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# IL-2-Mediated Upregulation of uPA and uPAR in Natural Killer Cells

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**Urokinase plasminogen activator (uPA) and its receptor uPAR play a major role in immune cell-mediated, including natural killer (NK) cell-mediated, degradation of extracellular matrices. Herein, we investigate the effects of IL-2 on NK cell uPA and uPAR. RNA and protein analyses showed upregulation of uPA and uPAR following IL-2 stimulation. Gel-shift assays and Western blots detected uPA and uPAR mRNA binding proteins (mRNABPs), previously shown to destabilize uPA and uPAR mRNA. Following IL-2 stimulation, a downregulation of uPAR mRNABP and a reciprocal induction of uPAR mRNA were noted. The increase in uPA following IL-2 stimulation appeared to be more transcriptionally regulated. These data suggest that IL-2 upregulates both uPA and uPAR in NK cells through posttranscriptional as well as transcriptional mechanisms, partially explaining increases in NK cell invasiveness following IL-2 stimulation.** 

***Key Words:*** **NK cells; urokinase; interleukin-2; cytokines; gene regulation.**

The urokinase plasminogen activator (uPA) system has been shown to play a major role in the extravasation and migration of leukocytes into areas of inflammation (1, 2). In the processes of inflammation, as leukocytes invade into diseased tissues and approach their target cells, various proteases such as uPA, matrix metalloproteinases (MMPs), human leukocyte elastase, and cathepsin G, each displaying a selective specificity for components of the extracellular matrix (ECM), cooperate to degrade ECM proteins that form barriers between the leukocytes and their target cells (3–7). The uPA system is capable of directly degrading glycoprotein components of the ECM such as laminin and fibronectin, as well as interacting with MMPs and their inhibitors in the degradation of various other ECM components (8, 9). One unique feature of the uPA system is the presence of a specific cellular receptor for uPA; uPAR, which is involved in cell migration and invasion independent of its role in the proteolytic pathways (8). uPAR has been shown to be involved in cellular adhesion by its ability to bind to the ECM protein vitronectin (10, 11), and has the capacity to initiate secondary signaling pathways through its interactions with the integrins, thereby promoting cellular movement and migration (12–14). Recently, we have demonstrated the expression of both uPA and uPAR by NK cells, have shown that NK cells employ the uPA system in their *in vitro* invasion through Matrigel (15), and have reported an increase in NK cell invasion through Matrigel following stimulation with IL-2 (16).

NK cells have been shown to exhibit high antitumor properties which has allowed for their successful use in cancer immunotherapy (17–20). Prior to their administration into tumor bearing animals, NK cells are stimulated with IL-2, one of the most potent activators of NK cells, and are termed activated NK (A-NK) cells. NK cells, including the human NK cell line (YT) used in our studies, have been shown to express both the high and moderate affinity IL-2 receptors, and to respond to IL-2 stimulation by increasing their proliferation and cytotoxicity toward other cells (21–23).

Although there have been numerous studies on the MMPs of IL-2 activated NK cells (16, 24, 25), to date there has been very little research on the effects of IL-2 on NK cell uPA and uPAR, and the concomitant effects on NK cell invasiveness. Understanding the effects of IL-2 on NK cell invasiveness is crucial for optimizing NK cell antitumor adoptive immunotherapy. NK cells must be able to traverse the basement membrane (BM)/ECM barriers and establish cell-cell contact with their target cells for effective cytolysis (26). Although the activation of NK cell cytotoxicity by IL-2 has been well established (23, 27–30), the effects of IL-2 on NK

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cell proteolytic enzymes, and hence their capacity to invade the BM/ECM following IL-2 stimulation, must be addressed to improve the therapeutic efficacy of A-NK cancer immunotherapy.

It has been shown that in lung fibroblasts, mesothelial, epithelial, and carcinoma cells, the regulation of uPA and uPAR expression occurs at a posttranscriptional level through mRNA binding proteins (mRNABPs) that destabilize uPA/uPAR mRNA following mRNA-mRNABP binding. In the case of uPA, a 30 kD protein was detected that binds to 66 nucleotides in the uPA mRNA 3′-untranslated region (UTR) (31). Likewise, the regulation of uPAR was also shown to be mediated through the interaction of uPAR mRNA with a 50 kD protein that bound to a 51 nucleotide fragment of the uPAR mRNA coding region (32–34).

