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A novel strategy for evasion of NK cell immunity by tumours expressing core2 *O*-glycans

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The *O*-glycan branching enzyme, core2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT), forms *O*-glycans containing an *N*-acetylglucosamine branch connected to *N*-acetylgalactosamine (core2 *O*-glycans) on cell-surface glycoproteins. Here, we report that upregulation of C2GnT is closely correlated with progression of bladder tumours and that C2GnT-expressing bladder tumours use a novel strategy to increase their metastatic potential. Our results showed that C2GnT-expressing bladder tumour cells are highly metastatic due to their high ability to evade NK cell immunity and revealed the molecular mechanism of the immune evasion by C2GnT expression. Engagement of an NK-activating receptor, NKG2D, by its tumour-associated ligand, Major histocompatibility complex class I-related chain A (MICA), is critical to tumour rejection by NK cells. In C2GnT-expressing bladder tumour cells, poly-*N*-acetyllactosamine was present on core2 *O*-glycans on MICA, and galectin-3 bound the NKG2D-binding site of MICA through this poly-*N*-acetyllactosamine. Galectin-3 reduced the affinity of MICA for NKG2D, thereby severely impairing NK cell activation and silencing the NK cells. This new mode of NK cell silencing promotes immune evasion of C2GnT-expressing bladder tumour cells, resulting in tumour metastasis.

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Introduction

The majority of deaths associated with solid tumours are caused by metastasis. The process of metastasis involves various steps, including tumour cell proliferation at primary sites, invasion into surrounding tissues, intravasation into lymphatic or blood vessels, dissemination through the circulation, and proliferation at distal sites (Gupta and Massague, 2006). Among the numerous factors involved in the process, a growing body of evidence has recently supported crucial roles for cell-surface carbohydrates in those steps of metastasis.

Cell-surface carbohydrates attached to proteins are classified by the nature of their linkages to the protein as either *N*-glycans (*N*-acetyllactosamine linked to asparagine) or *O*-glycans (*N*-acetyllactosamine linked to serine or threonine). Both *N*- and *O*-glycans have been shown to be involved in the process of metastasis (Fuster and Esko, 2005). For example, *N*-glycosylation of insulin-like growth factor 1 receptor is required for proliferation of melanoma and sarcoma cells (Girnita *et al*, 2000). Upregulation of the *N*-glycan branching enzyme β-1,6-*N*-acetylglucosaminyltransferase V reduces cell–cell interactions within a tumour, promoting cell detachment and invasion of tumour cells (Dennis *et al*, 2002). Also, expression of *O*-glycans containing an *N*-acetylglucosamine branch connected to *N*-acetylgalactosamine (GlcNAcβ1-6GlcNAc), which is designated the core2 branch (Figure 1A), is closely correlated with highly metastatic phenotypes of several tumour types (Yousefi *et al*, 1991; Shimodaira *et al*, 1997; Machida *et al*, 2001; Hagisawa *et al*, 2005).

In the case of patients with bladder tumours, the most common cause of the mortality is recurrence with metastasis. However, it has been reported that bladder tumours with the same grade and/or stage differ in recurrence and progression rates (Droller, 2004) and detailed molecular mechanisms of metastasis of bladder tumours are still unclear. We discover that the expression of C2GnT is more useful prognostic indicator of bladder tumour patients than pathological grade and stage and that C2GnT-expressing bladder tumour cells are malignant and highly metastatic. Based on this discovery, our primary goal is to understand the molecular mechanism how C2GnT expression increases the metastatic potential of bladder tumours to gain the insight into the roles for cell-surface carbohydrates in the process of tumour metastasis.

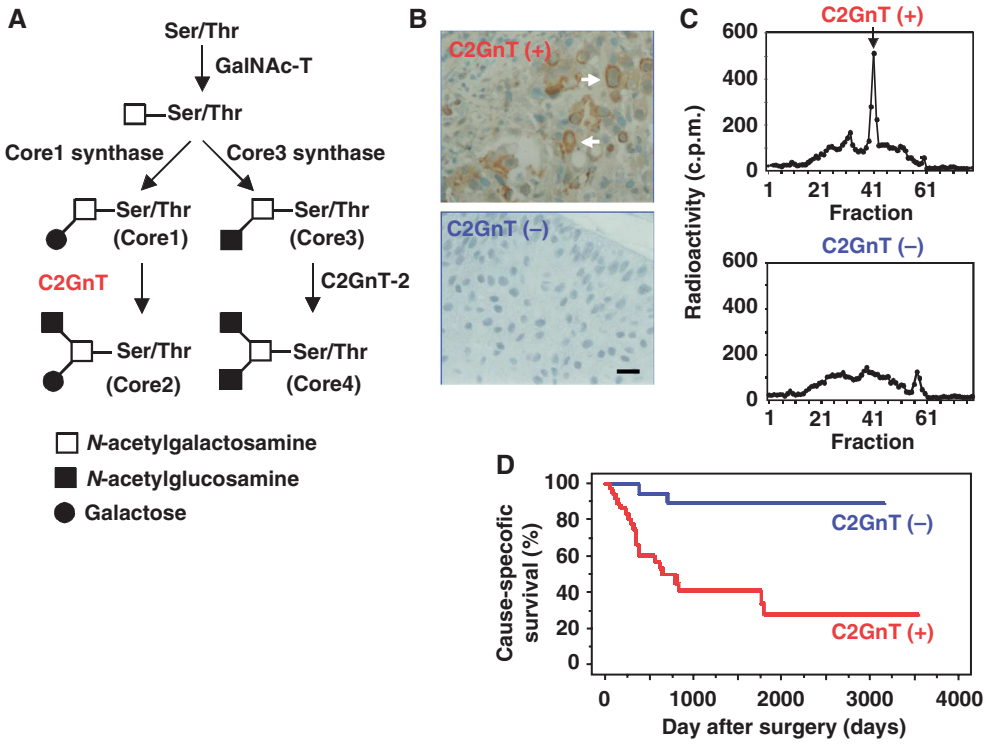

Figure 1 Expression of core2 *O*-glycans in bladder tumours. (**A**) Biosynthesis pathway of mucin-type *O*-glycan core structures, core1–4. *N*-acetylgalactosamine (GalNAc); *N*-acetylglucosamine (GlcNAc); Galactose (Gal). GalNAc is transferred to serine (Ser) or threonine (Thr) residues in a polypeptide by GalNAc-T, peptide GalNAc transferase. GalNAcα1-Ser/Thr is converted by Core1 synthase to Galβ1-3GalNAcα1-Ser/Thr (core1). Core1 is then converted by C2GnT to core2. Core1 is also converted by Core3 synthase to core3. Core3 is converted by C2GnT-2 to core4. Only C2GnT-2 is capable to synthesize core4 (Lee *et al*, 2009). (**B**) Immunohistochemistry of bladder tumours using anti-C2GnT antibody. C2GnT-positive tumour (upper panel); C2GnT-negative tumour (lower panel). C2GnT staining of a C2GnT-positive tumour specimen exhibited cytoplasmic pattern close to the nucleus. Two typical C2GnT-positive cells are denoted by arrows (upper panel). Bar, 10 μm. (**C**) Biosynthesis of core2 *O*-glycans in C2GnT-positive tumours. Oligosaccharide analysis was carried out on C2GnT-positive and C2GnT-negative tumours. ^{3}H-labelled *O*-glycans were isolated, desialylated and subjected to Bio-Gel P4 (1.0 × 100 cm) gel filtration. The elution profiles obtained from C2GnT-positive tumours (upper panel) and C2GnT-negative tumours (lower panel) were shown here. The elution position of a core2 branch containing tetrasaccharide, Galβ1-3(Galβ1−4GlcNAcβ1-6)GalNAcOH was indicated by an arrow. (**D**) Kaplan–Meier curve for the cause-specific survival of bladder tumour patients according to C2GnT staining status. Fifty-seven bladder tumour patients were divided into two groups according to the expression status of C2GnT, C2GnT positive ($n = 37$, red line) and C2GnT negative ($n = 20$, blue line).

Results

Expression of core2 O-glycans in bladder tumours

Core2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is a key enzyme for the formation of core2 *O*-glycans (Figure 1A). To evaluate the importance of the expression of core2 *O*-glycans in metastasis of bladder tumours, we immunohistochemically examined the expression status of C2GnT in radical cystectomy specimens from 57 patients with bladder tumours using anti-C2GnT antibody. The characteristics of the patients were described in Supplementary Table SI. The typical C2GnT-positive staining pattern showed C2GnT in the cytoplasm close to the nucleus, which is consistent with the intracellular localization of the Golgi apparatus, as previously observed (Skrincosky *et al*, 1997) (Figure 1B, upper panel, typical C2GnT-positive cells are denoted by arrows). Based on the staining status of Golgi apparatus, specimens with 10% or more positive cancer cells were judged as C2GnT positive. The patients were then divided into two groups, those with C2GnT-positive specimens (Figure 1B, upper panel) and those with C2GnT-negative specimens (Figure 1B, lower panel). The results from oligosaccharide analysis of bladder tumour specimens showed that a sharp peak of oligosaccharides containing the core2 branch, Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcOH, was detected in the oligosaccharides from C2GnT-positive bladder tumours (Figure 1C, upper panel, indicated by arrow), but not from C2GnT-negative bladder tumours (Figure 1C, lower panel), indicating that C2GnT-positive bladder tumour cells synthesize core2 *O*-glycans.

