Lienal polypeptide promotes NK cells to suppress PCa survival *in vitro*

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Abstract. As an important component in the innate immune system, natural killer (NK) cells have been demonstrated to be clinically associated with prostate cancer (PCa) progression and castration resistance. Therefore, the development of novel agents that may enhance the cytotoxicity of NK cells possesses promising therapeutic applications. In the present study, lienal polypeptide (LP) solution was supplemented into a co-culture system of NK and PCa cells, as it was previously demonstrated that LP are able to activate NK cells, which kill PCa cells based on an MTT cell viability assay. Mechanistic dissection demonstrated that LP enhanced androgen receptor degradation, which resulted in an upregulation of MHC class I polypeptide-related sequence A (MICA) and MICB. In turn, the induced expression of MICA and MICB was able to further trigger NK cell activation, forming a positive loop between NK cells and PCa cells in the presence of LP solution.

Introduction

Prostate cancer (PCa) remains a leading cause of cancer death in males (1). The mainstay treatment for this disease is androgen deprivation therapy (ADT), which has been practiced for many decades and showed promising therapeutic effect on PCa patients (2). Androgen receptor (AR) has been widely recognized as a key factor in determining PCa progression and continues to exert biological functions even in the castrated condition, in which androgen level is very low (3-6). Therefore, further suppression of AR activity remains a therapeutic goal. For instance, abiraterone and enzalutamide have been approved to treat metastatic castration-resistant prostate cancer (mCRPC) owing to their potent capacities to inhibit AR signaling (7-10).

PCa is a heterogeneous mass consisting of fibroblasts, endothelial cells, stem progenitor cells and immune cells, which together play profound roles in the pathogenesis of prostate cancer (11,12). It is widely accepted that immune cells are highly associated with PCa progression and clinical outcomes (13). Natural killer (NK) cells belong to innate immune system and exhibit antitumor function either by directly killing tumor cells or by influencing other immune cells via secreted cytokines such as TNFα and IFNr (14). Studies have been demonstrated that cytotoxic activity of NK cells is highly correlated with castration resistance and overall survival: patients with more NK cells infiltration would have better prognosis and take longer time to develop castration resistance (13). According to these clinical phenomenon, it is urgent to find new agents that can accelerate the proliferating rate of NK cells or enhance their activity to cure PCa.

Calf spleen-derived lienal polypeptide (LP) solution, consisting of small peptides, nucleic acids and polysaccharides, has been clinically used in China and proved to benefit patients who have leukemia, leukopenia, lymphoma and advanced tumors (15). Stimulation of innate immune response including NK cells is one of LP's pharmacologic effects. Nevertheless, the detailed mechanism(s) by which LP boosts the lysis activity of NK cells and how it affects PCa progression is still largely unknown.

In this study, we found that LP can enhance the cytotoxic activity of NK cells to kill PCa cells. LP-mediated NK activity bore the ability to downregulate AR expression levels, which in turn caused a released expression of MICA/MICB. Our original findings suggest that LP may be developed alone or in combination with other drugs to better suppress PCa growth.

Materials and methods

Cell culture. C4-2 and CRW22Rv1 cells (ATCC, Manassas, VA, USA) were cultured in 10% FBS RPMI-1640 with 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. NK-92MI cells (ATCC), were maintained in α-MEM (Invitrogen Life Technologies, Carlsbad, CA, USA)

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with 0.2 mmol/l inositol, 0.1 mmol/l 2-mercaptoethanol, 0.02 mmol/l folic acid, horse serum to a final concentration of 12.5% and FBS to a final concentration of 12.5%. Cells were maintained in humidified 5% CO_2 environment at 37˚C. For transient transfection experiments, Lipofectamine 3000 (Invitrogen, Grand Island, NY, USA) was used.

Plasmids construction and lentivirus generation. Twenty-one nucleotides against the target gene will be synthesized and cloned into the plKO vector. AR cDNA was cloned into PWPI vector. Lentiviral particles will be generated by transfection of lentiviral expressing plasmid, packaging plasmid psPAX2, and pMD2.G envelope plasmid into HEK293 cells. Lentiviral particles will be collected to transduce target cells according to Addgene's lentiviral protocol.

