International Immunopharmacology 63 (2018) 66–73

Contents lists available at ScienceDirect

International Immunopharmacology

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

CXCR3-deficient natural killer cells fail to migrate to B16F10 melanoma cells

Juyoung Kim[1], Ji Sung Kim[1], Hong Kyung Lee, Hyung Sook Kim, Eun Jae Park, Jeong Eun Choi, Yeo Jin Choi, Bo Ram Shin, Eun Young Kim, Jin Tae Hong, Youngsoo Kim, Sang-Bae Han*

College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 28160, Republic of Korea

ARTICLE INFO

Keywords:
Natural killer cells
Migration
CXCL10–CXCR3 axis

ABSTRACT

Natural killer (NK) cells eliminate cancer cells in a contact-dependent manner. However, how NK cells find cancer cells remain unclear. Here, using time-lapse imaging, we investigated how individual NK cells migrate toward cancer cells. Although naïve B16F10 cancer cells produce low levels of chemokines, IFN-γ-treated B16F10 cells secreted high levels of CXCL10, low levels of CCL5, but did not secrete CCL2, CCL7, or CXCL12. Wild-type NK cells migrated well toward cancer cells and killed them, whereas NK cells deficient in CXCR3 did not. CXCR3-deficient NK cells also showed slower migration speed than did wild-type NK cells. Taken together, our data show that NK cells find cancer cells, at least in part, by sensing CXCL10 produced by cancer cells and suggest that a strategy to increase CXCL10 secretion by cancer cells may improve the efficacy of NK cell–based immunotherapy.

1. Introduction

Natural killer (NK) cells are innate lymphocytes at the first line of defense against tumor cells and cells infected with viruses and microbes [1]. The effector functions of NK cells are regulated by the balance of activating and inhibitory signals [1]. Cancer cells tend to down-regulate the expression of MHC class I molecules for NK cell inhibitory receptors [2,3]. Ligands for NK cell activating receptors are typically not expressed or are present only at low levels on the surface of normal cells, but their overexpression is often induced by infection or malignant transformation [4,5]. Therefore, NK cells selectively eliminate target cells identified by activating signals but leave normal cells unharmed [6].

NK cell recruitment to solid tumors is essential for direct contact between NK and cancer cells and is regulated by chemokine networks. Chemoattractants regulate the distribution of NK cells in tissues under normal and pathological conditions [7, 8]. CXCR4 acts to retain immature NK cells in bone marrow [9]. Sphingosine 1-phosphate receptor 5 is upregulated upon NK cell maturation and induces egress from bone marrow to blood upon sensing sphingosine 1-phosphate [10]. Multiple chemokine receptors, such as CCR2, CCR5, CXCR3, CX3CR1, and ChemR23, regulate NK cell recruitment to solid tumors [11, 12]. CXCR3 is important for NK cell recruitment to tumor beds and inflamed tissues [13, 14]. CXCR3 binds CXCL10, which is also known as interferon (IFN)-γ inducible protein 10 (IP-10), and is associated with infectious diseases, chronic inflammatory and autoimmune diseases, and cancers in humans [15]. CXCL10 is secreted from a variety of cells, such as neutrophils, monocytes, endothelial cells, and fibroblasts [15–17]. In addition, CXCL10 can be secreted from various tumor cell lines, such as melanoma, renal cell carcinoma, and thyroid carcinoma upon IFN-γ treatment [18].