We next examined the relationship between C2GnT expression and prognosis in patients with bladder tumours. A Kaplan–Meier survival analysis with the log-rank significance test showed that the C2GnT-positive patients ($n = 37$) survived for a significantly shorter time than the C2GnT-negative patients ($n = 20$; $P = 0.0004$, log-rank test; Figure 1D). We also used a Cox proportional hazards regression analysis to test the association of C2GnT expression with other clinical and pathological variables for the prediction of the cause-specific survival. Both univariate and multivariate analyses revealed that the expression of C2GnT was a significant and independent predictor of life expectancy of the patient ($P = 0.003$ and $P = 0.028$, respectively; Table I). The above statistical analyses indicate that C2GnT expression is a useful prognostic indicator and that many of patients with C2GnT-positive bladder tumours die due to tumour recurrence (Figure 1D; Table I). In the case of the bladder tumours after radical cystectomy, the majority of deaths with tumour recurrence are caused by metastasis. Thus, these results strongly suggest that bladder tumours

Table I Cox proportional hazard model for predicting cause-specific survival

	HR	95% CI	*P*-value
Univariate analysis			
Age	1.043	1.003–1.085	0.035
Gender	1.004	0.371–2.717	0.993
Pathological stage	2.899	1.185–7.029	0.020
Lymph node	2.585	1.085–6.158	0.032
Grade	0.042	0.000–19.837	0.314
C2GnT expression	8.927	2.081–38.304	0.003
Multivariate analysis			
Age	1.045	1.003–1.085	0.053
Pathological stage	1.818	0.672–4.902	0.239
Lymph node	1.475	0.568–3.831	0.425
C2GnT expression	5.376	1.199–24.390	0.028

The association of C2GnT expression with other clinical and pathologic variables was tested for prediction of the cause-specific survival using Cox proportional hazard regression analysis. Univariate analysis showed that age, pathological stage, lymph-node involvement and C2GnT expression were significant predictors of recurrence ($P=0.035$, 0.020, 0.032, and 0.003, respectively). Mulitvariate analysis revealed that only C2GnT expression was an independent predictor of recurrence ($P=0.028$).

expressing core2 *O*-glycans are malignant and highly metastatic. We hypothesized that C2GnT-expressing bladder tumours acquire a highly metastatic phenotype by modifying cell-surface carbohydrates.

Evasion from NK cell tumour rejection responses by C2GnT

To test our hypothesis, we investigated the role of core2 *O*-glycans in metastasis by focusing on the process after the tumour cells disseminated into circulation, since all the patients analysed above have muscle invasive bladder tumours. We used two types of bladder tumour cell lines. One was KK-47 which derives from a non-aggressive and non-metastatic bladder tumour and barely expresses C2GnT endogenously (Hisazumi *et al*, 1981). The other one was YTS-1 which derives from a malignant and metastatic bladder tumour and highly expresses C2GnT (Kubota *et al*, 1996). We established C2GnT-expressing KK-47 (KK-47-C2) and C2GnT knockdown YTS-1 (YTSC2KD) by transfection (Supplementary Figure S1). RT–PCR analysis of the expression of four glycosyltransferases involved in the biosynthesis of various core structures of mucin-type *O*-glycans (Figure 1A) showed that both Core1 synthase and C2GnT were expressed at high levels in KK-47-C2 and YTS cells, but that the expression levels of both Core3 synthase and C2GnT-2 were very low (Supplementary Figure S2), suggesting that KK-47-C2 and YTS cells synthesize core2 *O*-glycans but not core4 *O*-glycans.

To determine the effect of C2GnT expression on metastatic potential of bladder tumour cells after dissemination into circulation, we injected cells into the tail veins of Balb/c nude mice and evaluated the metastatic potential of each cell line by examining lung metastasis. C2GnT-non-expressing KK-47 cells barely produced metastatic foci in lungs, whereas C2GnT-expressing KK-47-C2 cells produced a large number of foci (Figure 2A). And also, C2GnT-expressing YTS cells formed significantly more tumours than YTSC2KD cells with reduced C2GnT expression (Figure 2B). These results strongly

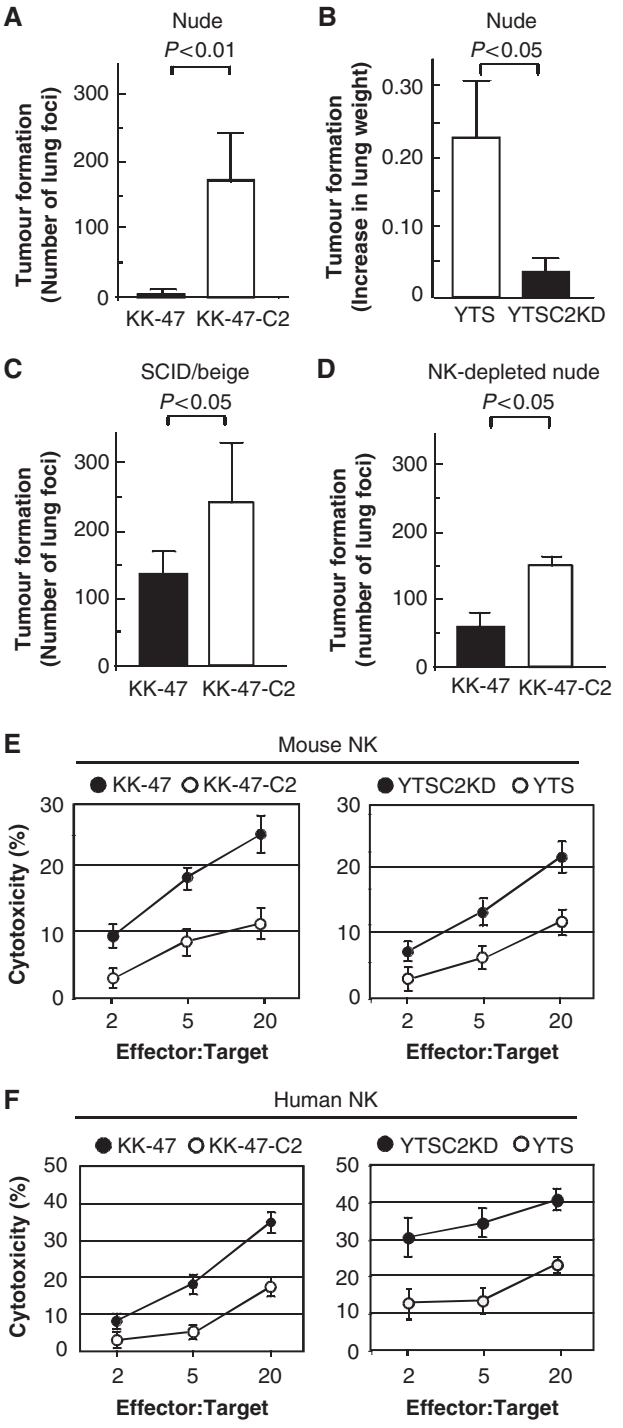

Figure 2 Evasion from NK cell tumour rejection responses by C2GnT-expressing tumour cells. (**A**, **B**) Tumour formation by bladder tumour cells in nude mice. Bladder tumour cells were intravenously injected into nude mice. After 3 weeks, the lungs were examined for tumour formation by counting metastatic foci or measuring lung weights. KK-47, closed bar ($n=8$); KK-47-C2, open bar ($n=6$) (**A**). YTS, open bar ($n=7$); YTSC2KD, closed bar ($n=7$) (**B**). (**C**, **D**) Tumour formation by bladder tumour cells in mice with impaired NK cell cytotoxicity. Bladder tumour cells were intravenously injected into SCID/beige mice ($n=5$ for each cell) (**C**) and NK cell-depleted nude mice ($n=5$ for each cell) (**D**). After 3 weeks, the lungs were examined for tumour formation by counting metastatic foci. KK-47, closed bars and KK-47-C2, open bars. (**E**, **F**) Effect of C2GnT expression on NK cell cytotoxicity. Cytotoxicity of mouse NK cells (**E**) and human NK cells (**F**) against bladder tumour cells was assayed. Mean values ± s.e. of three independent experiments.

suggest that the expression of C2GnT increases the metastatic potential of bladder tumour cells in the process after dissemination.

Because there were no significant differences in proliferation of the tumour cell lines regardless of the C2GnT expression level (Supplementary Figure S3), the metastatic potentials of the bladder tumour cells after dissemination should largely depend upon how well the injected tumour cells survive in circulation. We first characterized the immune cell types involved in rejecting the bladder tumour cells using SCID/beige (with impaired NK cell cytotoxicity) and NK cell-depleted nude mice. KK-47 cells produced significantly more metastatic foci in SCID/beige mice (Figure 2C) and NK cell-depleted nude mice (Figure 2D) than in nude mice (deficient in T cells) (Figure 2A). And also, there was no significant difference in foci produced by KK-47-C2 cells among these three kinds of mouse (Figure 2A, C, and D). These results taken together with Figure 2B suggest that C2GnT-non-expressing tumour cells were mainly killed by NK cells but that C2GnT-expressing cells were more resistant to NK cell attack than C2GnT-non-expressing cells *in vivo*.