Site-directed gene mutagenesis. The promoter of MICA/MICB was cloned into basic pGL3 using and site-directed gene mutagenesis was performed as previously described (16). Briefly, PCR was conducted in 20 μl volume system: DNA template (50 ng) (pGL3-promoter-MICA or pGL3-promoter-MICB), 2 μl 10X phusion buffer, 0.25 μl phusion enzyme, 1 μl 10 μM forward or reverse primers. The reaction was done as follows: 98˚C for 5 min, 30 cycles of: 98˚C, 30 sec; 60˚C, 30 sec; 72˚C, 2 min. PCR products was digested with *Dpn*I and subjected to PNK treatment, then was ligated with T4 ligase before transformation. Mutated clone was confirmed by *Hind*III restriction enzyme. The primers used in mutagenesis are:

pGL3-promoter-MICA forward, 5'-GGGGTACCGGGA T TATAGTCATGAACCACTG and reverse, 5'-CCCTCG AGCT CAGAATGCGGTGACAGC; pGL3-promoter-MICB forward, 5'-GGGGTACCCCAGTCTCTGAAGTCACTG TCA and reverse, 5'-CCCTCGAGCCTCGGCGCCCGA AAGCTTT; pGL3-promoter-MICA-mut forward, 5'-AGA TCTTCCCAAT AAGATGATTTA and reverse, 5'-TTGCAT GCAATAAACT GCATCT; pGL3-promoter-MICB-mut forward, 5'-AGATCTTCCCAATATGATGATTTA and reverse, 5'-TTGCATGCAATAAACTGCATCT.

MTT cell viability assay. C4-2 or CRW22Rv1 cells ($1X10^4$) were loaded into 24-well plates and treated with LP with or without NK-92MI cells. After 24 h later, NK-92MI cells were washed away and 0.5 ml MTT (0.5 mg/ml) was added to each well and incubated for another 2 h. The absorbance at 570/630 nm was detected.

RNA extraction and qRT-PCR assay. Total RNAs (1 μg), which were extracted using TRIzol reagent (Invitrogen Life Technologies), were subjected to reverse transcription using SuperScript III Transcriptase (Invitrogen Life Technologies). qRT-PCR was conducted using a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR-Green to determine the mRNA expression level of genes of interest. GAPDH was used as control.

Western blotting. Cells were lysed in ice-cold RIPA lysis buffer at 4˚C for 30 min. After centrifugation, equal amounts of the protein were loaded for electrophoresis on 8-12% denaturing SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After being blocked, membranes were incubated with appropriate dilutions of specific primary antibodies (1:1,000) overnight before their one-hour incubation with HRP-conjugated secondary antibodies. Signal was visualized using ECL system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). AR (N20), and GAPDH (8C2) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used in this study.

Chromatin immunoprecipitation (ChIP). Briefly, protein/DNA complexes were cross-linked by 1% formaldehyde for 10 min, then quenched using 125 mM glycine for 10 min. Cells were lysed and subjected to sonication to get chromatin DNA fragments (~500 bp in length). After centrifugation, the supernatant was incubated with AR antibody overnight at 4˚C. Next, pre-blocked A/G beads were added to AR/DNA complex for another 1 h, and chromatin DNA was purified using gel extraction kit (Invitrogen Life Technologies) and subjected to semi-quantitative PCR analysis.

Luciferase assay. Cells were plated in 24-well plates and transfected with MICA-pGL3/MICB-pGL3 or its corresponding mutant (100 ng/well) using Lipofectamine 3000 (Invitrogen Life Technologies) according to the manufacturer's instructions, pRL-TK (1 ng/well) was co-transfected as control. Two days later, cells were lysed for luciferase activity detection by the dual luciferase assay. Each Luciferase reading was performed in triplicate.

Statistical analysis. All values are reported as the mean ± SD and all comparisons were analyzed with a t-test or a one way ANOVA followed by t-test.

