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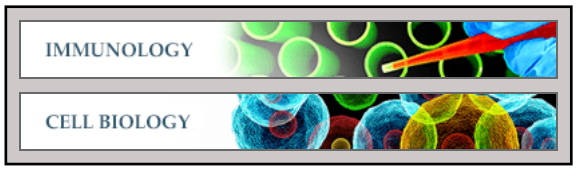

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Tumor-released Galectin-3, a soluble inhibitory ligand of human NKp30, plays an important role in tumor escaping from NK cell attack

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*Running title: *Galectin-3 inhibits NK cell activation*

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Keywords: NK cells; NKp30; Galectin-3; tumor; Immunosuppression

Background: Galectin-3, a β-galactoside-binding protein, expressed by many types of tumor cells.
Results: Galectin-3 secreted from the tumor works as a soluble inhibitory ligand of NK cell receptor NKp30 to inhibit NK cell immune responses against tumors.
Conclusion: Galectin-3 regulates the anti-tumor immunity of human NK cells.
Significance: The novel mechanism may provide a new therapeutic target for tumor treatment.

ABSTRACT

Human Galectin-3, a β-galactoside-binding protein expressed by tumor cells, was reported to act as an immune regulator in anti-tumor T cells; however, its effect on NK cells is elusive. Using a recombinant human NK cell activating receptor NKp30 fusion protein (NKp30-Fc), we found that soluble NKp30-Fc could immunoprecipitate Galectin-3. The direct interaction between NKp30 and Galectin-3 was further confirmed using surface plasmon resonance (SPR) experiments. Because Galectin-3 was mainly released from tumor cells in a soluble form in our study, the binding assay was performed to show that soluble Galectin-3 specifically bound to NK cells and NKp30 on the surface of the NK cells. Functionally, when soluble Galectin-3 was added to the NK-tumor cell co-culture system, the NKp30-mediated, but not NKG2D-mediated, cytolysis and CD107a expression in the NK cells were inhibited, and these phenotypes could be restored by pre-incubation of soluble Galectin-3 with NKp30-Fc fusion protein or the addition of anti-Gal-3 antibody alone. Moreover, genetic downregulation of Galectin-3 (shGal-3) resulted in tumor cells being more sensitive to NK cell lysis, and reversely, Galectin-3-overexpressing HeLa cells (exGal-3) became less sensitive to NK cell killing. The results of these *in vitro* experiments were supported by studies in shGal-3-HeLa or exGal-3-HeLa xenograft NOD-SCID mice after NK cell adoptive immunotherapy, indicating that Galectin-3 strongly antagonizes human NK cell attack against tumors *in vivo*. These findings indicate that Galectin-3 may function

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as an immune regulator to inhibit NK cell function against tumors, thus providing a new therapeutic target for tumor treatment.

Galectin-3 (Gal-3), one of the 15 members of the β-galactoside-binding lectin family, contains either one or two carbohydrate recognition domains (CRDs), which have a high affinity for β-galactosides (1). The overall homology of human intergalectins is approximately 20%, but their CRDs are relatively conserved (2). Previous studies showed that Gal-3 is highly expressed in many types of cancer cells (3-5). According to the cell type and proliferative status, Gal-3 can exist in the cytoplasm, within the nucleus, on the cell surface, and in the extracellular compartment (6-8). Published data obtained from different tumor cells, such as human breast cancer (9,10) and melanoma (11), have shown that intracellular Gal-3 promotes tumor growth, survival, and metastasis, and extracellular Gal-3 may facilitate metastasis by promoting tumor cell adhesion (12), invasiveness (13), and even immune escape (1,14), the latter of which is relatively rarely investigated.

More than ten years ago, it was noted that Gal-3 negatively regulates the T cell response and plays an important role in host homeostasis. The most recognizable mechanism by which Gal-3 regulates the immune response is through the induction of apoptosis in T cells. Fukumori et al. reported that the secretion of extracellular Gal-3 from tumor cells can activate apoptosis in both human and murine T cells after its binds to the cell surface glycoconjugate receptors CD7 and CD29, providing new insight about the mechanism by which cancer cells escape the immune system (15). Wang's group further confirmed this conclusion in both human and mice by showing that colorectal tumor-reactive T cells became apoptotic in response to Gal-3 stimulation, leading to enhanced tumor growth *in vitro* and *in vivo*(11). A human study also demonstrated that Gal-3 was significantly downregulated in biopsies of inflamed tissue from IBD patients; however, Gal-3 was expressed at comparably high levels in recovered inflammatory bowel disease (IBD) patients. Genetic deficiency in Gal-3 rescued the apoptosis phenotype of the T cells and induced autoimmunity. In contrast, exogenous Gal-3 led to reduced proliferation of blood T cells. This finding illustrates that constitutive expression of epithelial Gal-3 may help to prevent inappropriate immune responses, providing solid evidence to support the hypothesis that Gal-3 is an immune regulator (16). Based on these findings, blockade approaches against Gal-3 have been explored. It was reported that treatment with N-acetyllactosamine, a galectin ligand, or an anti-Gal-3 antibody restored the impaired secretion of IFN-γ by tumor-infiltrating CTLs. Moreover, GCS-100, a polysaccharide that may detach Gal-3 from TILs, boosted cytotoxicity and the secretion of IFN-γ, leading to tumor rejection in mice (17). Recently, Guha et al. found that TFD100, a glycopeptide from cod that binds Gal-3 with picomolar affinity, inhibited the apoptosis of activated T cells following induction with either recombinant Gal-3 or prostate cancer patient serum-associated Gal-3 at nanomolar concentrations (18). Collectively, Gal-3 may work as an immune regulator to induce apoptosis in activated T cells.