We then assayed NK cell cytotoxicity against those bladder tumour cells. C2GnT-expressing KK-47-C2 cells were less efficiently killed by mouse NK cells than C2GnT-non-expressing KK-47 cells at several effector:target ratios (Figure 2E). C2GnT-expressing YTS cells were less efficiently killed by mouse NK cells than YTSC2KD cells with reduced C2GnT expression (Figure 2E). These results are consistent with the results from the *in vivo* tumour formation experiments (Figure 2A–D). The same tendency was observed in human NK cell killing of the bladder tumour cells (Figure 2F). Figure 2E and F indicates that C2GnT-expressing KK-47-C2 and YTS cells are more resistant to NK cells than KK-47 and YTSC2KD cells, suggesting that tumour cells with high C2GnT expression possess a high ability to evade NK cell immunity.

O-glycans of MICA

We asked how C2GnT-expressing tumour cells evade NK cell immunity. A series of activating receptors expressed by NK cells recognize tumour ligands and have a major role in eliminating tumour cells. We hypothesized that the modification of the tumour ligands with C2GnT is involved in evasion of the C2GnT-expressing tumour cells from NK cell attack. To test this hypothesis, we investigated tumour ligands for NK-activating receptors.

To determine the most critical activating receptor for eliminating the bladder tumour cells, we first blocked the NK-activating receptors with their blocking antibodies and then assayed NK-mediated cytotoxicity against the bladder tumour cells (KK-47 and KK-47-C2). The most efficient inhibition was observed when natural killer group 2 member D (NKG2D) was blocked (Supplementary Figure S4).

Next, we evaluated the expression levels of NKG2D tumour ligands by RT–PCR and performed the blocking experiments for NKG2D ligands. Expression of a ligand, Major histocompatibility complex class I-related chain A (MICA), was higher than any other ligand and the most efficient inhibition was observed when MICA/B was blocked (Supplementary Figure S5). Supplementary Figures S4 and S5 suggest that the NKG2D–MICA interaction has a major role in the rejection response of the bladder tumour cells. We confirmed this by using a clonal NK cell line, KHYG-1 (Supplementary Figure S6). Based on these results, we decided to focus on the NKG2D–MICA interaction to investigate how C2GnT-expressing bladder tumour cells evade NK cell immunity.

C2GnT acts on *O*-glycans to form the GlcNAc-GalNAc branch (Figure 1A). To determine the effect of C2GnT expression on NK-mediated killing of bladder tumours, we analysed *O*-glycans on MICA. We treated cells with benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG). BAG inhibits the formation of Galβ1-3GalNAcα1-Ser/Thr, the core1 structure of *O*-glycans (Figure 1A) and membrane proteins from BAG-treated cells are poorly *O*-glycosylated (Kuan *et al*, 1989). MICA from untreated KK-47 cells exhibited a larger molecular weight than MICA from BAG-treated KK-47 and KK-47-C2 (Figure 3B, lanes 1–3), indicating that MICA from KK-47 cells was *O*-glycosylated. This suggests that MICA from KK-47 cells carries core1 *O*-glycans, because KK-47 cells express Core1 synthase at high levels but barely express Core3 synthase, C2GnT and C2GnT-2 (Figure 1A). MICA from KK-47-C2 cells exhibited a larger molecular weight than MICA from KK-47

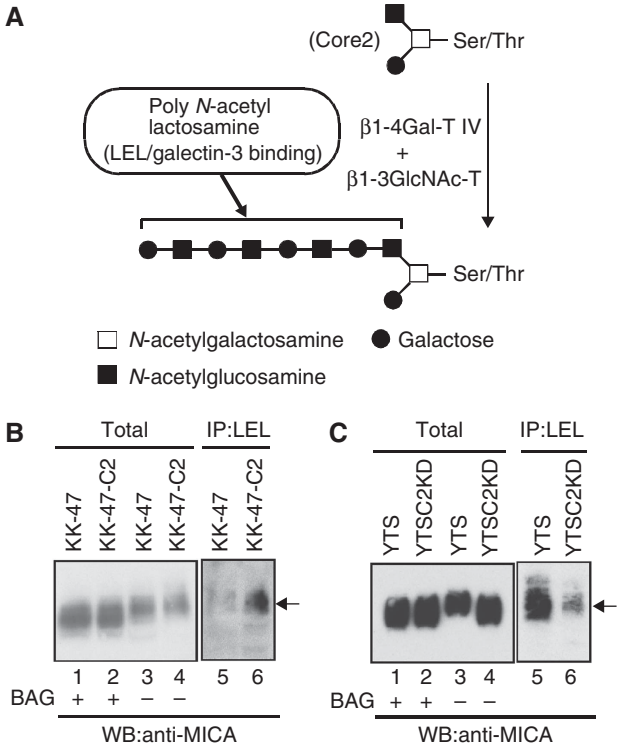

Figure 3 C2GnT-expressing tumour cells express MICA carrying poly-*N*-acetyllactosamine. (**A**) Biosynthesis of poly-*N*-acetyllactosamine on *O*-glycans. β-1,4-Galactosyltransferase IV (β1-4Gal-T IV) together with β-1,3-*N*-acetylglucosaminyltransferase (β1-3 GlcNAc-T) synthesize poly-*N*-acetyllactosamine in core2 branched oligosaccharides. *Lycoperiscon esculentum* (tomato) lectin (LEL) and galectin-3 bind specifically to poly-*N*-acetyllactosamine with at least three lactosamine unit repeats. (**B**, **C**) MICA from KK-47-C2 (**B**) and YTS (**C**) carries poly-*N*-acetyllactosamine. Total lysates from benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG, 2 mM)-treated or untreated cells (5×10^6 cells) were analysed by western blotting with anti-MCA (clone BAMO1) (lanes 1–4). Total lysates from tunicamycin (0.5 μg/ml)-treated cells were immunoprecipitated with LEL-agarose followed by western blot analysis with anti-MICA (lanes 5 and 6).

cells (Figure 3B, lanes 3 and 4). These results suggest that MICA from KK-47-C2 cells carries core2 *O*-glycans, because KK-47-C2 cells highly express C2GnT (Supplementary Figure S2). MICA carrying core2 *O*-glycans was designated MICAC2.

The core2 branch is a scaffold for the subsequent production of lactosamine disaccharide repeats, poly-*N*-acetyllactosamine (Galβ1-4GlcNAc)*n* on *O*-glycans in a wide variety of cells (Figure 3A; Maemura and Fukuda, 1992). To determine if MICAC2 carries poly-*N*-acetyllactosamine on *O*-glycans, we excluded poly-*N*-acetyllactosamine carried on *N*-glycans from bladder tumour cells by treatment of tunicamycin, an *N*-glycosylation inhibitor. Tunicamycin treatment efficiently prevented MICA in bladder tumour cells from *N*-glycosylation (Supplementary Figure S7). We then analysed cell lysates by immunoprecipitation using the *Lycoperiscon esculentum* (tomato) lectin (LEL), which binds specifically to poly-*N*-acetyllactosamine with at least three lactosamine unit repeats (Nachbar and Oppenheim, 1982). MICA was barely detectable in the LEL immunoprecipitates from KK-47 cells, but MICAC2 was detected in the LEL immunoprecipitates from KK-47-C2 cells (Figure 3B, lanes 5 and 6). Similarly, C2GnT-expressing YTS cells express MICAC2 (Figure 3C, lanes 1–4), and MICAC2 was detected in the LEL immunoprecipitates (Figure 3C, lane 5). MICAC2 was also detected in the LEL immunoprecipitates from YTSC2KD cells with reduced C2GnT expression, but in lower amounts than MICAC2 from YTS cells (Figure 3C, lane 6). These results indicate that MICAC2 carries poly-*N*-acetyllactosamine on *O*-glycans. The anti-MICA antibody (clone BAMO1) that we used for western blotting recognizes both MICA and MICB. We confirmed that MICB was also core2 *O*-glycosylated and carried poly-*N*-acetyllactosamine by using anti-MICB-specific antibody (clone 236511) (Supplementary Figure S8).

Galectin-3 binds MICA through poly-N-acetyllactosamine on O-glycans

Poly-*N*-acetyllactosamine is a ligand for galectins (Sato and Hughes, 1992; Knibbs *et al*, 1993). Among 15 members of galectin family, we focused on galectin-3 which has immunomodulatory effects (Partridge *et al*, 2004). We first examined galectin-3 on the surface of bladder tumour cells. MICA and MICAC2 were expressed on the bladder tumour cell surface at the similar levels (Figure 4B and G). We then analysed tunicamycin-treated bladder tumour cells for the existence of galectin-3 on the cell surface. Galectin-3 was detected on the surface of C2GnT-expressing cells (KK-47-C2 and YTS) (Figure 4D and I). Treatment with endo-β-galactosidase which cleaves the Galβ1-4GlcNAc linkage diminished the cell-surface galectin-3 (Figure 4E and J).