Results

LP enhances the cytotoxicity of NK cells to kill PCa cells. In China, LP solution has been applied to treat advanced cancer alone or in combination with chemotherapy drugs. To explore whether LP can alter the capacity of NK cells to lyse PCa cells, we applied co-culture system as indicated in Fig. 1A to end this. Interestingly, LP alone failed to influence CWR22Rv1 cell viability while combined with NK-92MI cells dramatically reduced the cell number of CWR22Rv1 after 24-hour co-culture (Fig. 1B), indicating that LP indeed could enhance the killing ability of NK-92MI cells. We also obtained similar result when CWR22Rv1 cell line was replaced by C4-2 cell line (Fig. 1C). One hallmark of NK activation is cytokine production. To investigate this, we collected NK-92MI cells after their co-culture with CWR22Rv1 cells and extracted RNAs for the measurement of TNFα and IFNr, two important cytokines determining the cytotoxic activity of NK cells. Consistently, the expression levels of TNFα and IFNr were much more abundant in cells which were treated by LP compared to these in non-treated ones (Fig. 1D). Taken together, all these data validate the notion that LP could activate NK cells to kill PCa cells.

AR renders LP-mediated NK cytotoxicity to PCa cells. Previous study has documented that NK cells could suppress

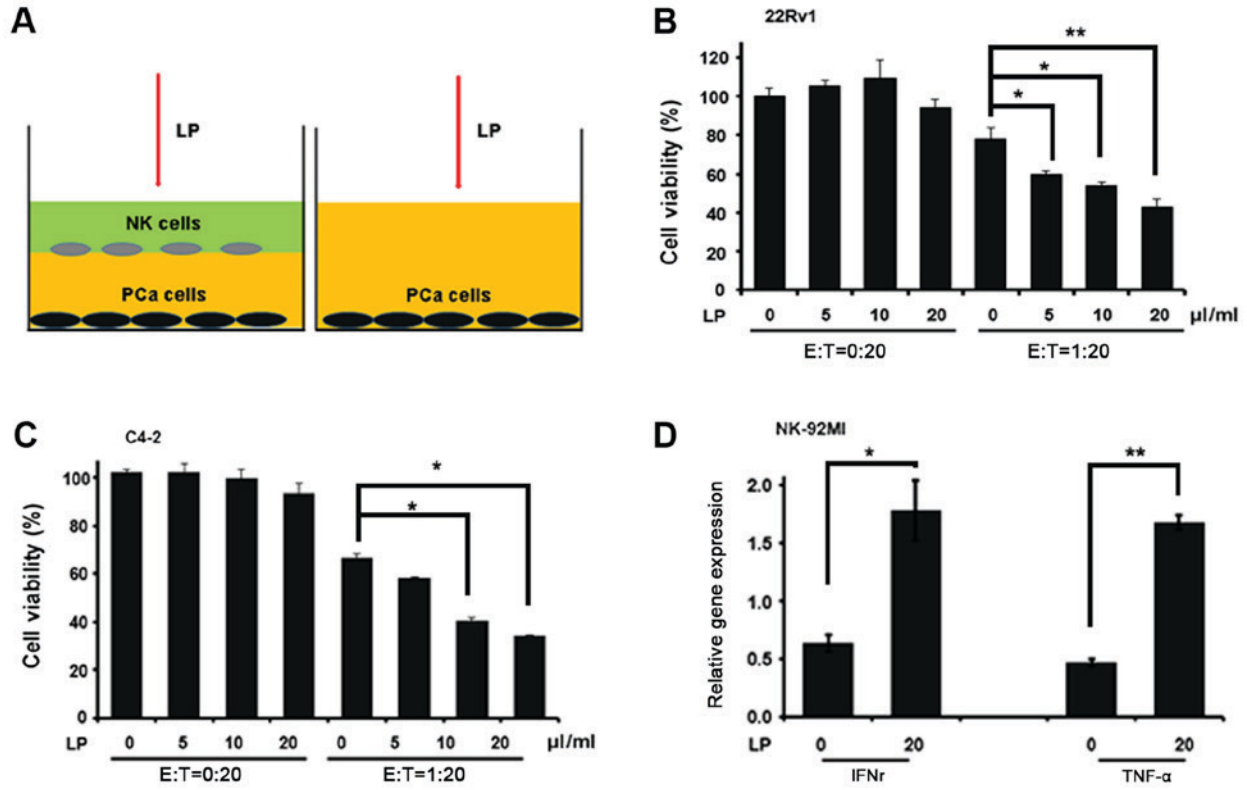

Figure 1. LP enhances the cytotoxicity of NK cells to kill PCa cells. (A) Cartoon showing the co-culture system of NK-92MI cells and PCa cells with LP solution. (B) LP enhances the cytotoxic activity of NK-92MI cell against CWR22Rv1 cells and (C) C4-2 cells. PCa cells (targets:T) were seeded into 24-well plate and incubated with NK-92MI cells (effectors:E) at 20:1 ratio (T:E=20:1) with various concentration of LP solution. Cell viability was measured by MTT assay. (D) Cytokine production was stimulated by LP solution. After co-culture, NK-92MI cells were collected for TNFα and IFNr detection using qPCR. RNAs were normalized to GAPDH, $^{*}P<0.05$, $^{**}P<0.01$. LP, lienal polypeptide; NK, natural killer; PCa, prostate cancer.