In this report we demonstrate an increase of both uPA and uPAR following stimulation of NK cells with IL-2. uPA and uPAR mRNABPs were detected in NK cells, and the noted increases in uPA and uPAR following IL-2 stimulation correspond to changes in uPA and uPAR mRNA-mRNABP interactions. Herein we document for the first time the presence of posttranscriptional regulation of both uPA and uPAR in NK cells following IL-2 stimulation.

## MATERIALS AND METHODS

*Cells and cell culture.* YT cells (human non-ATL leukemic cell line, a kind gift from Dr. Porunelloor Mathew, University of North TX Health Science Center (35)) were cultured in RPMI 1640 with 10% FBS, 55 $\mu$M 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin sulfate, 2 mM glutamine, 0.1 mM MEM nonessential amino acids, and 1 mM sodium pyruvate. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air at a density of approximately $10^6$ cells/ml.

*Reverse transcriptase (RT)-PCR.* Total RNA was isolated from YT cells using TRI Reagent-chloroform (Molecular Research Center, Inc., Cincinnati, OH). cDNA synthesis was performed using the RT-PCR kit from Stratagene (La Jolla, CA). For each cDNA synthesis, total RNA (5 $\mu$g) from NK cells was reverse transcribed using random hexamer or oligo $(dT)_{16}$ primer in a volume of 50 $\mu$l each, according to the protocol supplied by Stratagene. The two reactions were combined after heat inactivation of reverse transcriptase, and 2 $\mu$l of the cDNA were used for each PCR amplification. uPAR primers (5′-3′) forward CCAATGGTTTCCACAACGA and reverse GGTCACACAGCAAGTCTGTA, uPA primers (5′-3′) forward GTGGCCAAAAGACTCTGAGG and reverse ATTTTCAGCTGCTCCGGATA, and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (5′-3′) forward TAGACGGGAAGCTCACTGGC and reverse AGGTCCACCACCCTGTTGCT were added to the PCR reactions at a final concentration of 30 $\mu$M. The reactions were carried out using HotStart Taq polymerase (Qiagen, Valencia, CA).

*Ammonium sulfate precipitation.* Approximately $1 \times 10^8$ cells were serum starved for 12 h in OPTI-MEM serum free media (Gibco BRL Life Technologies, MD), and stimulated with IL-2 (1000 IU/ml) for 3, 6, and 12 h. At each time point, the cells were harvested and sonicated in 10 ml of dialysis buffer (2.5 mM Tis-HCl pH 7.9, 0.05 mM EDTA pH 7.45, 0.1 mM PMSF). Cell lysates were centrifuged at 30,000*g* for 15 min at 4°C. The pellet was discarded and to the supernatant solid $(NH_4)_2SO_4$ crystals (Sigma, St. Louis, MO) were added to yield a final $(NH_4)_2SO_4$ saturation of 40%. The $(NH_4)_2SO_4$-supernatant mixture was incubated for 30 min at 4°C with continuous stirring. The 40% $(NH_4)_2SO_4$ solution containing precipitated proteins was centrifuged at 30,000*g* for 15 min at 4°C. The pellet containing proteins was discarded, solid $(NH_4)_2SO_4$ crystals were added to the 40% $(NH_4)_2SO_4$ supernatant to yield a final $(NH_4)_2SO_4$ saturation of 60%, and again the 60% $(NH_4)_2SO_4$ supernatant was incubated for 30 min at 4°C with continuous stirring. The 60% $(NH_4)_2SO_4$ solution containing precipitated proteins was centrifuged at 30,000*g* for 15 min at 4°C. The supernatant was discarded and the 40–60% $(NH_4)_2SO_4$ precipitated proteins were collected, redissolved, and exhaustively dialyzed against extraction buffer containing 10% glycerol.

*Cytosolic protein extraction.* Approximately $5 \times 10^7$ cells were harvested, washed 3× with PBS, resuspended in 5 ml of hypotonic buffer (10 mM Tris-HCl pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM PMSF), and kept on ice for 15 min. The cells were centrifuged at 500*g* for 8 min, and resuspended in 200–400 $\mu$L of hypotonic solution. The cells were lysed using a motorized homogenizer, and were centrifuged at 6000*g* for 8 min. The post-nuclear supernatants were collected and used as the cytosolic extracts following protein estimation.

