL1, XCL2, CCL1, CCL3, CCL4, CCL5, CCL3L1), 3 growth factors (TGFB2, INHBC and INHBE), metalloproteinase inhibitor 1 (TIMP1), a tumor necrosis factor ligand superfamily member 4 (TNFSF4) and SECTM1. 'RNA polymerase II transcription factor activity' related molecules are another enriched molecular function, there were 13 DEGs enriched in this module: KLF2, NR4A2, JUNB, BATF2, EGR1/2, RARG, FOS, NR4A3, RELB, REL, ATF3, NR1I2. Another significantly enriched GO molecular function term was 'molecular function regulator', including 29 genes differently expressed, besides the 12 molecules cytokine activity related, another 17 DEGs were RGS1, RGS2, WNT10B, SEMA4C, SLC30A1, IL2RA, TRIB1, PDGFRB, USE1, ANXA1, SH2D3C, RGS16, TANK, BIRC3, PPP2R3A, NOTCH1, CISH.

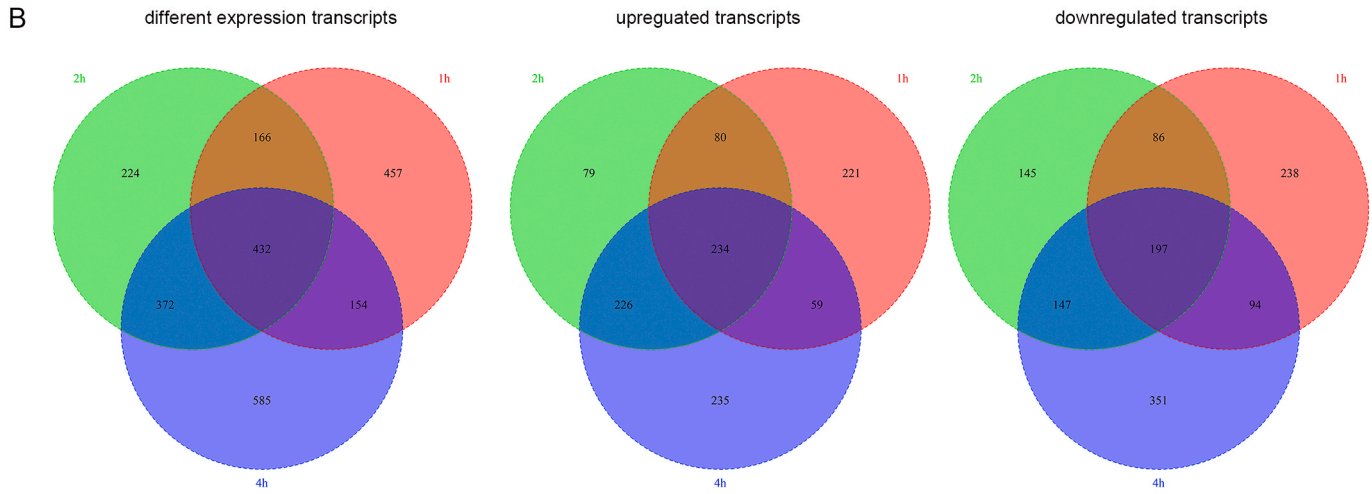
As GO and KEGG (Fig. 3C and Table S1.) showed that the transcription signature at different incubation time demonstrated NK-92 cells undertook different biological functions: at early target contact stage, major molecular functions focused on 'receptor ligand activity' and 'receptor regulator activity' (more than 40 genes enriched in these two GO terms), activity took place at membrane (almost 40 genes at site of membrane) and positive regulation of cellular biosynthetic process (40 genes) (Fig. 3C, Table S3.). At 2 h and 4 h, DEGs significantly enriched in 'SRP-dependent co-translational protein targeting to membrane' and 'structural constituent of ribosome' GO terms related ribosome activity. KEGG analysis also suggested these DEGs which were involved in different pathways at the three incubation timepoints (Table S4). At 1 h, different genes enriched in 'Allograft rejection', 'TNF signaling pathway', 'T cell receptor signaling pathway'. At 2 h, DEGs enriched in 'TNF signaling pathway', 'Ribosome', 'Oxidative phosphorylation'. And at 4 h, 'Ribosome' and 'Oxidative phosphorylation' are two of significantly enriched pathways.