AR expression (17). Consistently, we also found that the protein levels of AR in both CWR22Rv1 (Fig. 2A) and C4-2 (Fig. 2B) were markedly suppressed when LP was present in the co-culture system. However, AR mRNA levels are indiscriminate in this system (data not shown), suggesting NK cells/LP mediated AR decrease is independent of transcriptional regulation. To validate the involvement of AR signaling in conferring the cytotoxicity of NK cells to PCa cells, we exogenously introduced AR cDNA into CWR22Rv1 cells and found that the cytotoxicity of NK cells to PCa cells was attenuated even when LP was present in the co-culture system (Fig. 2C). Similar phenomenon was observed in C4-2 cells (Fig. 2D). Collectively, these data indicate that AR signaling in PCa cells is one of the effectors rendering NK cytotoxicity to PCa cells, which is mediated by LP solution in this case.

MICA and MICB are directly regulated by AR. Given that fact that AR is implicated into LP-mediated NK cytotoxicity, we continue to explore the downstream genes those are responsible for LP-mediated NK activation. To end this, we first focused several candidates (MICA, MICB, ULBP1, ULBP2 and ULBP3) because they are NKG2D ligands and can stimulate NK cells activation (18). As shown in Fig. 3A and B, MICA and MICB were consistently elevated at mRNA levels when AR was suppressed in CWR22Rv1 and C4-2 cells. According to online software analysis indicating that there is one AR response element (ARE) in the proximal promoters of MICA and MICB (Fig. 3C, top), we postulate that AR transcriptionally regulates their expression levels. Indeed, ChIP assay revealed that AR directly bound the proximal promoters of MICA and MICB (Fig. 3C, bottom). To test whether AR could regulate the promoters' activities of MICA and MICB, we performed luciferase-based promoter's activity assay, which showed that AR could suppress the promoters' activities of MICA and MICB while the suppression on their corresponding mutants could not be observed (Fig. 3D). All these data suggest that AR transcriptionally suppresses MICA and MICB expression, which can be reversed by LP-mediated NK activation.

MICA and MICB are involved in LP-mediated NK activation. To further confirm the role of MICA and MICB in LP-mediated NK cytotoxicity, we first measured the expression levels of MICA and MICB in PCa cells upon NK treatment in the presence or absence of LP. Fig. 4A demonstrated that combination of NK cells and LP could elevate MICA and MICB expression in C4-2 cells. More importantly, LP-mediated NK cytotoxic activity against PCa cells was partially abolished by knockdown of MICA and MICB in C4-2 cells (Fig. 4B and C). All these data prove that MICA/MICB is indeed involved in LP-mediated NK activation, probably by binding to NKG2D receptor on the surface of NK cells.