*Cell homogenization.* Approximately $5 \times 10^7$ cells were harvested, washed 3× with PBS, and resuspended in 300 $\mu$L of lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 15% glycerol, 1 mM $Na_3VO_4$, 1 mM EDTA, and 1 mM PMSF. Cells underwent 5 freeze/thaw cycles using dry ice and 37°C with continuous vortexing. Cell lysates were then centrifuged at 1000*g* for 8 min. The pellet was discarded and the supernatants were used in Western blot analyses.

*Western blotting.* Cell homogenates and 40–60% $(NH_4)_2SO_4$ precipitated protein extracts were electrophoresed on 10% SDS-polyacrylamide gels under non-reducing conditions. Gels were then electroblotted onto nitrocellulose membranes, which were then blocked for 1 h with 1% BSA in wash buffer containing 0.6% w/v NaCl, 10 mM Tris-HCl pH 7.4, 0.025% Tween-20. For uPA detection, mouse anti-human uPA monoclonal antibody (American Diagnostica, Greenwich, CT) was used at a final concentration of 66 pg/ml. For uPAR mRNABP detection, guinea pig anti-rabbit uPAR mRNABP polyclonal antibody was used at a final concentration of 100 ng/ml. Goat anti-mouse peroxidase conjugate (Pierce Chemical, Rockford, IL) and goat anti-guinea pig peroxidase conjugate (Rockland, Gilgertsville, PA) were used as secondary antibodies. The resulting chemiluminescence was recorded on Fuji film (Medical Systems U.S.A. Inc., Stamford, CT).

*Gel mobility shift assays.* RNA-protein binding assays were performed using $^{32}P$ uniformly-labeled transcripts corresponding to the uPA mRNA 3′-untranslated region (UTR) (31–33). $^{32}P$-labeled uPA transcripts (30,000 cpm) were incubated with the cytosolic extracts (50 $\mu$g) in 15 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 0.25 mM dithiothreitol (DTT), 12 mM HEPES, pH 7.9, 10% glycerol, and *E. coli* tRNA (200 ng/$\mu$L) in a total volume of 60 $\mu$L at 30°C for 30 min. To degrade free $^{32}P$-labeled mRNA, reaction mixtures were then treated with 50 units of RNase $T_1$ (Life Technologies Inc., Grand Island, NY) for 30 min at 37°C. Heparin (final concentration 5 mg/ml) (Sigma, St. Louis, MO) was added to the reaction mixtures and incubated at room temperature for 10 min to eliminate nonspecific protein binding. Samples were loaded on to 5% native polyacrylamide gels with 0.25× TBE running buffer and separated by electrophoresis. The gels were then dried and autoradiographed at −70°C using Kodak X-AR film (Eastman Kodak, Rochester, NY).

*Steady-state mRNA assessment by transcription chase.* Following 12 h of serum starvation in OPTI-MEM serum free media, the cells in the experimental group were stimulated with IL-2 (1000 U/ml) for 12 h in OPTI-MEM, while those in the control group were incubated in OPTI-MEM for 12 h. Actinomycin-D (Sigma, St. Louis, MO) was then added to the cell cultures at a final concentration of 10 $\mu$g/ml, to inhibit ongoing transcription. RNA was extracted as described above immediately following the addition of actinomycin-D and at times 3,