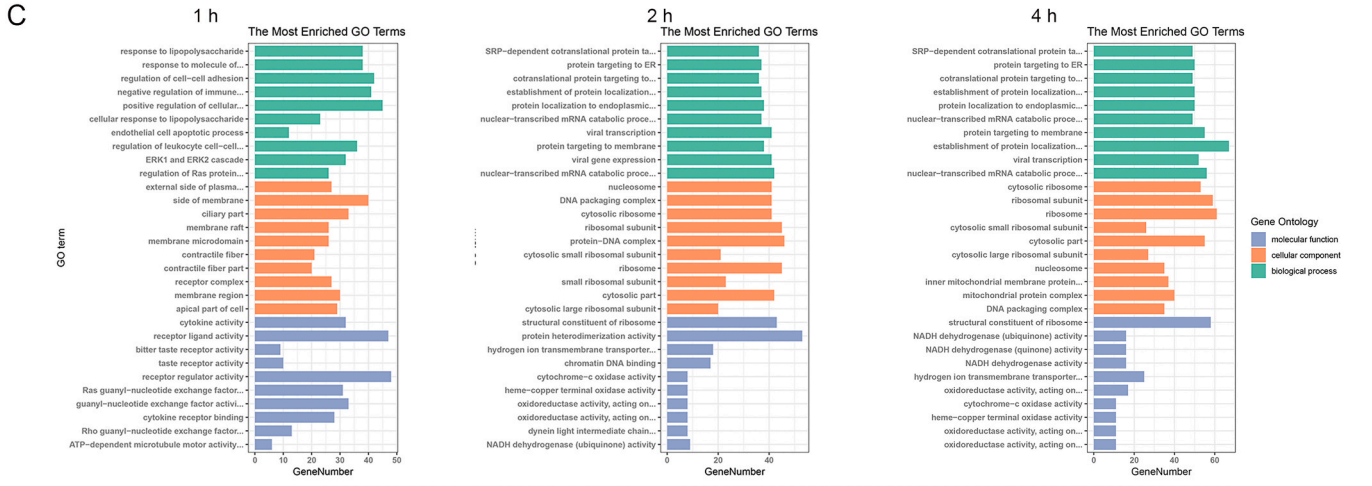
Second, five express patterns were observed (Fig. 3D): □ upregulation, keep increasing expression, for examples, CCL1 and BIRC3. ② upregulation, gradually down regulated expression level, such as NR4A2/3. ③ Keep steady high mRNA expression level like EGR2, CSF2 and CCL4. ④down-regulation keep continuous reduction expression as INPP4B, NEDD9 and LRRC18. ⑤ Reverse pattern like FOS, FOSB at early 2 h up-regulated and at late stage down regulated, and TNFRSF9 at early 1 h down regulated then up-regulated at late stage.

Third, unexpectedly, the transcription of CD69 and CD107a were not found in these DEGs list. IFN-γ increasing express by 2–4 times at 1 h and 2 h, but no be included in this list at 4 h (Table S1.).