We next examined whether galectin-3 interacts with MICAC2. Galectin-3 was detected in the total cell-surface proteins from the bladder tumour cells (Supplementary Figure S9). Next, tunicamycin-treated cells were subjected to dithio-bis-sulphosuccinimidyl propionate (DTSSP) (Pierce Biotechnology Inc.) crosslinking of cell-surface proteins to stabilize complexes. MICA and MICAC2 were immunoprecipitated from the cell lysates followed by western blotting for galectin-3. Galectin-3 co-immunoprecipitated with MICAC2 from KK-47-C2 cells, whereas the co-immunoprecipitation of galectin-3 with MICA was undetectable on KK-47 cells (Figure 4K, lanes 5 and 6). The MICA and MICAC2 immunoprecipitates were treated with endo-β-galactosidase. Upon extensive digestion, galectin-3 was not detected in both MICA and MICAC2 immunoprecipitates (Figure 4K, lanes 7 and 8), indicating that galectin-3 binds MICAC2 through poly-*N*-acetyllactosamine on *O*-glycans. We also confirmed that galectin-3 binds MICAC2 through poly-*N*-acetyllactosamine in YTS cells (Figure 4L).

To validate the results from bladder tumour cell lines, MICA from the bladder tumour patients' specimens (P1, P2, P3 and P4) (Supplementary Figure S10A) was analysed biochemically. The tumour from P1 was a low-grade tumour with very low C2GnT expression, but the tumours from P2, P3, and P4 were high-grade tumours with high C2GnT expression (Supplementary Figure S10B). MICA from C2GnT-expressing bladder tumours (P2, P3, and P4) exhibited higher molecular weights than MICA from C2GnT-non-expressing tumour (P1) (Figure 5A, lanes 1–4). This taken together with the expression profiles of glycosyltransferases (Supplementary Figure S10B) suggest that P2, P3, and P4 tumours express MICAC2 carrying core2 *O*-glycans. The MICA immunoprecipitates from specimens were treated with Glycopeptidase F (TAKARA Bio Inc.) to remove *N*-glycans, and then re-immunoprecipitated with LEL-agarose followed by western blotting with anti-MICA (clone BAMO1). MICAC2 was detected in the LEL immunoprecipitates from P2, P3, and P4, but not from P1 (Figure 5A, lanes 5–8), indicating that MICAC2 from P2, P3, and P4 carries poly-*N*-acetyllactosamine on *O*-glycans.

We then examined the MICA molecules from the bladder tumour specimens, P1, P2, P3, and P4 for interaction with galectin-3. The tumour tissues were treated with DTSSP, and then MICA was immunoprecipitated with anti-MICA followed by Glycopeptidase F treatment and western blotting using anti-galectin-3. Galectin-3 co-immunoprecipitated with MICAC2 from P2, P3, and P4, indicating that galectin-3 binds MICAC2 through the poly-*N*-acetyllactosamine on *O*-glycans in P2, P3, and P4 tumours.

To confirm the cell-surface expression of MICA, MICAC2, and galectin-3 in bladder tumours, we prepared primary culture from P1 and P2 and then stained primary cultured cells with anti-MICA (clone BAMO3) and anti-galectin-3. Both primary cultured cells express MICA but that galectin-3 was detected on the surface of cells from only C2GnT-expressing tumour, P2 (Supplementary Figure S11B and D). Endo-β-galactosidase treatment diminished galectin-3 on the cell surface of P2 (Supplementary Figure S11E). These results taken together showed that galectin-3 binds MICAC2 on the cell surface of C2GnT-expressing bladder tumours through the poly-*N*-acetyllactosamine on *O*-glycans as well as bladder tumour cell lines.

The MICA extracellular domain contains the α1, α2, and α3 regions and the structural analysis revealed that NKG2D-binding site resides in the α1 + α2 region (Groh *et al*, 1996; Li *et al*, 2001). We demonstrated that the α1 + α2 region carries poly-*N*-acetyllactosamine on its *O*-glycans and that galectin-3 binds this region by using a construct of an α3-truncated mutant MICA (Supplementary Figure S12). Binding of anti-MICA, BAMO1 (α1 + α2 specific) (Wu, 2004) to MICAC2 was slightly reduced compared with MICA (Supplementary Figure S13). In contrast, there was no difference in binding of BAMO3 (α3 specific) (Salih *et al*, 2003; Spreu *et al*, 2006) between MICA and MICAC2 (Figure 4B and G). This also supports that the α1 + α2 region carries poly-*N*-acetyllactosamine on its *O*-glycans.

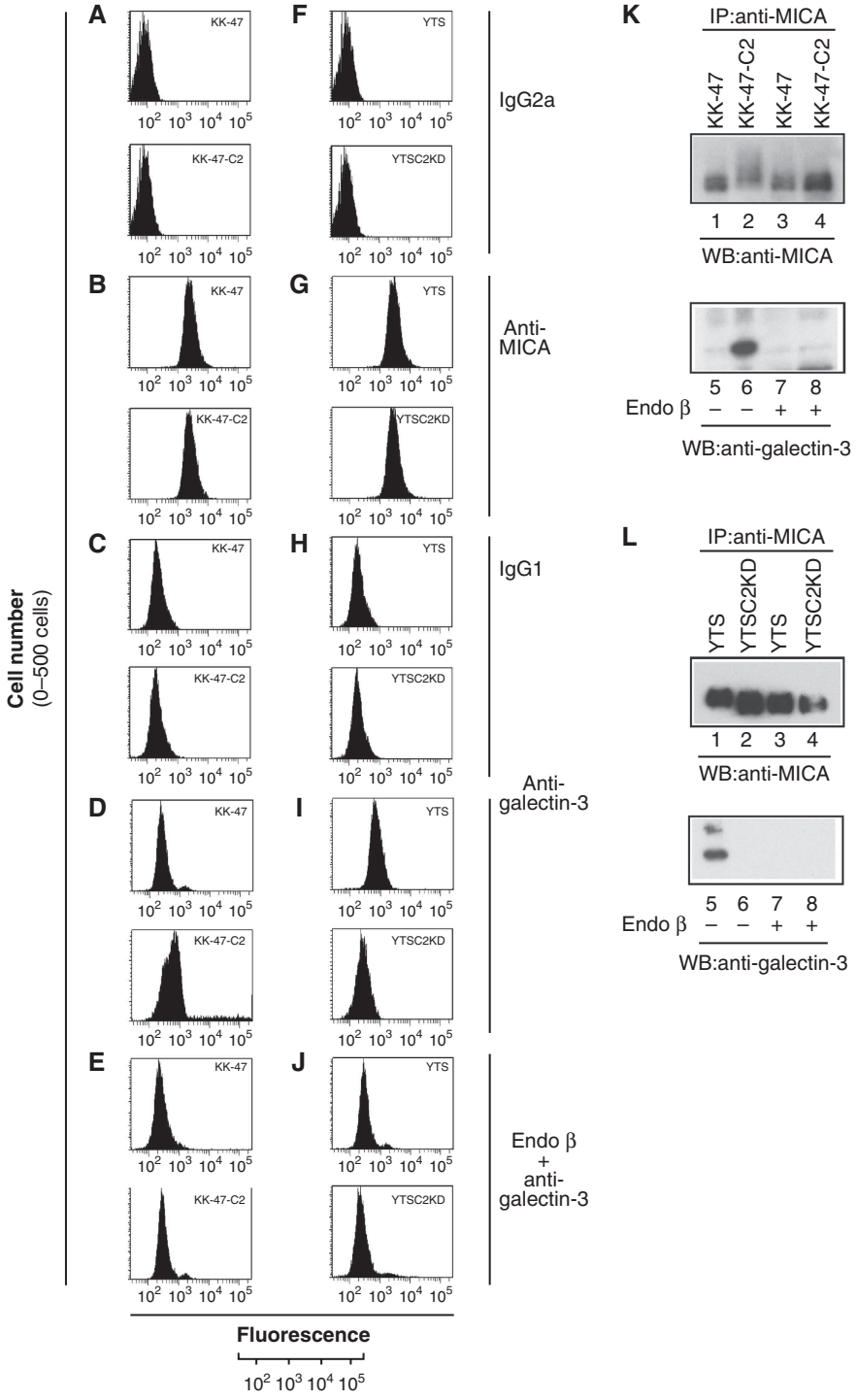

Figure 4 MICA and galectin-3 on the surface of bladder tumour cells. (**A**–**J**) Flow cytometric analyses of bladder tumour cells. Cells were stained with control IgG2a (**A**, **F**) and anti-MICA (clone BAMO3) (**B**, **G**). Tunicamycin-treated cells were stained with IgG1 (**C**, **H**) and anti-galectin-3 (**D**, **I**). Tunicamycin-treated cells (1×10^6) were incubated with endo-β-galactosidase (80 mU/ml, 37°C, 8 h), and then stained with anti-galectin-3 (**E**, **J**). (**K**, **L**) Galectin-3 binds MICAC2. Cells were subjected to dithio-bis-sulphosuccinimydyl propionate (DTSSP) crosslinking of cell-surface proteins and the total lysates were immunoprecipitated with anti-MICA (clone BAMO3) followed by western blotting with anti-MICA (clone BAMO1) (lanes 1–4) and anti-galectin-3 (lanes 5–8). Galectin-3 co-immunoprecipitated with MICAC2 from C2GnT-expressing KK-47-C2 (lane 6) (**K**) and YTS cells (lane 5) (**L**). Binding of galectin-3 to MICAC2 was disrupted by endo-β-galactosidase treatment (lanes 7 and 8) (**K**, **L**).