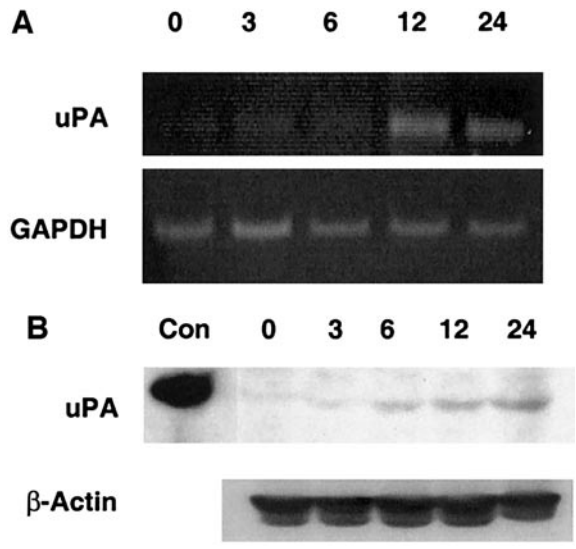


**FIG. 1.** Effects of IL-2 on uPA expression. (A) RT-PCR showing an increase in uPA mRNA following 3, 6, 12, and 24 h of stimulation with IL-2 (1000 U/ml). GAPDH was used to account for the variations in the mRNA extracted. (B) Western blot analysis demonstrating the effects of IL-2 stimulation on the uPA protein levels in YT cells at 3, 6, 12, and 24 h. $\beta$-Actin protein levels are used as loading controls. Time 0 refers to unstimulated control cells. Experiments were performed in duplicate. Con = 2.0 $\mu$g uPA positive control (American Diagnostica, Greenwich, CT).

6, 12, and 24 h following the addition of actinomycin-D. The RNA was then reverse transcribed and PCR amplified using the uPA primers shown above.

## RESULTS

### *Effects of IL-2 on uPA Expression*

The effects of IL-2 on uPA mRNA expression were studied using RT-PCR and Western blotting. Stimulation of YT cells with IL-2 for 3, 6, 12, and 24 h resulted in a time-dependent increase in uPA mRNA as detected by RT-PCR (Fig. 1A). The maximal uPA induction occurs at 12 h following IL-2 stimulation, and very little upregulation is seen between 12 and 24 h. Western blot analysis using anti-uPA monoclonal antibody was used to show the effects of IL-2 on uPA protein level (Fig. 1B). Consistent with the RT-PCR data, there also appears to be a time dependent upregulation of uPA protein levels with a peak induction 12 h following IL-2 stimulation.

### *Effects of IL-2 on uPA mRNABP*

We investigated the presence of uPA mRNABP in YT cells to determine whether or not the uPA mRNA-mRNABP interactions caused the noted increase in uPA expression following IL-2 stimulation. YT cytosolic extracts were analyzed using gel shift assays to determine the effects of IL-2 on uPA mRNA-mRNABP interactions. Following the stimulation of YT cells with IL-2 (1000 U/ml), the capacity of the uPA mRNABP to bind to uPA mRNA decreases (Fig. 2). This decrease appears to be time dependent with a maximum decrease occurring 24 h following IL-2 stimulation. Cold competition assays using 200-fold molar excess unlabeled probe confirmed the specificity of the uPA mRNA-mRNABP interactions (data not shown).

### *Decay of uPA mRNA*

Transcription chase experiments were conducted to determine the effects of transcriptional inhibition on uPA mRNA stability. Figure 3 compares the degradation of uPA mRNA in the IL-2 stimulated cells with the uPA mRNA degradation in unstimulated cells. The half-life of uPA mRNA following IL-2 stimulation ($\approx$7 h) appears to be slightly longer than the half-life in the unstimulated group ($\approx$5 h).

### *Effects of IL-2 on uPAR mRNA*

Figure 4 demonstrates the effects of IL-2 on uPAR mRNA of YT cells. Cells were serum starved in OPTI-MEM serum free media for 12 h. Following IL-2 (1000 U/ml) stimulation for 3, 6, 12, and 24 h, RNA was extracted and RT-PCR was conducted as described above. The results (Fig. 4) show a time-dependent increase in the uPAR mRNA compared with unstimulated cells (time 0). GAPDH levels were examined to control for variations in the RNA used in the RT-PCR.

### *Effects of IL-2 on uPAR mRNABP*

Western blot analysis was used to determine whether or not YT cells expressed uPAR mRNABP,