At last, we should be careful to explain the up-regulated genes when







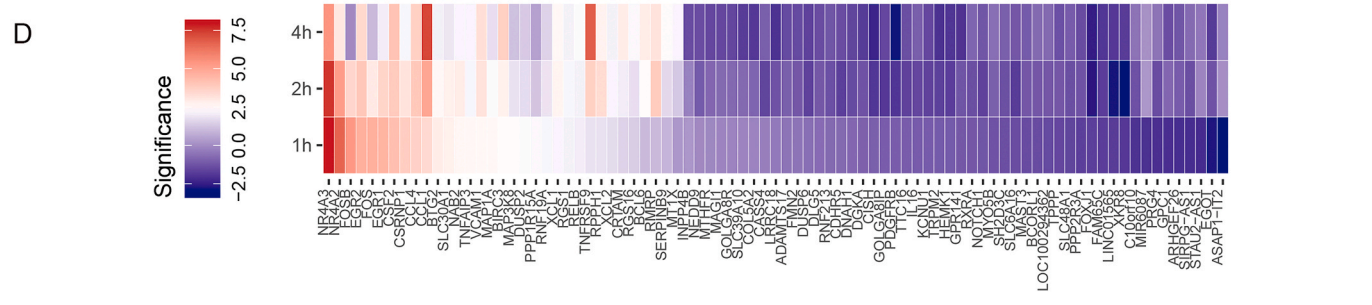


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Fig. 3. Whole transcriptome analysis of functional NK-92 cells incubated with K562 for 0 h (control), 1 h, 2 h, 4 h. A. Volcano plots representing up-regulated and down-regulated transcripts of functional NK-92 cells at different K562 stimulation duration. Size of each data point is calculated as −log10 (p-value) × log2 (FC), p-value cutoff $p < 0.05$, fold change cutoff > 2 or <1/2. Red dots represent up-regulated transcripts, blue dots represent down-regulated transcripts. B. Venn diagrams displaying different expression and up-regulated (red) and down-regulated (blue) transcripts of functional NK-92 cells across 1 h, 2 h and 4 h incubation. C. The most enriched Gene Ontology (GO) terms at 1 h, 2 h and 4 h incubation time. D Heat map comparison of common genes expression for top up (red) - and down-regulated (blue) genes ranked top 20 at 1 h, 2 h and 4 h incubation time. Data from one of three independent experiments is represented. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

NK-92 incubated with K562, which were indeed associated with the cytolysis progress, however it may be not functional mechanismly associated with cytotoxicity of NK-92, for an example, we found IL-10, the negative regulator of immunity, also increased expression by 4 (1 and 2 h)~2.7 (4 h) times.

3.4. RNAseq validation by real time PCR (RT-PCR)

RNAseq results were verified by quantitative RT-PCR (qRT-PCR) analysis of several DEGs. First, we validated 4 DEGs expression with K562 stimulation. Second, we checked these DEGs expression level under a negative condition to confirm whether DEGs expression was functionally correlated to the cytolysis process.

We quantified the four representative genes expression (BIRC3, CSF2, VCAM1 and TNFRSF9) on NK-92 which were stimulated by K562 at E:T 10:1 for 1 h, 2 h, 4 h. Results demonstrated that all these four genes increased expression in NK-92 stimulated by K562 compared with no stimulation control during 1–4 h. BIRC3 and TNFRSF9 continuously elevated expression during 4 h, while CSF2 expression declined by 2/3 (Fig. 4Aa-d) to 1/3 (Fig. 4C–e) compared to 1 h.

Besides BIRC3, CSF2, VCAM1, and TNFRSF9, we further verified 3 genes (CD69, CD107a, IFN-γ) expression by comparing expression level in NK-92 under K562 stimulation condition with K562 blocked by anti-CD54 and anti-CD102 (Fig. 4C). It is reported that NK-92 killing K562 potency decreased when K562 was blocked by CD54 and CD102 antibody [26]. We found that 5 μg/mL CD54 antibody blocking decreased K562 lysis by 30% after 2 h NK-92 incubation at 10:1 E:T ratio, and using CD102 antibody alone could not decrease K562 lysis, but using both antibodies decreased killing lysis by 50%, so we using 5 μg/mL anti-CD54 and anti-CD102 to prevent K562 from NK-92 killing (Fig. 4B) and check the genes expression under this blocking condition.