Effect of poly-N-acetyllactosamine and galectin-3 on NK cell function

On the basis of the above results (Figures 3–5), we asked if galectin-3 binding to MICAC2 affects the interaction of MICA molecules with NKG2D. We first examined the interaction of NKG2D with its ligands on the cell surface. More NKG2D-Fc bound to KK-47 than KK-47-C2 cells and to YTSC2KD than YTS cells (Figure 6B and E), indicating that C2GnT expression reduced NKG2D-Fc binding to the bladder tumour cells. Endo-β-galactosidase treatment increases NKG2D-Fc binding to KK-47-C2 and YTS cells to the same level as KK-47 and YTSC2KD cells (Figure 6C and F), suggesting

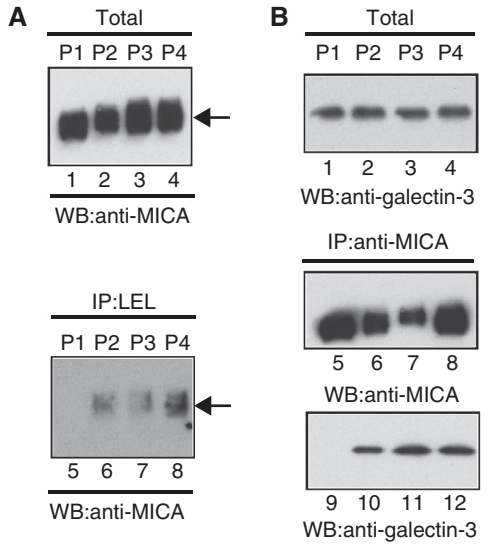

Figure 5 Galectin-3 binds MICAC2 from C2GnT-positive bladder tumour specimens. (**A**) MICA from C2GnT-positive bladder tumour specimens carries poly-*N*-acetyllactosamine. Total lysates from patients' tumour specimens (P1, P2, P3, and P4) were analysed by western blotting with anti-MICA (clone BAMO1) (lanes 1–4). The MICA immunoprecipitates from tumour specimens (0.1 g) with anti-MICA (clone BAMO3) were treated with Glycopeptidase F (20 μU/μl, 37°C, 16 h) and then re-immunoprecipitated with LEL-agarose followed by western blotting with anti-MICA (clone BAMO1) (lanes 5–8). (**B**) Bladder tumour specimens from patients (P1, P2, P3, and P4) were subjected to DTSSP crosslinking of cell-surface proteins and the total lysates were analysed by western blotting with anti-galectin-3 (lanes 1–4). The MICA immunoprecipitates from tumour specimens with anti-MICA (clone BAMO3) were treated with Glycopeptidase F and then analysed by western blotting with anti-MICA (BAMO1) (lanes 5–8) and anti-galectin-3 (lanes 9–12).

that poly-*N*-acetyllactosamine and galectin-3 reduce NKG2D-Fc binding to C2GnT-expressing bladder tumour cells. Next, we conversely examined MICA binding to the NK cell surface. We prepared MICA-IgG and MICAC2-IgG chimeras using KK-47 and KK-47-C2 cells, respectively (Supplementary Figure S14A), and analysed binding of MICA-IgG and MICAC2-IgG to human and mouse NK cells. MICA-IgG bound to NK cells and this binding was not affected by adding galectin-3 (Figure 6H and I). However, MICAC2-IgG bound less efficiently to NK cells than MICA-IgG and MICAC2-IgG binding was further reduced by adding galectin-3 (Figure 6J and K). We directly made a comparison of the binding affinity for NKG2D between MICA-IgG and MICAC2-IgG using a surface plasmon resonance-based biosensor. The affinity of MICAC2-IgG for NKG2D was lower than that of MICA-IgG (Supplementary Figure S14B), suggesting that the attachment of poly-*N*-acetyllactosamine to MICAC2 reduces the affinity of MICAC2 for NKG2D. Galectin-3 lowered binding of MICAC2-IgG to NKG2D in a dose-dependent manner, but did not affect binding of MICA-IgG to NKG2D (Figure 6L). These results indicate that galectin-3 binding to MICAC2 further reduces the affinity of MICAC2 for NKG2D in addition to the effect of poly-*N*-acetyllactosamine.

We next asked whether galectin-3 binding to MICAC2 affects NK cell functions. NK cells are activated through the interaction of NK receptors with ligands on target cells and kill the target cells by the release of granular contents (degranulation), including perforin and granzymes, which induce apoptosis of the target cells. Engagement of NKG2D by MICA activates NK cells to induce cytokine secretion and degranulation, thereby promoting tumour rejection (Russell and Ley, 2002). We measured the secretion of interferon-γ (IFN-γ) and granzyme B from NK cells upon stimulation with plate-bound MICA-IgG molecules by assaying the concentration of IFN-γ and the protease activity of granzyme B in medium. MICAC2-IgG induced significantly less secretion of IFN-γ and granzyme B than MICA-IgG (Figure 7A and B). Galectin-3 had no effect on the secretion of IFN-γ and granzyme B induced by MICA-IgG, whereas galectin-3 greatly reduced their secretion induced by MICAC2-IgG (Figure 7A and B). These results taken together with Figure 6 suggest that galectin-3 binding to MICAC2 through poly-*N*-acetyllactosamine reduces the affinity of MICAC2-IgG for NKG2D on NK cells, thereby impairing NK cell activation and resulting in reduced secretion of IFN-γ and granzyme B.

To determine if galectin-3 binding to the bladder tumour cells affects NK cell cytotoxicity, we removed galectin-3 from tumour cells and then assayed cytotoxicity. KK-47-C2 cells were resistant to human NK cell attack compared with KK-47 cells. However, KK-47-C2 cells were killed by human NK cells as efficiently as KK-47 cells after endo-β-galactosidase treatment (Figure 7C). YTS cells were also killed by NK cells as efficiently as YTSC2KD cells (with reduced C2GnT activity) after endo-β-galactosidase treatment (Figure 7D). Considering endo-β-galactosidase treatment diminished galectin-3 binding to the bladder tumour cells (Figure 4E and J), these results strongly suggest that galectin-3 binding to MICAC2 through poly-*N*-acetyllactosamine impair NK cell activation and silence the NK cells, resulting in evasion from NK cell attack by C2GnT-expressing bladder tumour cells.

In vivo role of NKG2D and tumour cell-surface-bound galectin-3 in tumour rejection responses

We have shown that galectin-3 binding to MICAC2 through poly-*N*-acetyllactosamine is a novel immunoevasion strategy from NK cell immunity using *in vitro* systems (Figures 3–7). To validate the roles of NKG2D and galectin-3 in this immunoevasion mechanism by *in vivo* experiments, we performed tumour formation assay using nude mice and the bladder tumour cells.

An anti-mouse NKG2D blocking antibody (CX5) inhibited NK cell killing of KK-47 cells (Figure 8A). CX5 efficiently blocks mouse NKG2D *in vivo* (Ogasawara *et al*, 2004). We intraperitoneally injected CX5 into nude mice to block NKG2D and then examined if the blockade of NKG2D affects tumour formation. KK-47 cells barely produced foci in control rat IgG1-treated mice, but produced a large number of foci in CX5-treated mice compared with control, suggesting that C2GnT-non-expressing tumour cells were killed *in vivo* in an NKG2D-dependent manner. In contrast, KK-47-C2 produced significantly more foci in CX5-treated mice than KK-47 cells and there was no difference in tumour formation by KK-47-C2 cells between CX5-treated and control mice (Figure 8B). These results suggest that NKG2D has a major role in NK cell killing of C2GnT-non-expressing bladder tumour cells *in vivo* and that C2GnT expression renders the bladder tumour cells resistant to NKG2D-mediated NK cell attack *in vivo*.

To address the role of tumour cell-surface-bound galectin-3 in NK cytotoxicity, we removed cell-surface galectin-3 by