In conclusion, LP could activate NK cells to kill PCa cells. In return, PCa cells undergo an AR downregulation process

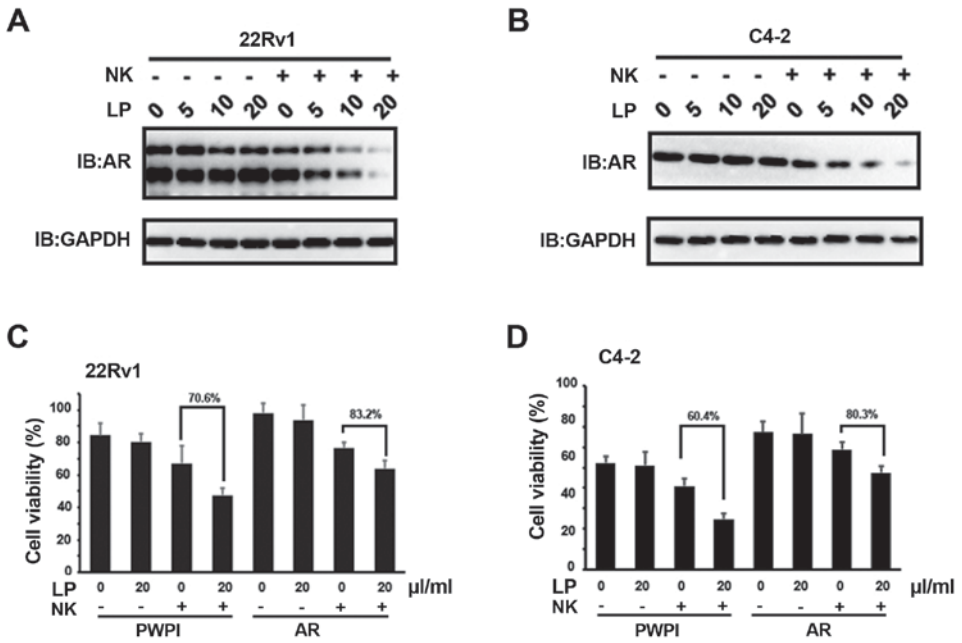

Figure 2. AR renders LP-mediated NK cytotoxicity to PCa cells. (A and B) AR expression levels were reduced in (A) CWR22Rv1 and (B) C4-2 by NK-92MI cells upon LP stimulation. GAPDH was used as loading control. (C and D) Overexpression of AR in (C) CWR22Rv1 and (D) C4-2 cells could partially attenuate LP-mediated NK-92MI activation. PCa cells were seeded into 24-well plate and incubated with NK-92MI cells at 20:1 ratio with 20 μl/ml LP solution. Cell viability was determined by MTT assay. AR, androgen receptor; LP, lienal polypeptide; NK, natural killer; PCa, prostate cancer.