Results showed all these 7 genes expression increased during K562 stimulation. In blocking groups, 7 genes expression decreased within 2 h compared with K562 not blocked counterpart. In line with Fig. 4A, 7 genes expression level had risen in NK-92 when stimulated by K562, while expression level dropped by 1/3–1/2 when K562 was blocked by CD54 and CD102 antibodies during first 2 h. These results suggest 7 genes transcription responded to K562 cytolysis. At 4 h, except BIRC3 and TNFRSF9, other 5 genes expression level equaled to or slightly expressed more than no antibody blocking. Whether there is a compensation mechanism of these genes expression at the later killing time under antibody blocking condition is unknown. In a word, at least,

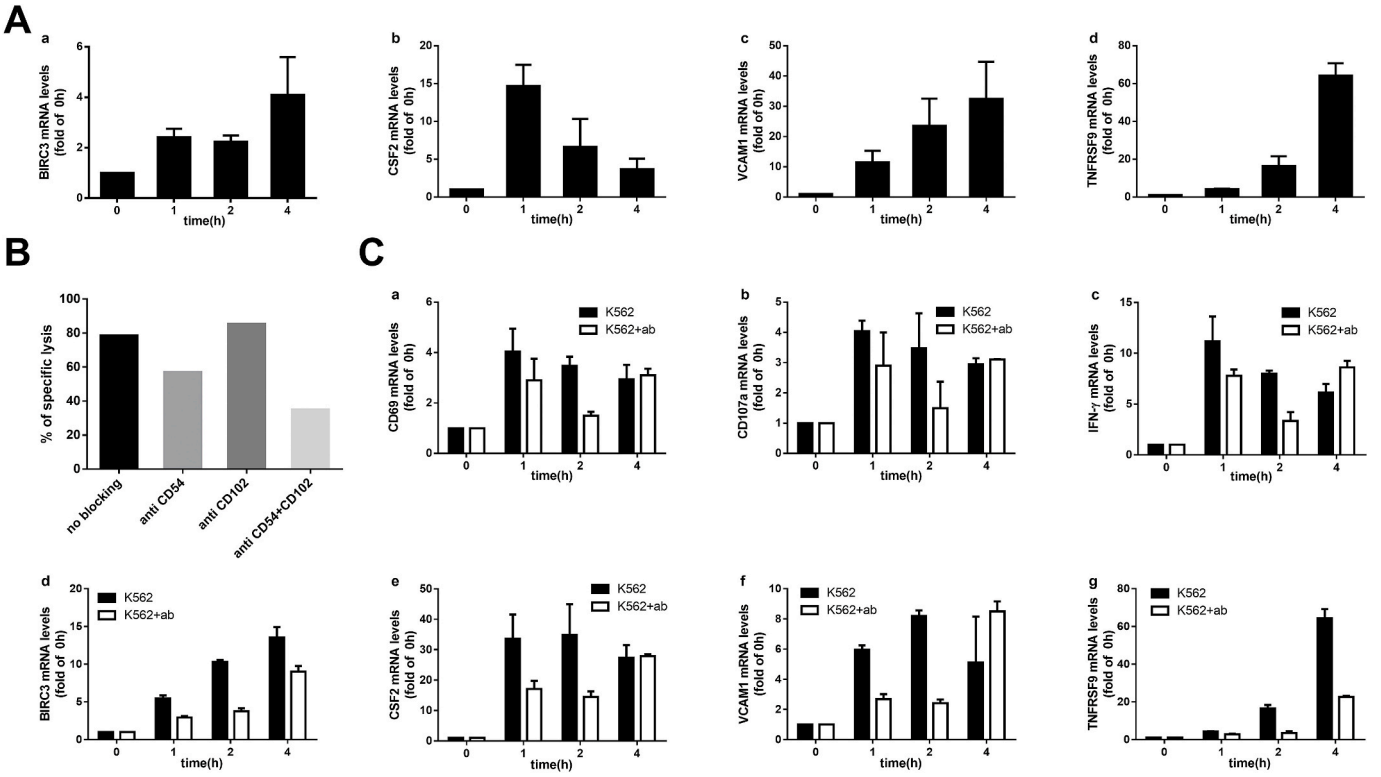

Fig. 4. The relative mRNA expression of candidate indicators on NK-92 stimulated by K562. A. Relative mRNA expression was validated by quantitive RT-PCR. The relative mRNA expression of BIRC3, CSF2, VCAM1 and TNFRSF9 on NK-92 stimulated by K562 at E:T 10:1 ratio during 1 h, 2 h, and 4 h. B. K562 blocked by CD54 and CD102 antibody decreased the NK-92 cytolysis. 2×10^5 cells/mL K562 cells blocked with 5 μg/mL CD54 and CD102 antibody at room temperature for 1 h. After blocking, K562 was labeled by BATAD, equal volume of 2×10^6 cells/mL NK-92 and labeled 2×10^5 cells/mL K562 cells were seeded into 96-well plate. Incubated for 2 h, cytolytic activity was determined with BATDA method. One result from three independent experiments is represented. C. The relative mRNA expression of CD69, CD107a, IFN-γ, BIRC3, CSF2, VCAM1 and TNFRSF9 under K562 stimulation or antibody preventing condition. 2×10^5 cells/mL K562 cells blocked with 5 μg/mL CD54 and CD102 antibody at room temperature for 1 h. After blocking, equal volume of 2×10^6 cells/mL NK-92 and 2×10^5 cells/mL K562 cells were seeded into 24-well plate co-cultured for 1 h, 2 h, 4 h. NK-92 cells was isolated with $CD56^+$ microbeads, and 7 genes relative expression were detected by qRT-PCR.

BIRC3 and TNFRSF9 gene responded to K562 stimulation and antibody inhibition.