Natural killer (NK) cells, which are effector lymphocytes of the innate immune system, provide the first line of defense against tumors. NK cells distinguish between normal healthy cells and abnormal cells using a sophisticated repertoire of cell surface receptors that control their activation, proliferation, and effect functions (19). For example, the natural cytotoxicity receptors (20), including NKp44 (21,22) , NKp46 (23), and NKp30 (24,25), as well as NKG2D, are involved in the anti-tumor response (26,27). Previous studies showed that Gal-3 is involved in the regulation of NK cell activation and function. Data from Dr. Gordana demonstrated that Galectin-3-deficient mice are more resistant to lung metastases of malignant melanoma, and tumor-bearing Gal-3-deficient mice exhibit higher serum

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levels of IFN-γ and IL-17 than control tumor-bearing mice. Interestingly, in this model, the cytotoxic activity of splenic NK cells, but not CTLs, was greatly enhanced in Gal-3-deficient mice, suggesting that the NK cells of tumor-bearing mice are preferentially affected by Gal-3. In contrast with the Gal-3- induced apoptosis of T cells in anti-tumor immunity, the mechanism of Gal-3 inhibition in NK cell tumor immunity involves shielding the ligands on the tumor cells from NK cell-activating receptors. For example, the NK-activating receptor NKG2D is critical for tumor rejection after recognition of its tumor-associated ligand, major histocompatibility complex class I-related chain A (MICA). Gal-3 can bind the NKG2D binding site of MICA, which is expressed on the tumor cell surface, through the core 2 O-glycans of MICA, thus shielding the interactive recognition of the bladder tumor cells by the NK cells and severely impairing NK cell activation and silencing the NK cells. (28). Because MUC-1, another ligand of the NK cell receptors, also contains the core 2 O-glycan structure, the binding of Gal-3 to MUC-1 attenuated the interaction between the tumor cells and the NK cells, leading to the tumor cells evading NK cell immunity (29).

In this study, we explored a novel mechanism by which Gal-3 regulates the anti-tumor immunity of human NK cells. Using the human cervical cancer and breast cancer model, we found that soluble Gal-3 released from tumor cells could specifically bind NKp30, thereby inhibiting the NKp30-mediated cytotoxicity of NK cells. Genetic down- or upregulation of Gal-3 in tumor cells led to tumor growth inhibition or enhancement in an NK cell-dependent manner, respectively, both *in vitro* and *in vivo*. These findings demonstrate that Gal-3 is a soluble inhibitory ligand of NKp30 and may function as an immune regulator to inhibit the NK cell immune responses against tumors, providing a new therapeutic target for tumor treatment.

Materials and Methods

Antibodies. Anti-human NKp30 (clone P30-15) was purchased from Biolegend, and anti-human Gal-3 (clone B2C10), PE-conjugated anti-human -NKp30, PE-conjugated anti-human CD107a, FITC/PE-Cy5-conjugated anti-human CD56, FITC-conjugated goat anti-human IgG, and PE-conjugated goat anti-mouse IgG were purchased from BD Bioscience. PE-CY7 -conjugated anti-human NKG2D was obtained from R&D Technologies.

Mice and cells. All experiments involving mice were approved by the Animal Care and Use Committee at the University of Science and Technology of China. The 6- to 8-week-old NOD-SCID mice were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and were bred in SPF conditions according to the experimental animal guidelines of the University of Science and Technology of China.

Human cervical cancer cell line HeLa cells, human breast cancer cell line MDA-MB-435 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamate. NK-92 cells were cultured in α-MEM (Gibco) containing 12.5% FBS (Gibco), 12.5% equine serum (HyClone), 2 mM L-glutamate, 0.1 mM 2-mercaptoethanol, and 100 U/ml rhIL-2 (Changchun Institute of Biological Products, China). Human NK cells were obtained from the peripheral blood mononuclear cells (PBMCs) of healthy donor buffy coats using Ficoll-Paque density gradient centrifugation (Solarbio, China). Non-NK cells were depleted using an NK Cell Isolation Kit according to the manufacturer's instructions (Miltenyi Biotech). The separated NK cells were cultured in complete α-MEM (Gibco). Cell culture was performed at 37℃ in a 5% CO_2 humidified atmosphere.

Soluble NKp30-Fc fusion proteins. The sequence encoding the extracellular portions of NKp30

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(Gene bank: AB055881) was amplified by PCR from cDNA isolated from human NK clones. The NKp30-specific primers were 5' primer (including an EcoR Ⅰ restriction site 5'- CCGGA ATTCCTCTGGGTGTCCCAGCCCC CTGAGATTCGTA-3'. 3' primer (including the Xho Ⅰ restriction site, 5'- CCGCTCGAGGGAC TGAAAATACAGGTTTTCCCCTAGCTGAGG ATGTTC-3'.The cDNA fragment encoding the Fc regions of human immunoglobulin G1 (Gene Bank:BC006402) was amplified by RT-PCR from RNA isolated from human peripheral blood lymphocytes. The Fc regions specific primers were 5' primer (including an Xho Ⅰ restriction site), 5'- CCGCTCGAGCCCAAATCTTGTGA CAAAACT-3', 3' primer (including the Xba Ⅰ restriction site), 5'-CTAGTCTAGATCATT TACCCGGAGACAGGGAGAGG-3'.The resulting amplified product was cloned into pcDNA3.0 vector (Invitrogen). Sequencing of the constructs revealed that NKp30 receptor ectodomain cDNA was in frame with the human Fc genomic DNA and were identical to the reported sequences. CHO-K1 cells were transfected with these expression vectors, G418-selected clones were screened for highest protein production and were further subcloned by limited dilution method for high producer clones. Supernatants were collected daily and purified on protein G columns. SDS-PAGE analysis revealed that both Ig fusion proteins were approximately 95% pure and of the proper molecular mass (approximately 55 kDa for NKp30-Fc, under reducing conditions).

Depletion and overexpression of Gal-3. For the depletion of Gal-3, lentiviruses expressing Gal-3 or control shRNAs were prepared. HEK 293T cells grown in a 6-cm dish were transfected with 2 μg of Gal-3 shRNA (cloned into the PLKO.1 vector) or control vector, 2 μg of pREV, 2 μg of pGag/Pol/PRE, and 1 μg of pVSVG. Twenty-four hours after transfection, the cells were cultured in DMEM containing 20% FBS for an additional 24 hours. The culture medium containing the lentivirus particles was centrifuged at 1,000 x *g* for 5 minutes. The viruses in the supernatant were used to infect tumor cells. The knockdown efficiency was evaluated using western blot and real-time RT-PCR analyses. The shRNA sequence targeting Gal-3 was 5'-CCGGGCTCACTTGTTG CAGTACAATCTCGAGATTGTACTGCAACA AGTGAGCTTTTT -3'.

For the overexpression of Gal-3, HeLa cells were transfected with pCMV6-Gal-3 or the control vector. Twenty-four hours after transfection, the cells were cultured in DMEM containing 20% FBS for an additional 24 hours. The cells were then screened with G418 for 4 d. The overexpression efficiency was evaluated using western blot and real-time RT-PCR analyses.