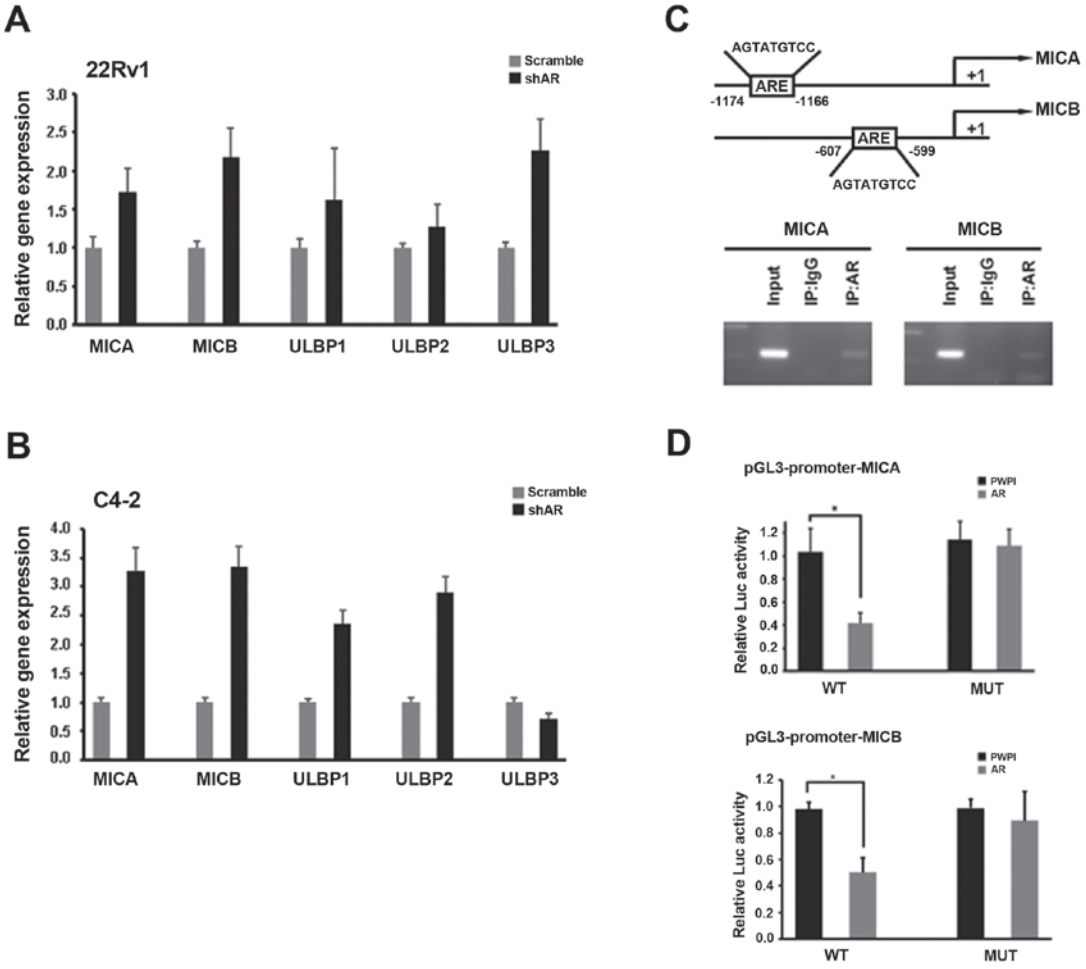

Figure 3. MICA and MICB are directly regulated by AR. (A and B) Knockdown of AR enhances the expression levels of MICA and MICB in both (A) CWR22Rv1 and (B) C4-2 cells. RNAs were normalized to GAPDH. (C) Top, AR binding site in the proximal promoters of MICA and MICB. Bottom, ChIP assay showed that AR directly binds to directly binds to the promoters of MICA and MICB. (D) Luciferase based promoter activity assay revealed that AR could suppress the promoter's activities of both MICA and MICB, $^{*}P<0.05$. MICA, MHC class I polypeptide-related sequence A; AR, androgen receptor.