Tumor-derived CXCL10 increases the infiltration of NK cells into solid tumors [18, 19]. In a xenograft mouse model, adoptively transferred human NK cells efficiently infiltrate CXCL10-transduced tumors and enhance regression of tumor growth compared with mock-transduced tumors [18]. Although this study showed that NK cells homed to solid tumor beds in a CXCL10-depedent manner, several questions remain. How do NK cells find cancer cells after extravasation? Which chemokines do B16F10 cells produce? Which chemokine receptors do NK cells use? How do cancer cell-derived chemokines affect NK cell motility? Do these chemokines directly affect NK cell cytotoxicity? To address these issues, we examined the migration dynamics of NK and cancer cells using time-lapse imaging. At the single-cell level, our data show that CXCR3$^{-/-}$ NK cells show slower movement, less directional migration, and weaker contact-dependent cytotoxicity than wild-type NK cells, although they have similar amount of weapons such as

* Corresponding author at: 194-31, Osongsaengmyung-1, Heungdeok, Cheongju, Chungbuk 28160, Republic of Korea.
E-mail address: shan@chungbuk.ac.kr (S.-B. Han).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.intimp.2018.07.026
Received 22 February 2018; Received in revised form 16 July 2018; Accepted 23 July 2018

perforin and granzymes.

2. Materials and methods

2.1. Mice and cells

C57BL6/J mice were purchased from OrientBio (Sungnam, Gyunggi, Korea) and CXCR3$^{-/-}$ mice (B6; 129P2-Cxcr3^{tm1Dgen}/J) from the Jackson Laboratory (Bar Harbor, ME, USA). All mice (6–10 weeks of age) were housed under specific pathogen-free conditions at 21–24 °C and 40–60% relative humidity under a 12 h light/dark cycle and were used in accordance to the guidelines of the Institutional Animal Care and Use Committee of Chungbuk National University. NK cells were isolated from mouse spleen cells by negative selection using an NK isolation kit (Miltenyi Biotec, Auburn, CA, USA). Purified NK cells were cultured in complete RPMI 1640 medium supplemented with 3000 U/ml recombinant human IL-2 (Bayer HealthCare Pharmaceuticals, Emeryville, CA, USA), 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. Cell proliferation and viability were determined by cell counting in a hemocytometer and trypan blue exclusion assay. Cell purity exceeded 90%. IL-2-activated NK cells were used from day 10 to 12.

Cancer cell lines, such as B16F10, Hepa-1c1c7, Colon 26, and MC57G, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM medium containing 10% fetal bovine serum and 100 μg/ml streptomycin. B16F10 cells were also treated with 100 U/ml rmIFN-γ (R&D Systems, Minneapolis, MN, USA) for 24 h to enhance their CXCL10 secretion (called IFN-B16 hereafter).

2.2. Flow cytometry

Cells were stained for 15 min at 4 °C with antibodies against mouse CD3, CD69, NK1.1, NKG2D (BD Biosciences, San Jose, CA, USA), or DNAM-1 (BioLegend, San Diego, CA, USA). For intracellular staining, NK cells were fixed using a CytoFix-CytoPerm Kit (BD Biosciences) according to the manufacturer's instructions and then were stained with anti-perforin–APC antibody and anti-granzyme B–FITC antibody (eBioscience, San Diego, CA, USA). Cells were analyzed using a FACSCalibur flow cytometer and the data were processed using Cell Quest Pro software (BD Biosciences).

2.3. Cytotoxicity assay

NK cells were incubated with cancer cells in 96-well plates at various effector-to-target cell ratios. Cytotoxicity was determined using a lactate dehydrogenase (LDH) release assay according to the manufacturer's instructions (Takara, Shiga, Japan). After 4 h of incubation, the plates were centrifuged and 100 μl of the supernatant was transferred to new 96-well plates. The release of LDH into the supernatant was quantified by recording the absorbance at 490 nm. The percentage of specific lysis was calculated from LDH as follows: (experimental release – target spontaneous release – effector spontaneous release) / (target maximum release – target spontaneous release) × 100% [20].

2.4. Western blotting

Cell lysates were prepared as previously described [21]. Detergent-insoluble materials were removed, and equal amounts of protein were fractionated by 10% SDS-PAGE and transferred to pure nitrocellulose membranes. Membranes were blocked with TBS/Tween 20 (TTBS) containing 5% bovine serum albumin (BSA) for 1 h and then incubated with an appropriate dilution of primary antibody in TTBS containing 5% BSA for 2 h. Blots were incubated with biotinylated secondary antibody for 1 h and then with HRP-conjugated streptavidin for 1 h. Signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Anti-mouse antibodies against STAT1, phospho-STAT1, CCR2, CCR5, and CXCR3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Table 1
Primers.