Immunoprecipitation and western blot analysis. Cells were collected and resolved in lysis buffer containing 1% Triton X-100, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 10 mM NaF, 1 mM PMSF (Sigma), 2 mM EDTA, and a protease inhibitor cocktail (Roche). The cell lysates were pre-incubated with soluble proteins (final concentration 10 μg/ml) at 4℃ for 2 h. Protein A/G plus agarose was pre-incubated with or without mAbs (final concentration 10 μg/ml) at 4℃ for 2 h. Then, the protein A/G agarose was washed two times with cell lysis buffer and was added to the cell lysates for 2 h or overnight at 4℃. For the biotinylation of the proteins, cell monolayers were incubated with 1 mg/ml EZ-Link Sulfo-NHSBiotin (Pierce) in PBS (pH 8.0) for 30 min at 25℃, followed by three washes with cold PBS. After extensive washing, the cells were lysed and incubated with streptavidin-coated agarose beads (Pierce) at 4°C for 2 h to precipitate the cell membrane proteins. The protein-bound beads were washed four times with cell lysis buffer and resuspended in PBS. Finally, the suspension was mixed with 2×SDS-loading buffer, boiled, and centrifuged to remove the pellet. The supernatant was separated using SDS-PAGE electrophoresis under reducing or non-reducing conditions on a 12%

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polyacrylamide gel and then electrotransferred onto PVDF membranes (Millipore) in vertical buffer tanks. The membranes were blocked with 5% non-fat milk in TBST buffer (50 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 0.1% Tween 20) and then incubated with primary antibodies for 2–3 h at room temperature or overnight at 4 ℃. After the addition of the HRP-conjugated secondary antibodies for 1 h, the bands on the membranes were detected using an enhanced chemiluminescence system (Pierce).

Surface plasmon resonance. Surface plasmon resonance(abb. SPR) analysis was performed on a BIAcore 3000 apparatus at 25 ℃. Reconbinant human (rh) Gal-3 (R&D Technologies) was covalently immobilized to the carboxyl groups in the dextran layer of a Biacore Sensor Chip CM5. Gal-3 was injected in 0.01 M sodium acetate buffer (pH 5). Solutions containing the different recombinant proteins were sequentially injected over the surface of the immobilized Galectin-3, or buffer was used as a blank. For the regeneration of the surface, we used PBS (pH 7.2), which also served as the running buffer. The flow rate was set at 10 μl/min. The data were analyzed using Biaevaluation 4.1 software.

Flow cytometric analysis. For cell surface staining, the cells were harvested, blocked with an anti-FcγR mAb, and incubated with the appropriate concentrations of the recombinant proteins or Abs for 30 min at 4 ℃. To stain intracellular proteins, the cells were fixed in 1% paraformaldehyde in PBS/0.05% BSA for 30 min on ice and washed in permeabilization buffer (eBioscience); the cells were permeabilized in permeabilization buffer containing 3% BSA for 30 min at 4 ℃, followed by staining with the primary antibodies. All proteins and mAbs were used at a final concentration of 10 μg/ml. All stained cells were gated for analysis using a FACSCalibur cytometer (Becton Dickinson), and the data were processed using FlowJo software (TreeStar, Ashland, OR).

ELISA. For assays determining the amount of Gal-3 protein secreted by the tumor cells, a direct enzyme-linked immunosorbent assay (ELISA) was used. Briefly, 150 μl of supernatant, which was collected at various time points, was added to microtiter plates, and the plates were incubated overnight at 4 ℃. After washing, the plates were blocked as described in the above experiments. An anti-human Gal-3 antibody (diluted 1:500 in 0.3% BSA/0.1% PBST) was then added to the plates for 2 h at 37 ℃. The plates were incubated with an HRP-conjugated anti-mouse antibody for 1 h, followed by incubation with TMB as a substrate for 5-10 min. The colorimetric signal was measured at an OD of 450 nm.

Cytotoxicity assay. The cytotoxicity of the NK-92 cells and human NK cells against the target cells was measured using a standard 4-hour ^{51}Cr release assay, as previously described (30). Briefly, the target cells were labeled with ^{51}Cr by incubation with $Na_2{}^{51}CrO_3$ (200 μCi/106 cells) for 1 h at 37 ℃ and then washed 3-4 times with PBS. The labeled target cells were combined with NK cells at different E: T cell ratios. For the blocking assay, the NK-92 cells or primary NK cells were preincubated with Gal-3 protein or an anti-NKp30 antibody for 30 min or 1h at 4 ℃ prior to the addition of the with target cells to prevent the NKp30 ligands on the target cells from binding the NKp30 activated receptor on the NK cells. In turn, the target cells were preincubated with the NKp30-Fc protein or an anti-Gal-3 antibody for 30 min at 4 ℃ prior to the addition of the NK cells to prevent the activation of NK cell killing by the NKp30 ligands derived from the target cells. To restore NK cell killing, NKp30-Fc was preincubated with Gal-3 protein for 30 min at 4 ℃ before the NK cells killed the target cells. Maximum ^{51}Cr release was determined by incubating the target cells with 2% Triton X-100. To measure the spontaneous ^{51}Cr release, the target cells were incubated

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without effector cells, in assay medium alone. All experiments were performed in triplicate. The plates were slightly spun and incubated at 37 ℃ for 4 h. After 4 h, the cells were centrifuged, and the amount of ^{51}Cr release in 100 µl of the supernatant was counted using a gamma counter. The percentage of specific ^{51}Cr release was calculated using the formula: ([experimental release-spontaneous release]/[maximum release-spontaneous release])× 100. Spontaneous release did not exceed 10% of the maximum release in all experiments.

Degranulation assays. To test the NK cell-derived degranulation, NK-92 cells (1x10^5) were incubated with different proteins or protein complexes (10 µg/ml) for 1 h at 37 ℃, and tumor cells were incubated with antibody or control mIgG (5µg/ml) for 1 h at 37 ℃. Then, the NK-92 and untransfected or transfected tumor cells were mixed at an effector:target ratio of 1:1, and the cells were incubated for 2 h at 37 ℃ in the presence of anti-human CD107a PE-conjugated antibodies, anti-human CD56 PE-Cy5/FITC conjugated antibodies, and isotype-matched control antibodies. As a positive control, NK-92 cells were incubated with 5 µg/ml PMA (final concentration 50 ng/ml; Sigma) and 1 µg/ml ionomycin (final concentration 10 µg/ml; Sigma). After incubation, the cells were washed and resuspended. CD56 $^+$cells were gated for analysis using a FACSCalibur flow cytometer (BD Bioscience).