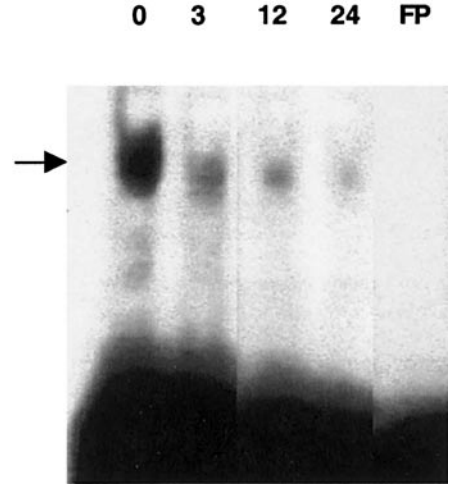


**FIG. 2.** uPA mRNA-mRNABP interactions. YT cytosolic extracts (50 $\mu$g) containing RNase $T_1$ resistant $^{32}$P-labeled uPA mRNA-mRNABP complexes were resolved on a 5% polyacrylamide gels, dried, and autoradiographed. The bands reflect the degree of uPA mRNA-mRNABP interactions following 3, 12, and 24 h IL-2 (1000 U/ml) stimulation compared with control unstimulated cells (time 0). FP refers to free probe without any sample. Experiments were performed in duplicate.

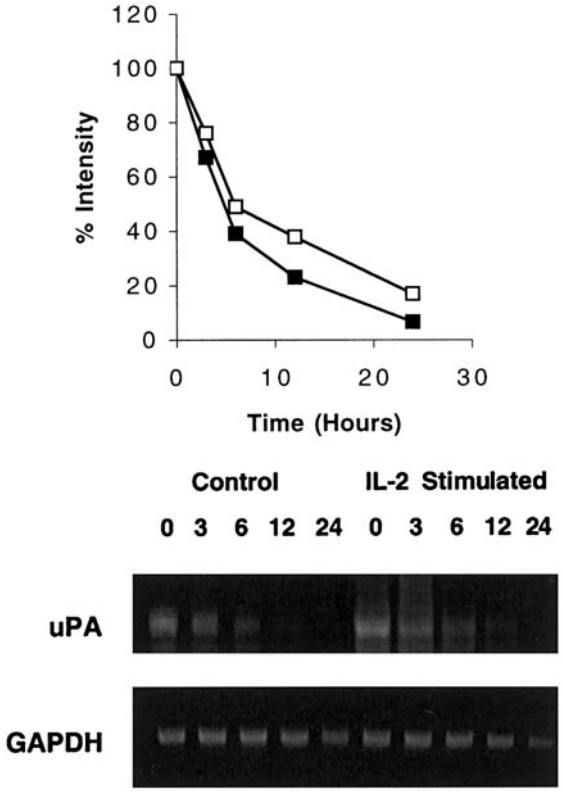


**FIG. 3.** Stability of uPA mRNA following IL-2 stimulation. Following serum starvation, IL-2 (1000 U/ml) stimulation, and actinomycin-D treatment, RNA was extracted and RT-PCR was conducted. (Top) Data were transformed and graphed over 24 h to compare the rate of uPA mRNA decay following IL-2 stimulation (□) to control unstimulated cells (■). (Bottom) RT-PCR showing the decay of the uPA mRNA over time 3, 6, 12, and 24 h following actinomycin-D treatment. GAPDH shows equal amounts of RNA used in the RT-PCR. Experiments were performed in triplicate.

and to investigate the effects of IL-2 on uPAR mRNABP. Following the stimulation of YT cells with IL-2 (1000 U/ml), Western blot analysis of protein samples precipitated from the 40%–60% $(NH_4)_2SO_4$ fraction, which has been shown to contain the uPAR mRNABP in human pleural mesothelial cells (33), was utilized to determine if the noted increases in the uPAR mRNA following IL-2 stimulation of YT cells coincided with changes in the expression of uPAR mRNABP. Consistent with the uPAR mRNA increases noted via RT-PCR (Fig. 4), Western blot analysis demonstrate a time dependent decrease in the level of expression of uPAR mRNABP following IL-2 stimulation. We also found similar results with gel mobility shift assays.

## DISCUSSION

The uPA system appears to play a major role in the accumulation of immune cells into inflamed pathologic tissues (1, 2). IL-2 activated NK cells have shown potential for use in antitumor therapy (17–19). Although

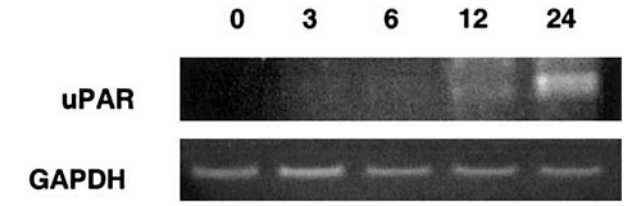


**FIG. 4.** Effects of IL-2 on uPAR expression. Cells were serum starved for 12 h and then stimulated with IL-2 (1000 U/ml) for 3, 6, 12, and 24 h. RNA was extracted and RT-PCR was used to analyze the uPAR mRNA levels following IL-2 stimulation. Time 0 refers to unstimulated control cells. GAPDH was used to account for the variations in the mRNA extracted. Experiments were performed in duplicate.