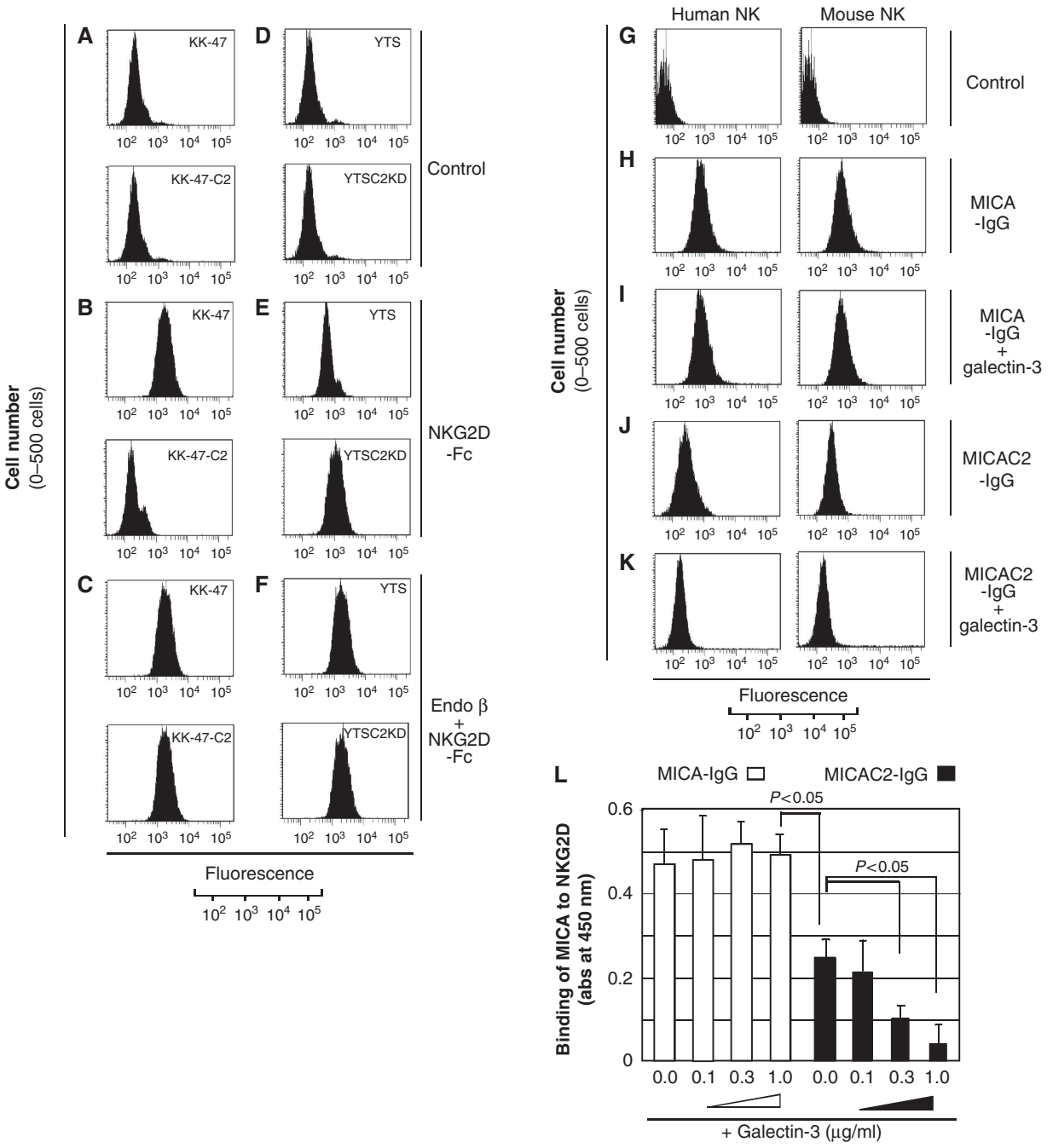

Figure 6 Galectin-3 binding to MICA reduces the interaction of NKG2D with MICA. (**A**–**H**) Interaction of NKG2D with MICA on the surface of bladder tumour cells. Flow cytometric analysis of bladder tumour cells (**A**–**F**) and NK cells (**G**, **H**). Bladder tumour cells were stained with control IgG chimera (CD43-IgG) (**A**, **D**) and NKG2D-Fc (**B**, **E**). Cells were treated with endo-β-galactosidase followed by NKG2D-Fc staining (**C**). (**G**–**K**) Human (left panel) and mouse (right panel) NK cells were stained with CD43-IgG, MICA-IgG, or MICAC2-IgG. (**L**) Effect of galectin-3 on MICA binding to NKG2D. MICA-IgG or MICAC2-IgG was incubated with galectin-3 at 37°C for 30 min, and then added to the plate coated with NKG2D-Fc by ELISA (MICA-IgG, open bars; MICAC2-IgG, closed bars). Mean values ± s.e. of three independent experiments.

cleaving poly-*N*-acetyllactosamine with endo-β-galactosidase. KK-47-C2 cells were resistant to NK cell killing, but galecin-3-removed KK-47-C2 cells were efficiently killed by NK cells during 4 h culture (Figure 8C). We confirmed that little of cell-surface-bound galectin-3 was restored by *de novo* biosynthesis of poly-*N*-acetyllactosamine and galectin-3 during the 4 h-culture (Supplementary Figure S15F). Figure 8C taken together with Supplementary Figure S15 suggest that galectin-3-removed KK-47-C2 cells become susceptible to NK cell attack due to removal of galectin-3.

We then evaluated the *in vivo* role of tumour cell-surface-bound galectin-3 in tumour rejection responses. KK-47-C2 cells produced a number of foci (Figure 8D, left panel and Figure 2A). Galectin-3-removed KK-47-C2 cells produced significantly less foci than KK-47-C2 cells (Figure 8D, right panel), suggesting that galectin-3-removed KK-47-C2 cells were killed by NK cells *in vivo*. Figure 8C and D strongly suggests that tumour cell-surface-bound galectin-3 has an important role in immunoevasion mechanism from NK cell immunity by C2GnT-expressing tumour cells *in vivo*.

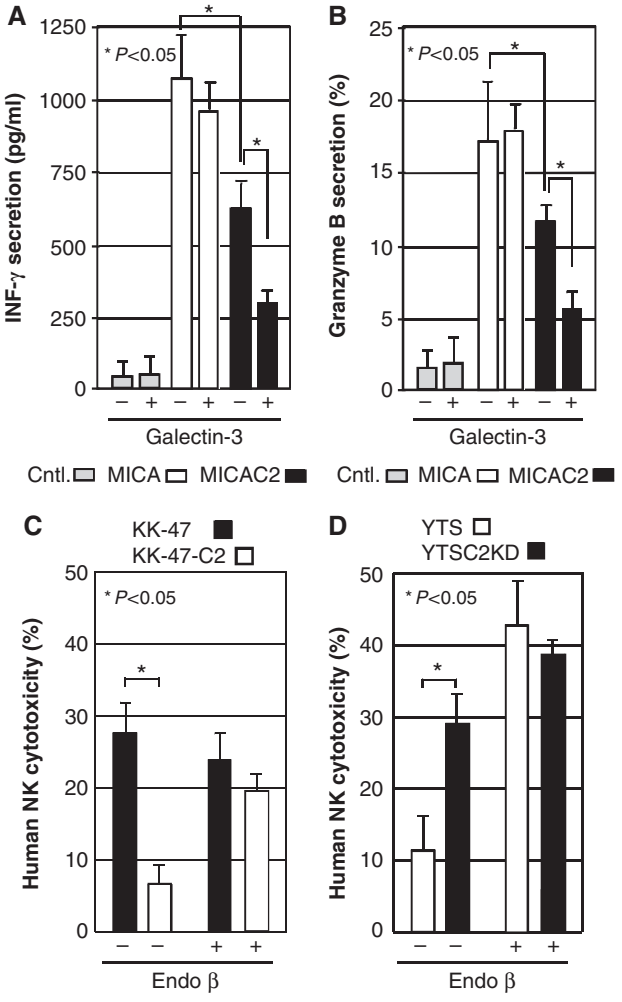

Figure 7 Galectin-3 binds MICAC2, impairing NK cell functions. (**A**, **B**) Effect of galectin-3 on secretion of INF-γ (**A**) and granzyme B (**B**). NK cells were stimulated with the plates coated with CD43-IgG (cntl.), MICA-IgG, or MICAC2-IgG in the presence (1.0 μg/ml) and absence of galectin-3. After stimulation, the supernatant was recovered and NK cell activation was evaluated by measuring secretion of INF-γ and granzyme B into the supernatant. (**C**, **D**) Removal of galectin-3 from bladder tumour cells enhances NK-mediated cytotoxicity. Tunicamycin-treated bladder tumour cells were treated with endo-β-galactosidase, and then human NK-mediated cytotoxicity was measured at an effector:target ratio of 5:1. Mean values ± s.e. of three independent experiments.