Cervical adenocarcinoma xenograft model. HeLa cells (4x10^6) were left untreated or were transfected with the different Gal-3 constructs used to deplete or overexpress the protein. The HeLa tumor cells were resuspended in 200 µl of PBS and were injected subcutaneously into NOD-SCID mice to establish tumors. NK-92 cells were injected subcutaneously into the tumor issue area (2 x 10^6/200 µl per animal) 5 days after tumor inoculation. The tumor volumes were measured on day 10 and day 20, and the formation of the tumors was observed on day 20.

Statistical analysis. Data were expressed as means ± SD, and Significance was denoted as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Calculations were performed using GraphPad-Prism software (San Diego, CA) with Student's *t*-test and PS-Power and Sample Size Software with power analysis for *t*-test (31).

Results

Identification of Galectin-3 as a ligand of human NKp30. Natural killer (NK) cells are important components of the innate immune system (32) and participate in the elimination of tumors (33). NKp30 is a human receptor that triggers anti-tumor NK cell cytotoxicity and cytokine secretion (25). To detect a novel ligand of NKp30, we generated soluble NKp30-Fc fusion proteins. When NKp30-Fc was immunoprecipitated from HeLa cell lysates, an extra band was observed in the NKp30-Fc precipitate (Fig. 1A). This protein band was subjected to in-gel tryptic digestion and was analyzed by mass spectrometry, and the results demonstrated that this band corresponded to Gal-3, a member of the β-galactoside-binding lectin family. Immunoblot analysis also showed that NKp30-Fc could interact with Gal-3 in HeLa cells (Fig. 1B). To confirm the direct interaction between NKp30 and Gal-3 and to rule out the possibility of Gal-3 binding to the Fc tag, surface plasmon resonance (SPR) experiments were performed. The results showed that Gal-3 specifically bound to the extracellular domain of NKp30, as it did not bind to human IgG (Fig. 1C). These results suggested that tumor-derived Gal-3 is a potential ligand of NKp30.

Cellular distribution of Galectin-3 in human tumor cells. Gal-3 is expressed in a variety of forms in tumor cells, depending on the cell type and proliferative status. A previous study showed that Gal-3 can be detected in the nucleus and

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cytoplasm of the HeLa cell line (34). Here, biotinylated membrane-bound proteins on HeLa cells were immunoprecipitated with streptavidin-coated agarose beads, followed by immunoblot analysis with an anti-Gal-3 antibody. Gal-3 was present in the total cell lysate but was not found in the membrane fraction of HeLa cells (Fig.2A). Flow cytometry also showed that Gal-3 was expressed intracellularly, but it was not found on the membrane of HeLa cells (Fig.2B). Additionally, it was previously reported that the concentration of circulating Gal-3 was markedly increased in the serum of patients with different tumor types (35,36). Therefore, an ELISA assay was performed to identify the soluble Gal-3 released from tumor cells. As shown in Figure 2C,D, there was a high level of Gal-3 in the HeLa and MDA-MB-435 cells culture supernatant, and this level peaked at 12h and 24h, respectively. The results showed that Gal-3 is present in a soluble form and could be released by tumor cells.

Galectin-3 specifically binds to NKp30 on NK cells. Flow cytometry analysis showed that Gal-3 protein, compared to BSA, was significantly bound to the NK cells (Fig.3A), resulting in a much higher mean fluorescence intensity (MFI) (Fig.3B). We next examined whether Gal-3 directly interacted with the NKp30 receptor. Total NK cell lysate was incubated with Gal-3 and co-precipitated with an anti-Gal-3 antibody or an immobilized NKp30 antibody, followed by immunoblot analysis for NKp30 or Gal-3. The results showed that Gal-3 could specifically bind NKp30 from NK cells (Fig. 3C).

Galectin-3 inhibits the NKp30-mediated cytolysis of NK cells. Because it was reported that Gal-3 might block the binding of NKG2D to MICA (28), we selected a NK-92 cell clone that does not express NKG2D to exclude the influence of Gal-3 interacting with NKG2D (Fig. 4A). As shown in Figure 4, the addition of Gal-3 into the NK-92-HeLa co-culture system significantly reduced the expression of CD107a, a marker of NK cell degranulation during the initiation of cytolysis, whereas CD107a expression was restored by pre-incubating the Gal-3 with the NKp30-Fc fusion protein (Fig. 4B, C); this result was further confirmed using a 4 hour ^{51}Cr release assay to examine the direct cytolysis of the tumor cells by the NK cells. The results showed that the susceptibility of the tumor cells to NK-92 (NKG2D- NK92 clone) cells cytolysis was impaired by the addition of Gal-3, whereas this effect of Gal-3 was attenuated by pre-incubating the Gal-3 with the NKp30-Fc fusion protein (Fig. 4D). Moreover, the cytotoxicity of primary human NK cells was also impaired by the addition of Gal-3, and these results were the opposite of what was observed following incubation with an anti-Gal-3 antibody (Fig. 4E). Not only HeLa cells but also MDA-MB-435 cells could induce NK-92 cells to express CD107a, and which was significantly increased in the presence of anti-Gal-3 antibody (Fig. 4F). Therefore, the results suggested that the Gal-3 that is released from tumor cells could impair NK cell-mediated cytotoxicity by directly antagonizing NKp30 function.

Galectin-3 is an inhibitory ligand that blocks human NK cell activity against tumor *in vitro* and *in vivo*. We constructed a Gal-3-specific lentiviral-based shRNA to knock down the endogenous expression of Gal-3 in HeLa tumor cells, and the results showed that genetic downregulation of Gal-3 (shGal-3) resulted in the HeLa cells becoming more sensitive to NK cell lysis (Fig.5A); in contrast, HeLa cells that overexpressed Gal-3 (exGal-3) became less sensitive to NK cell killing (Fig.5B). This result was further found using the Gal-3-silenced MDA-MB-435 cells to examine the CD107a expression of NK-92 cells (Fig.5C). Therefore, the evidence suggested that Gal-3 expreesion of tumor cells directly affects NK cell function.