Meanwhile, we found the mRNA expression of CD69, CD107a and IFN-γ were all upregulated by qRT-PCR (Fig. 4Ca-c), it is slightly different from the results described earlier. FACS and RNAseq results suggested that CD69 and CD107a expression had no response to K562 stimulation: CD69 and CD107a expressing population were not increasing (Fig. 2), and both genes were not included in DEGs list (Table S1.) in RNA-seq test. When validated by qRT-PCR, the mRNA transcription of CD69 and CD107a were 3–4 times upregulated compared with no K562 stimulation group. This difference may be caused by different state of NK-92, which has impact on results calculated by $2^{-\triangle\triangle Ct}$ method.

4. Discussion

In this study, we found and validated several potential biomarkers associated to NK-92 cytotoxicity by quantitative analysis of cytotoxicity effector molecules secretion or expression and transcriptome of cytolytic NK-92. We assumed that ideal potential indicators used for immunotherapy regulation should meet at least three requirements: First, indicators are important for cytotoxicity ability. Second, indicators can be easily monitored by current techniques. Third, it is better that NK cytolytic activity is dose dependent on indicator molecules. IFN-γ meets all three requirements of an ideal indicator accordingly to our results. As we all know, IFN-γ plays a key role in host defense by promoting the development and activation of a variety of immune cells. It also exhibits anti-proliferative and apoptotic effects. IFN-γ detection technology is accessible. Importantly, as demonstrated in this study, the concentration of IFN-γ secreted by NK-92 exponentially increased after 75 min incubation with target cells, and this cytokine secretion is highly correlated with cytolytic activity of NK-92, the correlation coefficient is 0.9678. Thus, IFN-γ is a candidate indicator for evaluation of cytolytic efficacy of NK-92. IFN-γ could be easily evaluated by ELISA with cultured media, easier to operate than target cells labeling depended cytolysis test. It is of great significance to statistically determine the minimum concentration of IFN-γ at which can ensure the killing efficacy of NK-92 in the future, it is useful for elimination of invalid products.