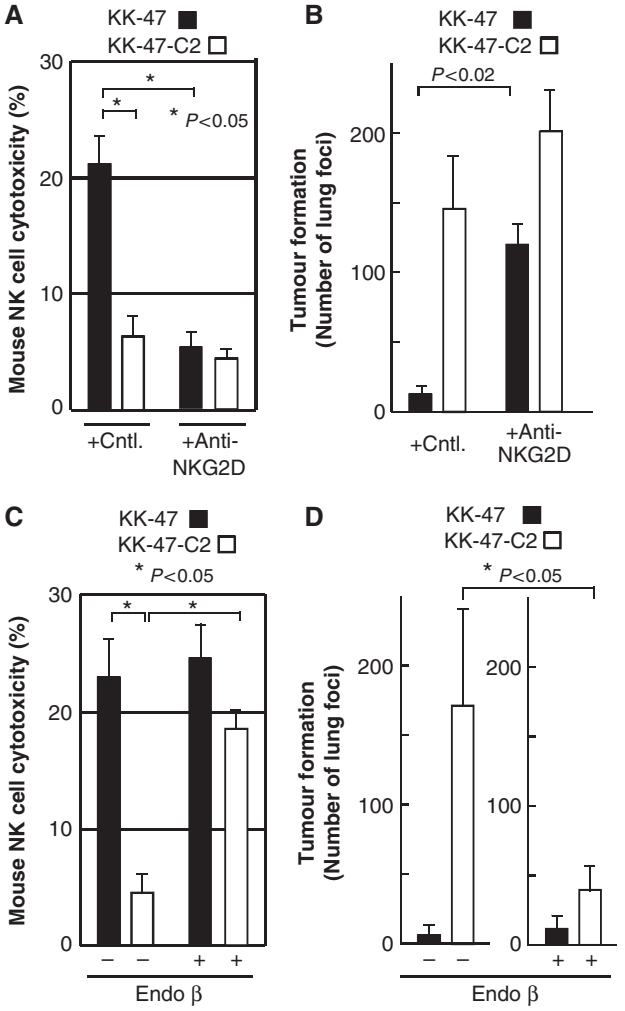

Figure 8 *In vivo* role of NKG2D and tumour cell-surface-bound galectin-3 in rejection responses of the bladder tumours. (**A**) Mouse NK cells were analysed for cytotoxicity against bladder tumour cells in the presence of indicated antibodies at an effector:target ration of 5:1. Mouse NK cells were pre-incubated for 30 min with saturating concentration of rat IgG1 (+cntl.) or anti-mouse NKG2D blocking antibody (CX5). (**B**) Bladder tumour cells (5×10^6 cells) were intravenously injected into nude mice. The nude mice were pre-treated with anti-mouse NKG2D (CX5) ($n = 5$ for each cell) or control rat IgG1 (+cntl.) ($n = 3$ for each cell). After 3 weeks, the lungs were examined for tumour formation by counting metastatic foci. (**C**) Mouse NK cells were assayed for cytotoxicity against bladder tumour cells after endo-β-galactosidase treatment at an effector:target ratio of 5:1. Tumour cells were pre-treated with (+) (80 mU/ml, 37°C, 8 h) or without endo-β-galactosidase treatment (−). (**D**) Endo-β-galactosidase-treated bladder tumour cells (5×10^6 cells) (KK-47, closed bars ($n = 4$); KK-47-C2, open bars ($n = 4$)) were intravenously injected into nude mice. After 3 weeks, the lungs were examined for tumour formation. The results from the tumour formation by untreated cells (Figure 2A) were re-displayed as a left panel of Figure 8D for the comparison purpose.

Discussion

By analysing bladder tumour cell lines and patients' specimens and using animal models, we have clarified one of the most critical factors increasing the metastatic potential of bladder tumours. We provided evidence that MICA, a ligand for an NK-activating receptor (NKG2D), from C2GnT-expressing bladder tumour cells carries poly-*N*-acetyllactosamine on *O*-glycans and that galectin-3 modulates the NKG2D–MICA interaction by binding MICA through poly-*N*-acetyllactosamine (Figures 3–6). The immunomodulation effect of galectin-3 reduced NK cell activation and degranulation (Figure 7), impairing the rejection of C2GnT-expressing tumours (Figures 2 and 8). Analyses of NKG2D knockout mice revealed that the NKG2D–MICA interaction is critical to tumour rejection responses (Guerra *et al*, 2008; Zafirova *et al*, 2009) and MICA functions as a ligand for mouse NKG2D *in vivo* (Friese *et al*, 2003; Elsner *et al*, 2007). These observations support the immunoevasion mechanism that we reported here. Thus, C2GnT-expressing bladder tumour cells evade NK cell immunity by silencing NK cell activation, resulting in longer survival of the tumour cells disseminating in the circulation during the process of metastasis.

Previous studies reported that some tumour cells modulate NKG2D-mediated tumour immunosurveillance in the following three ways: (i) large amount of soluble MICA shed by

tumour cells downregulate NKG2D expression (Groh *et al*, 2002; Doubrovina *et al*, 2003; Clayton *et al*, 2008); (ii) tumour cells sustain the expression of NKG2D ligands (Oppenheim *et al*, 2005); (iii) tumour cells decrease the cell-surface expression of MICA by retaining MICA in cells (Fuertes *et al*, 2008). Downregulation of NKG2D or NKG2D ligands then allowed the tumour cells to evade the tumour immunosurveillance systems. In fact, we also observed that NKG2D expression was downregulated by the soluble form of MICA (Supplementary Figure S16C and D). However, substantial amount of NKG2D (~70% of unstimulated NK cells) was still present at the cell surface when NK cells were stimulated with MICA-IgG or MICAC2-IgG (Supplementary Figure S16D–F). On the other hand, there was no difference in the expression level of MICA among bladder tumour cells and also among patients' specimens (Figure 4; Supplementary Figure S11). These results taken together suggest that the NKG2D downregulation by soluble MICA molecules is not a primary immunoevasion system by C2GnT-expressing bladder tumour cells. Our results revealed a novel strategy for immunoevasion from tumour immunosurveillance systems. This novel immunoevasion strategy allows C2GnT-expressing tumour cells to survive longer in the host and to metastasize much more efficiently than C2GnT-non-expressing tumour cells. This is probably a major cause of poor prognosis in patients with C2GnT-expressing tumours (Figure 1D). Mechanisms of C2GnT expression in some tumour cells remain to be explored, but suppression of C2GnT expression in tumour cells might be a new therapeutic way to reduce bladder tumour metastasis.

We observed that binding of MICAC2-IgG to NKG2D-Fc was inhibited by galectin-3 (Figure 6L), suggesting the possibility that galectin-3 binds soluble MICAC2 shed by tumour cells through poly-*N*-acetyllactosamine. We examined the effect of galectin-3 on the ability of NKG2D downregulation by MICAC2-IgG. NKG2D downregulation by MICAC2-IgG was slightly reduced in the presence of galectin-3 compared with MICA-IgG (Supplementary Figure S17). However, the difference was small and substantial amount of NKG2D was still present at the NK cell surface after stimulation with either MICA-IgG or MICAC2-IgG in the presence of galectin-3. This suggests that the effect of galectin-3 binding on this immunoevasion mechanism is small.

It has been reported that poly-*N*-acetyllactosamine on *N*-glycans modulate the cell–cell interaction to suppress excessive immune responses (Togayachi *et al*, 2007), which is consistent with our results showing that the attachment of poly-*N*-acetyllactosamine on the binding site of MICA for NKG2D reduces the MICA–NKG2D interaction to impair NK cell activation (Figures 6L and 7). Poly-*N*-acetyllactosamine provides a backbone structure for sialyl-Lewis^x (SLe^x) which is involved in tumour metastasis by mediating adhesion between tumour cells, platelets, and leukocytes (Nakamori *et al*, 1999; Borsig *et al*, 2002). However, these bladder tumour cells do not utilize SLe^x for metastasis, because we were unable to detect expression of fucosyltransferases (FucT III, FucT IV, and FucT VII) that are responsible for the formation of SLe^x by RT–PCR (Supplementary Figure S18).

Galectins are β-galactoside binding proteins that have been implicated in numerous biological processes, including immunity, inflammation, and tumour progression (Leffler *et al*, 2004; Dumic *et al*, 2006). Among 15 members of galectin family, galectin-1, -2, -3, -4, -7, -8, and -9 are known to be involved in tumour progression and metastasis. Above all, galectin-1 and -3 are widely distributed in normal tissues and many tumour types, although the expression of the other galectins is limited to the certain tissues such as gastrointestinal tract (galectin-4) and skin (galectin-7) (Rabinovich *et al*, 2002; Liu and Rabinovich, 2005). Increased expression of galectin-1 and -3 has been found to correlate with the malignant potential of many tumours (Rabinovich *et al*, 2007; Demydenko and Berest, 2009; Abdou *et al*, 2010; Zaia Povegliano *et al*, 2010; Kim *et al*, 2011). Galectin-3 has been reported to provide a critical determinant for survival of disseminating cancer cells in the circulation during metastasis and has been studied as a potential target of anti-cancer drugs (Fukumori *et al*, 2007; Thijssen *et al*, 2007; Yang *et al*, 2008; Glinsky and Raz, 2009), but the detailed mechanisms were unclear. We, therefore, concentrated our investigation on the role of galectin-3 in cell survival of disseminating bladder tumour cells in the circulation by focusing on cell-surface-bound galectin-3. We here provide evidence that galectin-3 binds the NKG2D-binding site of MICA through poly-*N*-acetyllactosamine, thereby inhibiting the NKG2D–MICA interaction to reduce stimulation of NK cells with MICA. This is a newly identified function of cell-surface-bound galectin-3.

Immunomodulatory functions of galectin-3 are supported by several recent studies, including the modulation of T-cell responses by tumour-associated galectin-3 (Peng *et al*, 2008) and the contribution of galectin-3 to the barrier functions of the cell-surface mucin MUC1 (Argueso *et al*, 2009). The present study also suggests that inhibition of the immunomodulatory functions of galectin-3 may improve the therapeutic potential of cancer immunotherapy. Our investigation will provide a new insight into the roles of cell-surface carbohydrates in tumour metastasis and contribute to better understanding the process of tumour metastasis.

Materials and methods

Cells, reagents, and antibodies

KK-47, a low-grade and non-metastatic bladder tumour (Hisazumi *et al*, 1981), was provided by Dr T Masuko, Tohoku University (Japan) and YTS-1, a high-grade and metastatic bladder tumour (Kubota *et al*, 1996), was a generous gift by Dr H Kakizaki, Yamagata University (Japan). A clonal NK cell line, KHYG-1 (Yagita *et al*, 2000), was purchased from Japanese Collection of Research Bioresources (JCRB) (Osaka, Japan). Cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Australia). All the biochemical reagents were purchased from Sigma, unless otherwise noted. Endo-β-galactosidase (*Escherichia freundii*), Glycopeptidase F, and galectin-3 were provided by Seikagaku Kogyo Co. (Tokyo, Japan), TAKARA BIO Inc. (Tokyo, Japan), and Myltenyi Biotech (Auburn, CA, USA), respectively. The following reagents for immunological experiments were used: anti-FLAG M2, anti-FLAG M2-agarose, anti-mouse IgG-agarose (Sigma-Aldrich), and FcR Blocking Reagent (for mouse and human) (Myltenyi Biotech). The antibodies used in this study were listed in Supplementary Table SII.