It has been reported that Gal-3 expression by tumor cells might intrinsically contribute to tumor

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progression (9-11). Here, we wanted to elucidate whether Gal-3 negatively regulated NK cell-mediated anti-tumor immunity *in vivo*; therefore, we used NOD-SCID mice bearing xenografts of Gal-3-silenced HeLa (shGal-3) or Gal-3-overexpressing HeLa (exGal-3) tumor cells, and we treated these mice using adoptive immunotherapy with human NK-92 cells. As shown in Figure 6A, the NOD-SCID mice were left untreated or were subcutaneously injected with HeLa cells (exGal-3 or shGal-3), followed by intratumoral injection of NKG2D- NK-92 cells (see Fig. 4A) during the early stage (day 5) of tumor growth; tumor growth was then observed on day 10 and day 20. We found that the tumor volume was reduced after NK treatment in the WT HeLa-bearing mice on day 10 and day 20 (Fig. 6B, C), indicating that the HeLa tumor was sensitive to the NK cell therapy. Importantly, the shGal-3 HeLa tumors became much more sensitive to NK attack, whereas the exGal-3 HeLa tumors became more resistant to NK attack at both day 10 and day 20 (Fig. 6B, C). These results further suggested that Gal-3 secreted by the tumor antagonized human NK cell attack against the tumor *in vivo*. Previous reports also demonstrated that Gal-3 expression directly improved tumor growth in xenograft mice without NK cell therapy (9-11); however, the efficiency of NK cell therapy was still attenuated by Gal-3 expression from the xenograft tumor (data not shown). Taken together, these results imply that Gal-3 may function as an immune regulator to inhibit NK cell function against the tumor, and inhibition of Gal-3 expression may serve as a therapeutic target for cancer therapy.

Discussion

Using human cervical cancer, breast cancer cell lines and the xenograft tumor NOD-SCID model, we showed that tumors can escape from NK cell attack by releasing soluble Gal-3, which specifically binds to NKp30 and inhibits the NKp30-mediated cytotoxicity of NK cells. Genetic up- or downregulation of Gal-3 in tumor cells led to tumor promotion or inhibition, respectively, in an NK cell-dependent manner, both *in vitro* and *in vivo*. Our results revealed that Gal-3 is a soluble ligand of NKp30 and can function as an immune regulator to mediate tumor escape from NK cell immunity.

Previous studies have shown that Gal-3 may facilitate tumor metastasis through immune escape. For example, data from Zubieta et al. demonstrated that Gal-3 expression correlated with the apoptosis of tumor-associated lymphocytes in human melanoma biopsies (37). A recent study also demonstrated that Gal-3 secreted from tumors directly interferes with the formation of the immune synapses of anti-tumor T cells and thereby inhibits tumor-reactive $CD8^+$ T cells, leading to fast tumor growth in a mouse model of colorectal cancer (11). Until now, the mechanisms by which Gal-3 induces immune suppression were grouped into two categories. One category includes the stimulation of apoptosis by the binding of Gal-3 to the anti-tumor T cells (11,37), and the other includes the shielding of ligands on the surface of tumor cells from the NK cell activating receptors (28,29). NK cells are critically important in destroying tumor cells, and their anti-tumor function is performed mainly through NK cell receptor-tumor ligand interactions, resulting in the release of cytotoxic granules containing perforin and granzymes (26,27). One important approach is the engagement of an NK-activating receptor, NKG2D, by its tumor-associated ligand MICA, to reject tumor by NK cells (38). Interestingly, a study from Tsuboi et al. demonstrated that Gal-3 could bind the NKG2D-binding site of MICA and reduce the affinity of MICA for NKG2D in bladder tumor cells, thereby evading NK cell immunity (28). This finding suggests that the Gal-3 released by tumor cells may possibly alter the NK cell responses by modulating their recognition of the tumor.

In our study, a new mechanism was observed, in which tumor-associated Gal-3, as a soluble

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ligand of NKp30, directly bound to NKp30 and blocked NKp30-mediated cytotoxicity of NK cells. Similar to NKG2D, NKp30, which is an important NK-activating receptor, is also involved in NK lytic activity against tumor cells by interacting with their physiological ligands. Several tumor ligands of NKp30 have been found. For example, the nuclear factor HLA-B-associated transcript 3 (BAT3) is released from tumor cells in membrane vesicles, such as exosomes (39), and B7-H6 is selectively expressed on some tumor cells (40); both of these ligands can engage NKp30 on NK cells and induce NKp30-dependent cell activation and cytotoxicity. In contrast to these ligands, soluble Gal-3, which was released from tumors in our study, could directly inhibit NK cell activation by binding to NKp30. To exclude Gal-3's influence on MICA/NKG2D, we selected a NKG2D$^-$NK-92 cell clone, and we found that Gal-3 absolutely inhibited NK cells in the absence of NKG2D (see Fig. 4A), demonstrating that the NKp30-mediated cytolysis of NK cells was impaired in the presence of Gal-3.

Our *in vivo* results clearly demonstrated the regulatory effect of Gal-3 on the anti-tumor NK cells. In our study, the cytotoxicity of NK cells was almost completely lost when a blocking anti-NKp30 antibody was added into the NK-HeLa co-culture system *in vitro* due to the complete blockade of all NKp30-ligand interactions (data not shown); these results demonstrate that NKp30 is critical for the anti-tumor capability of NK cells. Moreover, when Gal-3 was depleted or overexpressed in human tumor cells, the anti-tumor function of the adoptively transferred human NKG2D- NK cells was enhanced or reduced, respectively, further indicating that Gal-3 inhibits NK cell function by binding to NKp30 (Fig 6). We demonstrated that after incubation of the NKp30-Fc protein with Gal-3 *in vitro*, the NKp30-Fc protein could compete with Gal-3 and attenuate the Gal-3 effect on NK cells; however, we inferred that the *in vivo* data did not support this conclusion, as NKp30-Fc could bind to all of its activating and inhibiting ligands, leading the NK cells to become unresponsive to both positive and negative stimulation. In our study, the *in vivo* results showed that Gal-3 secreted from the tumor cells contributes to tumor escape from NK-mediated innate immune defense. Taken together with the results shown in Figure 4, it is likely that the effect of the Gal-3-impaired, NK cell-mediated tumor rejection was through its interaction with the NKp30 receptor on the NK cells.