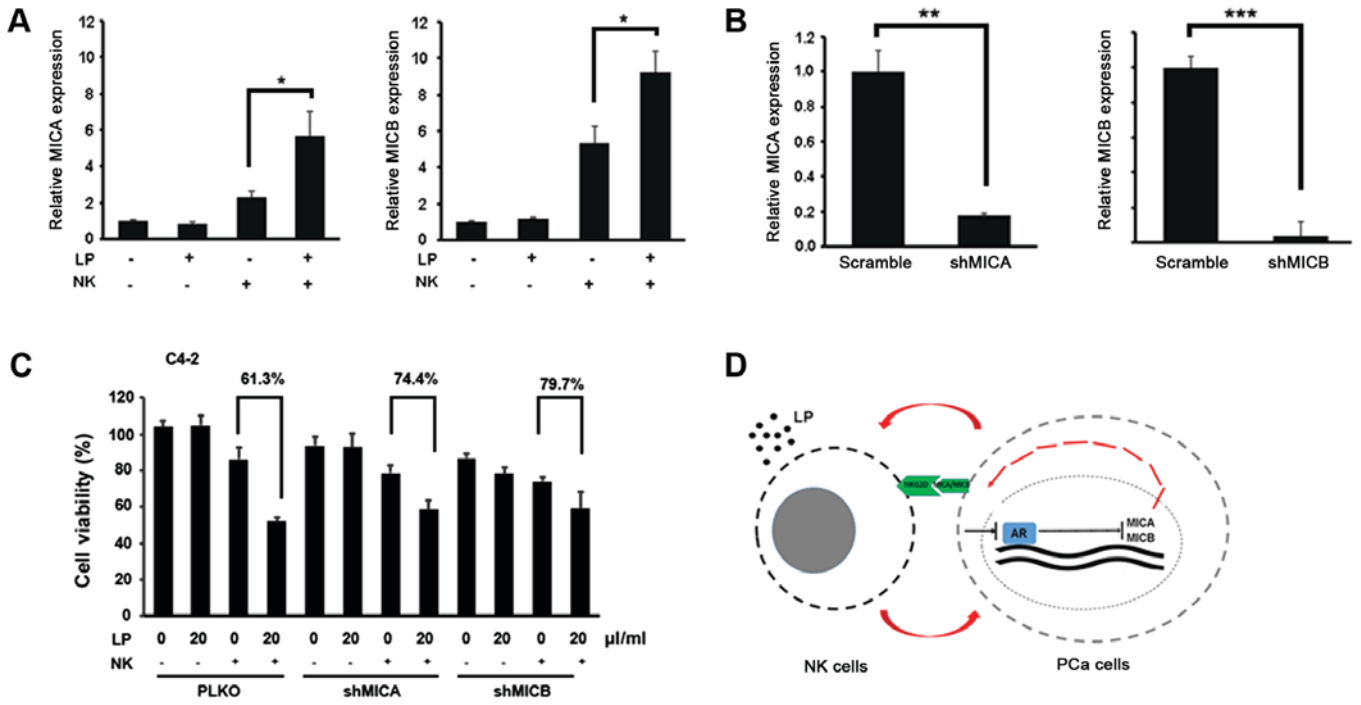

Figure 4. MICA and MICB are involved in LP-mediated NK activation. (A) NK-92MI cells could further enhance the expression levels of MICA and MICB in PCa cells when LP solution was supplied into co-culture medium. RNAs were normalized to GAPDH. (B and C) Knockdown of MICA or MICB in (B) C4-2 cells blocked LP-mediated killing ability of (C) NK-92MI cells. (D) Schematic depiction the interaction between NK and PCa cells in the presence of LP solution, *P<0.05, **P<0.01, ***P<0.001. MICA, MHC class I polypeptide-related sequence A; LP, lienal polypeptide; NK, natural killer; PCa, prostate cancer.

to boost MICA/MICB expression, which further triggers NK cells activation to form a positive loop (Fig. 4D).

Discussion

PCa continues to threaten male's health for many decades. Although drugs targeting AR signaling have shown promising results in the treatment of PCa patients, the complex micro-environment surrounding tumor is one of the obstacles that affects their therapeutic efficacy. As a key player in innate immune response, NK cells play fundamental roles in the progression of PCa and their infiltration is highly associated with clinical outcomes. Here, we found that LP solution can boost the cytotoxic activity of NK cells to lyse PCa cells. Interestingly, we also observed that LP-mediated NK activation can downregulate AR expression, leading to the released expression of its downstream genes: MICA and MICB. Overexpression of AR or knockdown of MICA/MICB could attenuate LP-mediated cytotoxicity of NK cells to both C4-2 and CWR22Rv1 cells, suggesting that AR-MICA/MICB pathway is the downstream effector of NK cells and LP solution.

The population of NK cells in PCa microenvironment is highly related to disease progression. Study has demonstrated that patients who harbor higher amount of NK cells would have much delayed castration resistance. Since LP solution can activate the cytotoxicity of NK cells, we believe that LP solution, if applied clinically, would synergize ADT treatment. More importantly, amplification of AR signaling due to gene amplification, gene mutation and occurrence of AR-v7 in castration resistant stage are the major concerns for current treatments (19-22), which would be alleviated by LP solution because it enhances the capacity of NK cells to downregulate both AR-FL and AR-v7. All these data suggest that LP is the potential agent that can alter castration resistance via stimulating the activity of NK cells.

We also found that LP can upregulate MICA/MICB expression via degrading AR. MICA and MICB, which interact with their common receptor NKG2D on NK cells to trigger cytotoxic event or cytokine production, are frequently observed in PCa patients, indicating its potential role in linking PCa tumor to NK cells (23). The upregulation of MICA and MICB mediated by LP would provide a positive feedback loop between PCa and NK cells. In this case, PCa cells undergo a self-destroyed mechanism to recruit more NK cells to kill themselves. Also, in castration resistant stage, the amplification of AR signaling probably suppresses MICA/MICB expression so that the interaction between NK cells and PCa cells is marginal, resulting in insufficient cytotoxicity of NK cells to PCa cells. In summary, our data provide strong rational to develop LP solution as new agent to battle PCa.

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