Analyses of bladder tumour patients

The cohort consisted of 57 patients with muscle invasive bladder tumours treated with radical cystectomy at the Department of Urology, Akita University Graduate School of Medicine, Akita, Japan. Supplementary Table SI lists the demographic data on those patients. Tumour stages were determined according to the American Joint Committee for Cancer Staging, 2002. Histopathological

grading was performed according to the World Health Organization System (Eble *et al*, 2004). The bladder tumour specimens were fixed with 10% buffered formalin for 12 h. The paraffin-embedded samples were cut at 3 μm and subjected to haematoxylin and eosin staining and immunohistochemistry using an affinity-purified rabbit anti-C2GnT antibody. Anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase (Nichirei, Tokyo, Japan) was used as a secondary antibody and peroxidase activity was visualized with aminoethylcarbazol (AEC) solution (Nichirei). Based on the staining status of Golgi apparatus, specimens with 10% or more positive cancer cells were judged as C2GnT positive. The cause-specific survival of the patients was compared according to the results of immunohistochemistry. To analyse MICA glycosylation in bladder tumour specimens, tumours from four genetically independent patients at Department of Urology, Hirosaki University School of Medicine were used. Supplementary Figure S10A shows characterization of the patients (P1, P2, P3 and P4). Written consents were taken from all the patients. The institutional ethics committees of Akita University Graduate School of Medicine and Hirosaki University Graduate School of Medicine approved this study.

Analysis of O-glycans from bladder tumour specimens

Glycoproteins were isolated from bladder tumour specimens with PBS containing 1% Igepal CA-630 and proteinase inhibitors and digested with Pronase at 37°C for 48 h. The digested samples were applied to a column (1.0 × 110 cm) of Sephadex G-50 equilibrated with 0.1 M NH_4CO_3. High molecular weight glycopeptides were pooled, desalted, and subjected to β-elimination in 0.05 M NaOH, 1 M $NaBH_4$ containing [^{3}H]-$NaBH_4$ at 45°C for 16 h. After β-elimination, the released *O*-glycans were isolated by G-50 (1.0 × 110 cm) gel filtration and desialylated with mild acid hydrolysis (2 M acetic acid, 100°C, 1 h). The desialylated *O*-glycans were applied to a column (1.0 × 110 cm) of Bio-Gel P-4 (BIO-RAD, Hercules, CA, USA) equilibrated with 0.1 M NH_4CO_3. The amount of radioactivity in aliquots of the fractions was determined by scintillation counting.

Tumour formation in mice

Bladder tumour cells (5×10^6 cells for KK-47 and KK47-C2 cells; 1×10^7 cells for YTS and YTSC2KD cells) were suspended in 100 μl of serum-free RPMI-1640 medium and injected into the tail vein of 6- to 8-week-old mice. Three kinds of mouse, Balb/c nude (Clea Japan, Tokyo, Japan), SCID/beige (Charles River, Japan, Yokohama, Japan), and NK cell-depleted Balb/c nude mouse were used for tumour formation. To deplete NK cells, Balb/c nude mice were injected intraperitoneally with 25 μl of anti-asialo GM1 (Wako, Japan) (Brunda *et al*, 1993). To neutralize NKG2D in mice, Balb/c nude mice were injected intraperitoneally with 200 μg of anti-mouse NKG2D (CX5) (Ogasawara *et al*, 2004). Antibody injection was performed 1 day before tumour injection and every 3 days during experiments. After 3 weeks, lungs were harvested and fixed with Bouin's solution. Number of tumour foci was counted under a dissecting microscope or the weights of lungs were measured for evaluation of tumour formation. The committee for animal experiments of Oyokyo Kidney Research Institute approved these experiments.

Cytotoxicity assay

Human primary NK cells were purified from human peripheral blood mononuclear cells with NK cell isolation kit (Myltenyi Biotech). Mouse primary NK cells were purified from C57BL/6 mouse spleens with mouse NK cell isolation kit (Myltenyi Biotech). Cytotoxicity was measured by using the Cytotox 96 Non-radioactive Cyotoxicity Assay Kit (Promega, Madison, MI, USA). NK cells (1×10^6 cells/ml) were cultured for 3 days in RPMI-1640 medium supplemented with 10% FBS in the presence of 1000 units/ml of human recombinant IL-2 (Wako, Osaka, Japan) or mouse recombinant IL-2 (Myltenyi Biotech) (Takeda *et al*, 2000). Target cells (tumour cells) were incubated with IL-2-activated NK cells for 4 h at 37°C. Cytotoxicity against KK-47 cells by resting cells at effector:-target ratio of 5:1 is 1.1 ± 0.9% (human NK) or 2.0 ± 1.8% (mouse NK). The release of lactate dehydrogenase from lysed target cells was measured. All assays were performed in triplicate. Percent cytotoxicity = (experimental lactate dehydrogenase release−effector spontaneous release−target spontaneous release)/(target maximum release−target spontaneous release) × 100.

Western blotting

Total lysates of tumour cells and specimens were prepared by solubilization in 50 mM Tris–HCl buffer, pH 7.5, containing 1% Igepal CA-630, 150 mM NaCl, and proteinase inhibitors. The lysates were resolved by SDS–PAGE on an 8–16% gradient gel (Invitrogen), and transferred onto PVDF membrane. Western blotting analyses were performed using specific primary antibodies and a horseradish peroxidase-conjugated secondary antibody. Signals were visualized using the ECL + PLUS detection system (GE Healthcare, UK).

Flow cytometry

Bladder tumour cells and NK cells (1×10^6 cells) were incubated with antibodies or cell-surface receptor-IgG chimeras for 20 min at 4°C followed by the incubation with Alexa Fluor 488-labelled secondary antibodies (Invitrogen). NK cells were incubated with FcR Blocking Reagent (Myltenyi Biotech) for 10 min at 4°C before the incubation with antibodies and IgG chimeras. Cells were analysed on a FACSCanto II (BD Biosciences) and Cytomics FC500 (Beckman Coulter).

Immunoprecipitation

For lectin immunoprecipitation, lysates from patients' specimens and bladder tumour cells were incubated with LEL-agarose (Vector Laboratories, Burlingame, CA, USA). For immunoprecipitation of MICA, cell-surface proteins were biotinylated and then crosslinked using the homobifunctional crosslinker DTSSP (Pierce Biotechnology Inc.) (Partridge *et al*, 2004). Lysates were incubated with 2 μg/ml anti-MICA monoclonal antibody (clone BAMO3) and then incubated with anti-mouse IgG agarose. The resin binding the immune complex was eluted with 1× Laemmli's SDS–PAGE sample buffer.

Binding of MICA to NKG2D

To prepare MICA-IgG fusion proteins (MICA-IgG), a cDNA encoding the extracellular domain of MICA (Bahram *et al*, 1994) and the human IgG1 hinge plus constant region fragment (Tsuboi and Fukuda, 1997) were ligated into pcDNA3.1 to yield pcDNA3-MICA-IgG. Two days after transfection of KK-47 and KK-47-C2 with pcDNA3-MICA-IgG, the conditioned media were recovered. By using Protein A Sepharose column (Pierce Biotechnology Inc.), MICA-IgG and MICAC2-IgG were purified from the conditioned media of KK-47 and KK-47-C2 transfectants, respectively (Supplementary Figure S14A). Preparation of control IgG chimera (CD43-IgG) was previously described. CD43 is a T-cell marker, mucin-type protein which has no specific ligands (Sawada *et al*, 1994). To analyse binding of MICA to NKG2D, microtitre plates were coated with recombinant human NKG2D-Fc chimera (R&D Systems) at 10 μg/ml. Purified MICA-IgG was added to the microtitre plates and binding of MICA-IgG to NKG2D was measured with anti-MICA monoclonal antibody (clone BAMO3), anti-mouse IgG conjugated to horseradish-peroxidase and substrate solution (R&D Systems) using an ELISA microplate reader, EIA READER Model 2550 (BIO-RAD Laboratories).

INF-γ and granzyme B secretion

Microtitre plates were coated with IgG chimeras at 10 μg/ml. Human NK cells were stimulated with IgG chimera-coated plates in the presence of FcR Blocking Reagent (Myltenyi Biotech). Granzyme B in the supernatant was assayed by measuring serine proteinase activity with a colorimetric peptide substrate, IEPD-p-nitroanilide (BIOMOL International, Plymouth Meeting, PA, USA) after 5 h stimulation at 37°C. Specific granzyme B secretion was expressed as a percentage of the total cellular enzyme activity after subtracting the spontaneous release for each stimulation with IgG chimera. INF-γ secretion was measured in supernatant by ELISA using DuoSet Human INF-γ kit (R&D Systems) after 18 h stimulation at 37°C.

Statistical analysis

We used the statistical program SPSS 12.0 (SPSS, Chicago, IL, USA). Statistically significant differences were determined using the Student's *t*-test. Differences were considered significant, if $P < 0.05$.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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