Because it was established that Gal-3 interacts with crosslinked glycan molecules on the cell surface, such as MICA, to attenuate the interaction between the tumor cells and the immune cells, we observed that NKp30, as an essential glycoprotein, also contains two predicted N-glycosylation sites (N42 and N121) in its ectodomain. Taken together with a study from Tsuboi, it seems that the tumor-released Gal-3 may reduce the tumor cell sensitivity of NK cells by binding glycosylated NKp30, thus interfering with the binding of NKp30 to its ligands on tumor cells and resulting in the evasion of the tumor cells from NK cell attack in the human cervical cancer model. This speculation should be further examined. Taken together, although the functional details of how Gal-3 inhibits NK cell function by binding to NKp30 remain unknown, our data, for the first time, reveal that the Gal-3 secreted from the tumor work as a soluble inhibitory ligand to compromise NK cells, which helps the tumor escape from immune attack.

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FOOTNOTES

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Abbreviations used in this article: Ab, antibody; IFN, interferon (e.g., IFN-γ); IL, interleukin (e.g., IL-2); kDa, kilodalton (only with numbers); mAb, monoclonal Ab; μg, microgram (only with numbers); μl, microliter (only with numbers); NK cell, natural killer cell; RT-PCR, reverse transcriptase polymerase chain reaction; NOD, nonobese diabetic; SCID, severe combined immunodeficiency; PBS, phosphate-buffered saline;PCR, polymerase chain reaction; shRNA, short hairpin RNA; Gal-3,Galectin-3;NKp30-Fc, recombinant chimeric NKp30 receptor fused with the Fc portion of human IgG1.

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Figure Legends

FIGURE 1. Interaction of Gal-3 with the extracellular domain of NKp30. (**A**) The binding of tumor-associated proteins to soluble NKp30-Fc. The NKp30-Fc fusion protein coupled to protein-A/G Sepharose beads were incubated with HeLa cell lysate, and the proteins that bound to NKp30-Fc were resolved by SDS-PAGE and stained with Coomassie blue. An approximately 30-kDa cellular protein that interacts with NKp30-Fc was present in the cell lysate. NKp30-Fc alone served as a negative control. (**B**) The binding of tumor-derived Gal-3 to soluble NKp30-Fc. Gal-3 protein or the cell lysate was examined by immunoblot analysis with an anti-Gal-3 mAb in immune complex. (**C**) The kinetics of the interactions between recombinant human (rh) Gal-3 and NKp30-Fc. rhGal-3 was covalently immobilized to the carboxyl groups in the dextran layer of a BIAcore sensor chip. Solutions containing NKp30-Fc or human IgG were injected over the surface of immobilized rhGal-3, and PBS buffer was used as a control. SPR analysis was performed on a BIAcore 3000 apparatus at 25°C; under these conditions, binding of immobilized rhGal-3 to soluble NKp30-Fc was observed, but binding to human IgG was not. The data are representative of two independent experiments.

FIGURE 2. Gal-3 distribution in tumor cells. (**A**, **B**) Gal-3 distribution (intracellular, surface) in HeLa cells was examined by immunoprecipitation (**A**) or flow cytometry (**B**). (**A**) HeLa cells were biotinylated to label the cell surface proteins, and the biotinylated membrane proteins were then precipitated using streptavidin-coated agarose beads. Gal-3 from the cell lysate (left line) or the membrane protein fraction (right line) was detected using immunoblot analysis with an antibody against Gal-3. (**B**) HeLa cells were treated with permeabilization buffer (**left**) or without (**right**), followed by incubation with an anti-Gal-3 mAb and stained with a fluorescence-conjugated polyclonal antibody. (**C, D**) Gal-3 is released from tumor cells. ELISA plates were coated with 100 µl of culture supernatants (SN) collected from HeLa (**C**) or MDA-MB-435(**D**) cells at hr 12, 24, or 36 after cell recovered in fresh medium ; control medium served as a negative control. Gal-3 was detected using an anti-Gal-3 mAb and stained with anti-mouse HRP-linked secondary antibody. The data represent the absorbance at 450 nm after normalization to the background (nonspecific binding of the antibody to the plate). The data were representative of two independent experiments and were analyzed using Student's t-test. *, $P<0.05$; **, $P<0.01$;***, $p<0.0001$.

FIGURE 3. Binding of Gal-3 to NKp30 on NK cells. (**A**) The binding of Gal-3 to NK-92 cells. An anti-Gal-3 mAb was preincubated with the irrelevant protein BSA or soluble Gal-3, with or without an isotype- matched control antibody (gray histogram), and the solution was then incubated with NK-92 cells and stained with a fluorescence-conjugated polyclonal antibody. (**B**) The binding ratio of BSA or Gal-3 to the NK-92 cells was determined by statistical analysis of the mean fluorescence intensity from two independent experiments, as described in (**A**). (**C**) Soluble Gal-3 was precipitated with NKp30 from NK cells. NK-92 cell extracts were incubated with soluble Gal-3, and the protein complex was immunoprecipitated with an anti-Gal-3 mAb or an anti-NKp30 mAb immobilized on protein-A/G Sepharose beads. Immunoblot analysis was performed for NKp30 using an anti-NKp30 mAb (left panel) or for Gal-3 using an anti-Gal-3 mAb (right panel).

FIGURE 4. Gal-3 inhibits NK cell cytotoxicity by binding to NKp30. (A) The selection of the NKp30-expressing, but not NKG2D-expressing, NK-92 cell clone. NK-92 cells were stained with

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PE-conjugated anti-NKp30 antibody, PE-CY7-conjugated anti-NKG2D antibody or an isotype control (gray histograms). The data were representative of two independent experiments. (B, C) CD107a surface expression was measured by flow cytometry after co-incubation of NK-92 cells with HeLa cells in the presence of BSA, Gal-3, or Gal-3 plus NKp30-Fc. The gray histograms represent the isotype control. (D) Blocking soluble Gal-3 restored the cytotoxicity of the NK-92 cells. A 4 h, the cytotoxicity assay was performed in the presence of BSA, Gal-3, or Gal-3 plus NKp30-Fc at a 4:1 (E: T) ratio. Cytotoxicity of NK-92 cells against HeLa cells was normalized to 100% in the presence of BSA. (E) The cytotoxicity of primary human NK cells against HeLa cells was assayed. The effector NK cells were incubated with Gal-3 or BSA or with the anti-Gal-3 mAb or control mIgG1κ mAb for 1 h. Then, HeLa cells (target cells) labeled with ^{51}Cr were added at the indicated effector/target ratios (E: T). A 4 h cytotoxicity assay was performed, and the inhibition of NK cell cytotoxicity by soluble Gal-3 is indicated. Cytotoxicity of human NK cells against HeLa cells was normalized to 100% at the most E: T (10:1) ratios, The data of the cytotoxicity assay were representative of two independent experiments. (**F**) Anti-Gal-3 antibody restored the CD107a surface expression of NK-92 cells. The flow cytometry was performed to measure CD107a surface expression after co-incubation of NK-92 cells with HeLa cells or MDA-MB-435 cells in the presence of anti-Gal-3 mAb or control mIgG1κ**.** The data were representative of two independent experiments and were analyzed using Student's t-test**.**. *, P<0.05; **, P<0.01;***, p<0.0001.