Protein	Sequences (sense, anti-sense)
CCR2	5′-GGTCATGATCCCTATGTGG-3′, 5′-CTGGGCACCTGATTTAAAGG-3′
CCR5	5′-GCTGAAGAGCGTGACTGATA-3′, 5′-GAGGACTGCATGTATAATGA-3′
CXCR3	5′-GAACGTCAAGTGCTAGATGCCTCG-3′, 5′-GTACACGCAGAGCAGTGCG-3′
CCL2	5′-CCCAATGAGTAGGCTGGAGA-3′, 5′-GAACTGCCTTTGCCTTCTTG-3′
CCL5	5′-GCCAAGAGCAACAACTAGGC-3′, 5′-ATTTGGAACCCAGCATTGAG-3′
CCL7	5′-GCTCATAGCCGCTGCTTTC-3′, 5′-GCTTTGGAGTTGGGGTTTTC-3′
CXCL10	5′-GGATGGCTGTCCTAGCTCTG-3′, 5′-ATAACCCCTTGGGAAGATGG-3′
CXCL12	5′-CTTCATCCCCATTCTCCTCA-3′, 5′-GACTCTGCTCTGGTGGAAGG-3′
Granzyme A	5′-ATTCCTGAAGGAGGCTGTGAA-3′, 5′-GCAGGAGTCCTTTCCACCAC-3′
Granzyme B	5′-GCCCACAACATCAAAGAACAG-3′, 5′-AACCAGCCACATAGCACACAT-3′
Perforin	5′-AGCCCCTGCACACATTACTG-3′, 5′-CCGGGGATTGTTATTGTTCC-3′
FasL	5′-TACCACCGCCATCACAAC-3′, 5′-GAGATCAGAGCGGTTCCA-3′
β-actin	5′-TGGAATCCTGTGGCATCCATGAAAC-3′, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

2.5. PCR and ELISA assays

Total RNA was isolated using TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA), quantified using a spectrophotometer and stored at −80 °C at a concentration of 1 mg/ml. cDNA was synthesized from 1 μg total RNA using an RT kit (Bioneer, Daejon, Korea). The mRNA expression levels of chemokines and their receptors were examined by PCR (Roche Applied Bioscience, Indianapolis, IN, USA); primer sequences are shown in Table 1. PCR products were fractionated on 1% agarose gels and stained with 5 μg/ml ethidium bromide. Real-time quantitative PCR was performed using SYBR Green and a thermocycler (both from Applied Biosystems, Foster City, CA, USA) with denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 1.5 min and synthesis for 30 s at 56 °C. cDNA encoding β-actin was quantified as an internal standard to provide a reference point for comparing mRNA levels in cells. Cancer cells were incubated for 24 h, culture supernatants were collected, and the concentrations of CCL2, CCL5, and CXCL10 were measured by using commercial immunoassay kits (R&D Systems) according to the manufacturer's instructions.

2.6. Time-lapse imaging

Cancer cells (70 μl of 1.5 × 10^5 cells/ml) were seeded into the left chamber of a culture-insert μ-Dish^{35mm} culture dish (ibidi GmbH, Martinsried, Germany) and incubated for 24 h. Then, wild-type (WT) or CXCR3$^{-/-}$ NK cells (70 μl of 3 × 10^5 cells/ml) were seeded into the right chamber in complete RPMI medium containing propidium iodide (PI, 2 μM). Dishes were incubated for 2 h and then inserts were carefully removed. Time-lapse imaging was performed with a Biostation IM-Q microscope equipped with a 10× magnification objective (numeric aperture 0.5) and environmental chamber kept at 37 °C and 5% CO_2 (Nikon Inc., Melville, NY, USA) [22]. Images were acquired every 2 min for 8 h. Images were analyzed by using Imaris software version 7.2