IL-2 is primarily known for its ability to activate NK cell-mediated cytotoxicity (23, 27–30, 36), in the present study we report an increase in both uPA and uPAR mRNA and protein following stimulation of NK cells with IL-2, possibly contributing to the previously observed increases in NK cell invasion through Matrigel following IL-2 stimulation (16).

Previous reports have shown that uPA and uPAR are regulated posttranscriptionally through interaction of uPA and uPAR mRNA with their respective destabilizing mRNABPs (31–34). In this report, we document the presence of both uPA and uPAR mRNABPs in NK cells. The uPA mRNABP was detected in cytosolic extracts of YT cells. The interaction of the uPA mRNABP decreased with time following YT cell stimulation with IL-2 (Fig. 2). In spite of the changes detected in uPA mRNABP levels, transcription chase experiments showed that the upregulation of uPA by IL-2 is primarily controlled transcriptionally, although a slight posttranscriptional component is also noted. This dual regulation of components of the uPA system is not unique to NK cells. Maity *et al.* have also detected both transcriptional and posttranscriptional regulation of uPAR mRNA following hypoxia in human MCF7 breast carcinoma cells (37). As demonstrated by Western blot analysis (Fig. 5), there appears to be a downregulation of uPAR mRNABP expression following IL-2 stimulation. This downregulation of uPAR mRNABP may be a

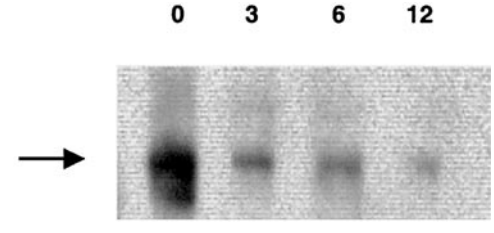


**FIG. 5.** Effects of IL-2 on uPAR mRNABP. Western blot analysis showing the effects of IL-2 on uPAR mRNABP expression. Protein samples (20 $\mu$g) were precipitated from YT cell 40%–60% $(NH_4)_2SO_4$ fractions 3, 6, and 12 h following IL-2 stimulation. Time 0 refers to control unstimulated cells. Experiments were performed in duplicate.

major contributor to the noted increase in uPAR mRNA following IL-2 stimulation of YT cells (Fig. 4). Even though we infer that IL-2 downregulates the production of uPAR mRNABP by YT cells, we can not exclude other mechanisms such as structural modifications to the uPAR mRNABP that could also contribute to the noted increase in uPAR mRNA following IL-2 stimulation.

Our data indicate that the posttranscriptional regulation of uPAR in YT cells following IL-2 stimulation appears to be controlled at least in part through destabilizing mRNABP interactions. Furthermore, in contrast to T-lymphocyte posttranscriptional regulation of uPAR mRNA, the destabilizing protein detected in YT cells has been shown to interact with nucleotides within the uPAR mRNA coding region (195–246), rather than the AU rich motif of the uPAR mRNA 3′UTR (32, 34). An understanding of these events may allow for the design of better approaches for cell-mediated immunotherapy, e.g. for the development novel approaches that will allow the generation of potent NK cells without the use of toxic immunostimulants and cytokines (e.g. IL-2) for their activation.

In view of reports showing the capacity of ECM proteins and protease inhibitors within the tumor microenvironment to form barriers preventing NK cell entry into tumors (38, 39), we speculate that under such conditions, modifying uPA and/or uPAR mRNABP levels in NK cells prior to their administration, can potentially lead to enhanced tumor infiltration by NK cells, and consequently more effective tumor destruction. Since the infiltration of NK cells into tumor tissues may be required for effective antitumor therapy, and since NK cell-target cell contact may be essential for NK cell killing (26, 40–42), increasing NK cell invasiveness prior to NK cell administration for cancer immunotherapy, by upregulating uPA and/or uPAR, may lead to increased tumor infiltration and eradication by NK cells.

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