FIGURE 5. Effect of Galectin-3 downregulation and overexpression on NK cytotoxicity. HeLa cells were transfected with the empty vector (EV), Gal-3-specific lentiviral- based–shRNA (shGal-3) (**A**) or Gal-3 expression vector (exGal-3) (**B**), followed by cell lysate was prepared and analyzed for Gal-3 expression using immunoblot analysis. GAPDH (**A**) or β-actin (**B**) was used as control. Then, transfected HeLa cells were incubated with NK-92 cell clones at effector:target ratios between 8:1 and 4:1, and the lysis of the target cells was determined using a 4-h europium release assay. The NK cells cytotoxicity was normalized to 100% at the most E: T (8:1) ratios.(C) MDA-MB-435 cells were transfected with the empty vector (EV) or Gal-3-specific lentiviral- based–shRNA (shGal-3), immunoblot analysis was performed as described previously. And the flow cytometry was performed to measure CD107a surface expression after co-incubation of NK-92 cells with EV- or shGal-3-MDA-MB-435 cells. The data are representative of two independent experiments and were analyzed using Student's t-test. **, P<0.01; ***, p<0.0001.

FIGURE 6. Gal-3 blocks human NK cell activity against tumor in vitro and *in vivo*. (**A**) Xenograft tumor model. Untreated or transfected (exGal-3 or shGal-3) HeLa tumor cells were i.p. injected into NOD-SCID mice,followed by injection with PBS or NK cells into the tumor tissue area 5 days after tumor inoculation. (**B**, **C**) The tumor volumes were measured on days 10 and 20 (**B**), and the formation of the tumors was observed on day 20 (**C**) after tumor inoculation. The data were representative of two independent experiments with five animals in each group, and the maximum and minimum of tumor volumes were excluded in the statistics. The data were analyzed using Student's *t*-test and power analysis for *t*-test (abb.pwr). *, p<0.05; **, P<0.01; ***, p<0.0001.