As for other cytotoxicity effector molecules or new found DEGs, three conditions cannot be satisfied at the same time. Literatures suggested exocytosis perforin, granzymes, CD69 and CD107a related to NK high lytic activity. Our results demonstrated that mRNA expression of CD69 and CD107a increased by 3–4 times, and antibody blocking K562 reduced the expression level within 2 h, this result indicated CD69 and CD107a transcripts respond to K562 stimulation. However, the secretion of perforin, granzymes, and surface expressing cells of CD69 and CD107a were not upregulated which indicated that NK-92 cytotoxic activity was not dose dependent on these functional molecules. The difference may be attributed to different types of NK cells. On NK and NKT, CD69 expression increase was induced by incubation with K562 [23] or on primary NK cells from PBMC, IL-2 treatment also induced IFN-γ and CD69 dramatically increase expression [21]. $CD69^{+}$ NK-92 cell frequencies did not increase during K562 incubation, might because CD69 positive NK-92 cells has been maximized by IL-2 stimulation. Result suggested that the de-granulation molecule CD107a is neither upregulated by incubation with K562. This phenomenon might be mechanistically explained as following, individual NK cell only needed two to four de-granulation events on average to mediate target cell death, despite one NK cell containing almost 200 granules [27], it is inferred that NK-92 has been overloaded by granules, excess stimulation by K562 could not induce more cells expressing CD107a. As for granzymes, it is reported that NK-92 has the highest levels of Granzyme A compared to new freshly isolated NK cells [28]. Combined the fact that the Granzyme B expression has not been elevated during K562 stimulation, which suggests that granzyme has been fully stimulated even there is no target cells present in the NK-92. In a word, NK-92, a highly cytotoxic, IL-2 actived human NK cell line expressed high level of CD69, CD107a and Granzyme B, and these genes in NK-92 did not change when directly interacted with K562.

Collectively, the production of cytokines, the enhanced expression of a glycoprotein associated with de-granulation, and the combined ability to produce perforin have all been partially correlated with NK cells cytotoxicity, but none is a robust surrogate for efficacy control or measures cell-mediated killing directly [29], as well as the results revealed by this study, several well known potential indicators of NK-92 cytotoxicity are necessary but insufficient for cytotoxic function. So it is of great importance to survey the entire dynamic transcriptome of NK-92 stimulated with K562 in attempt to better understand NK-92 cell cytolysis activity. Here we unmasked dynamic transcriptomic fingerprints of NK-92 cell activity in response to a direct cell cytolysis model.

Some of identified DEGs are involved in NK-92 cytotoxicity, which is consistent with other report [30]. It is reported that TNF receptor super family 9 (TNFRSF9, also named CD137 or 4-1BB, an activation marker for antigen-specific Tregs) and BIRC3 were upregulated at all stimulation conditions, combined our study, we can draw the conclusion that TNFRSF9, BIRC3 are NK-92 cytolysis responsive markers. However, to confirm whether these DEGs could be qualified for the biomarker, there is the most important question to be answered what roles these molecules play in NK-92 cytotoxicity.

NK and T mediated cancer cytolysis are promising therapies and growing fast in recent years. It is in great need of biomarkers or indicators to establish regulatory technology for assessing the efficacy and safety of the cell therapeutic products. We provided several potential markers, however, the contribution of such as TNFRSF9 and BIRC3 expression to NK-92 cytolysis and potential therapeutic implications is unknown. Further mechanisms studies are needed to be determined the functional roles the molecules play in cytolysis process, and whether these molecules expression respond to cytolysis in other NK cells such as NK cells derived from PBMC and LAK or CIK cells.

Authors' contributions

MS conceived the study. SX and XC carried out the experiments, interpreted the data; XC prepared the manuscript and made the critical revision of the manuscript. FJ, ZX and WX provide critical analysis of the data. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2020.08.009.

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