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Figures

Figure1

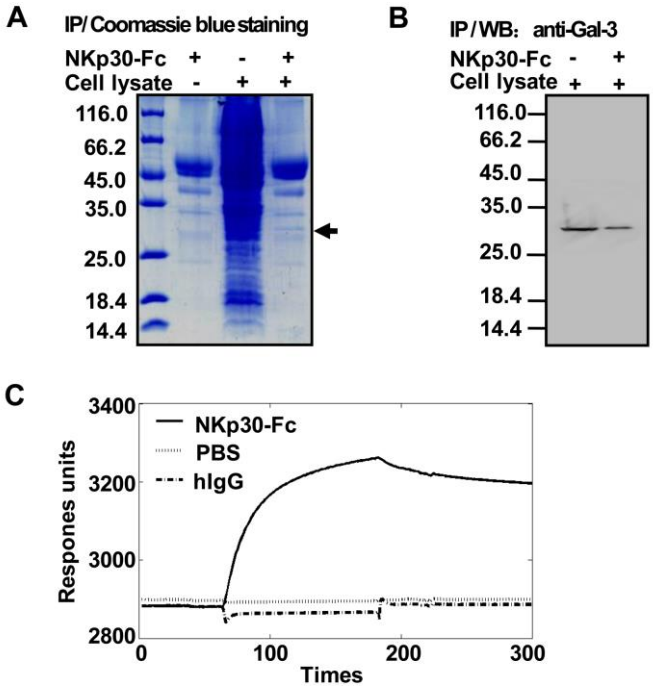

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Figure2

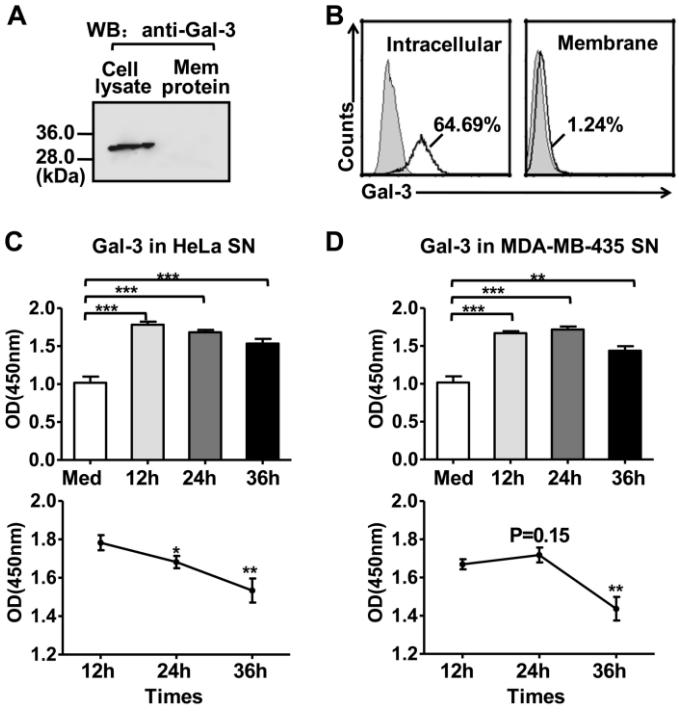

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Figure 3

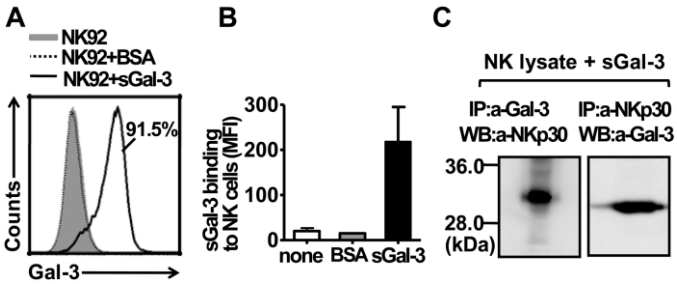

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Figure 4

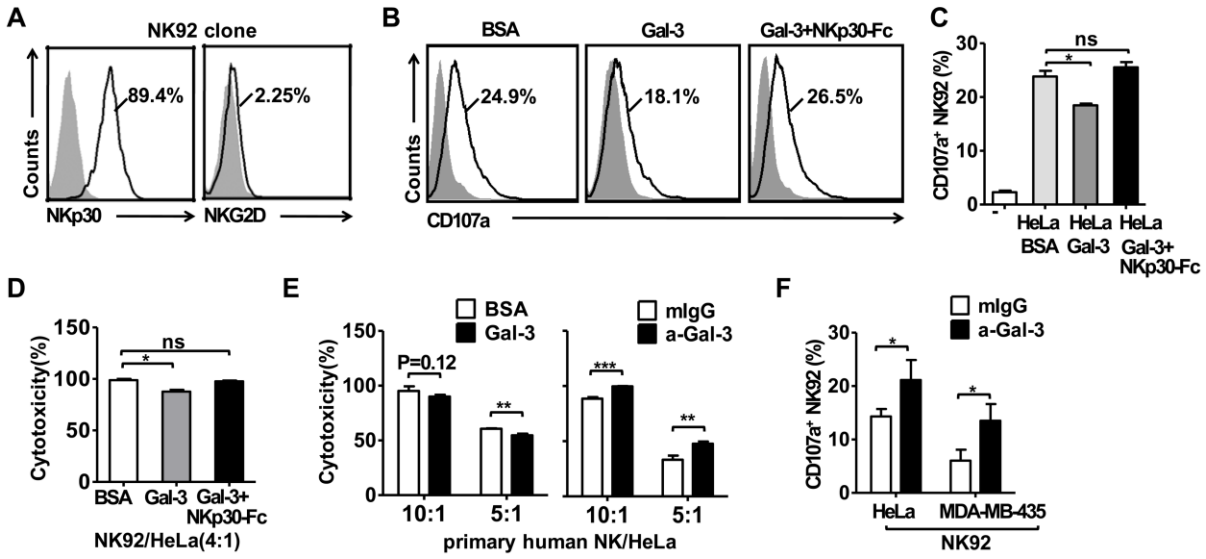

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Figure 5

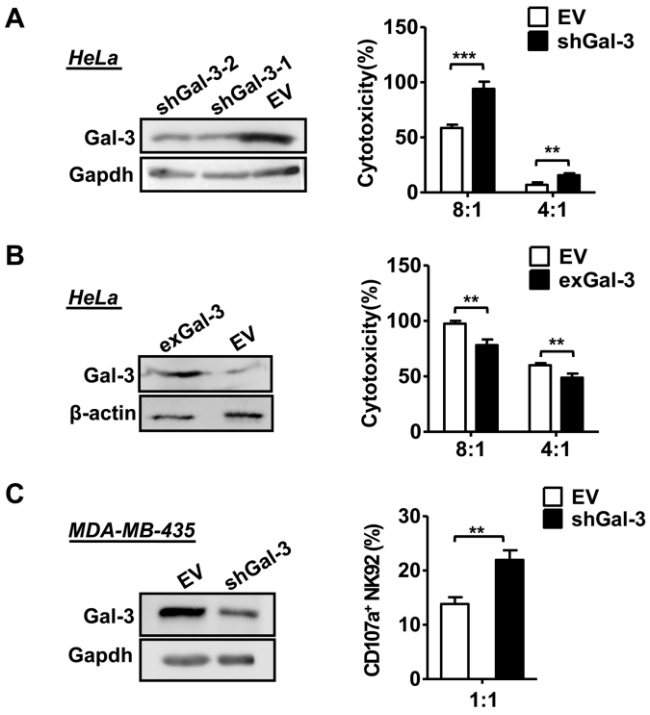

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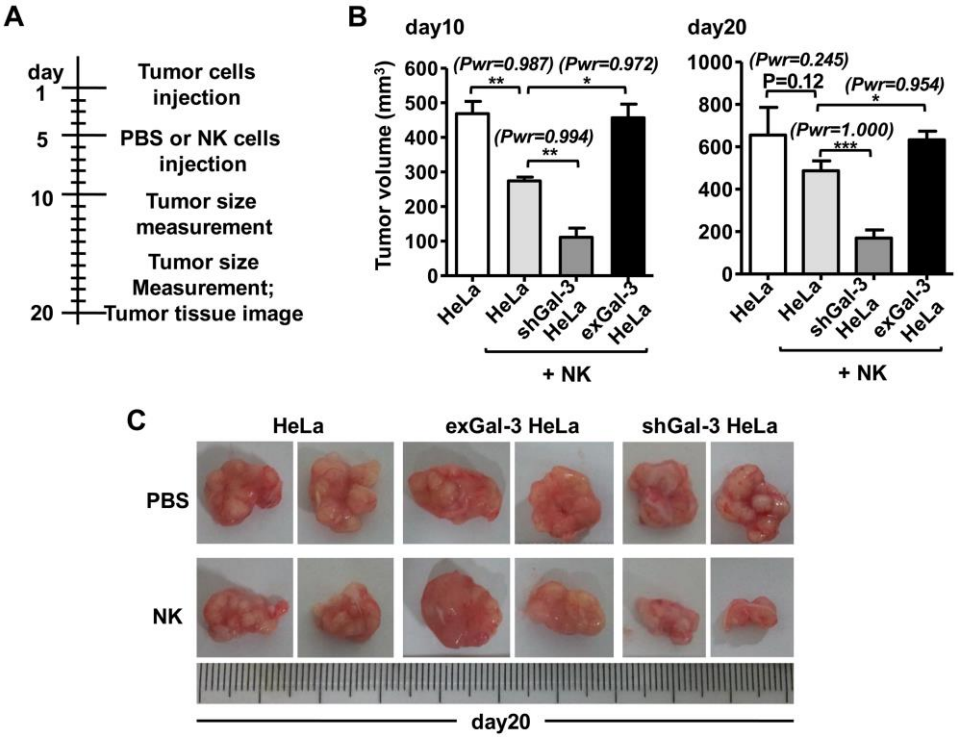
Figure 6



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