Obstructing Shedding of the Immunostimulatory MHC Class I Chain–Related Gene B Prevents Tumor Formation

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Abstract

Purpose: Clinical observations have suggested that shedding of the MHC class I chain–related molecule (MIC) may be one of the mechanisms by which tumors evade host immunosurveillance and progress. However, this hypothesis has never been proven. In this study, we tested this hypothesis using a prostate tumor model and investigated the effect of shedding of MIC on tumor development.

Experimental Design: We generated a shedding-resistant noncleavable form of MICB (MICB.A2). We overexpressed MICB.A2, the wild-type MICB, and the recombinant soluble MICB (rsMICB) in mouse prostate tumor TRAMP-C2 (TC2) cells and implanted these cells into severe combined immunodeficient mice.

Results: No tumors were developed in animals that were implanted with TC2-MICB.A2 cells, whereas all the animals that were implanted with TC2, TC2-MICB, or TC2-rsMICB cells developed tumors. When a NKG2D-specific antibody CX5 or purified rsMICB was administered to animals before tumor implantation, all animals that were implanted with TC2-MICB.A2 cells developed tumors. In vitro cytotoxicity assay revealed the loss of NKG2D–mediated natural killer cell function in these prechallenged animals, suggesting that persistent levels of soluble MICB in the serum can impair natural killer cell function and thus allow tumor growth.

Conclusions: These data suggest that MIC shedding may contribute significantly to tumor formation by transformed cells and that inhibition of MIC shedding to sustain the NKG2D receptor–MIC ligand recognition may have potential clinical implication in targeted cancer treatment.

Expression of murine NKG2D ligands on tumor cells has been shown to be effective in activating natural killer (NK)-mediated tumor elimination experimentally (1–4). In murine systems, identified NKG2D ligands include the retinoic acid early inducible family of proteins RAE-1 (1, 2), the minor histocompatibility antigen H60 (1, 2), and the murine ULBP-like transcript 1 (4, 5). Cells expressing these molecules are sensitive to the cytotoxicity of mouse NK cells. Ectopic expression of RAE-1 and H60 results in rejection of tumor cell lines expressing normal levels of MHC I molecule (2–4). Immunodepletion and other experiments showed that the tumor rejection is due to NK and CD8 T cells (2, 3). NKG2D neutralization in vivo enhances host sensitivity to carcinogen-induced spontaneous tumor initiation (6). These studies have proven the principle function of the NKG2D ligand receptor-mediated NK cell immunity in tumor rejection.

In humans, the MHC class I chain–related molecule MICA and MICB (generally termed as MIC) are the most investigated NKG2D ligands, which were proposed to play roles in tumor rejection (7–9). MIC is rarely expressed by normal human tissues but induced in most human epithelial tumors (10–13). Expression of MIC on the tumor cell surface can markedly enhance the sensitivity of tumor cells to NK cells in vitro and has been shown to inhibit the growth of human gliomas or small lung carcinomas in experimental models (14, 15). These studies suggested that NK cells can potentially eliminate MIC-positive tumor cells in cancer patients. However, as clinically observed, most of the human epithelial tumors are found to be MIC-positive rather than MIC-negative (10–13), which suggests the functional compromise of the MIC ligand–NKG2D receptor system in cancer patients to permit the growth of MIC-positive tumor cells. We and others have shown that tumor-derived soluble MIC as a result of tumor shedding is one of the factors causing the ineffectiveness of NKG2D-mediated immunity in cancer patients (13, 16–21). In vitro studies have shown that engagement of soluble MICA to NKG2D results in marked reduction in surface NKG2D expression on NK and T cells (13, 16, 21). Thus, soluble MIC is believed to induce down-modulation of NKG2D expression on systemic and tumor-infiltrated NK and T cells and thus result in functional impairment of NK and T cells in MIC-positive cancer patients (13, 16, 17). Reduction in the density of MIC expressed on the tumor cell surface due to MIC shedding from tumors is also proposed to be one of the mechanisms for tumor evasion (21).
Shedding of MIC in Tumor Formation

Translational Relevance

In humans, the MHC class I chain-related molecules MICA and MICB (generally termed MIC) are frequently found expressed on epithelial-originated tumor cells. MIC is a ligand for the activating NK cell receptor NKG2D. Engagement of tumor-expressed MIC to NKG2D can activate NK cell tumor-lytic activity in vitro. Thus, expression of MIC on tumor cells is proposed to play a significant role in tumor immunosurveillance. However, as observed in many cancer patients, majority of the tumors remain MIC positive, suggesting the ineptness of the NKG2D-mediated NK cell function. We and others have shown that shedding of MIC by tumor cells can impair NK cell function in cancer patients. These clinical studies prompted the hypothesis that tumor shedding of MIC may be one of the mechanisms by which MIC-positive tumors evade NK cell immunosurveillance and progress in cancer patients. However, it is impossible to test this hypothesis clinically. Taking the advantage that human MICB can be recognized by mouse NKG2D (22, 23) and that only the extracellular α1α2 domain of MIC interacts with NKG2D (24–26), here we test the hypothesis experimentally that shedding of MIC permits tumor growth and that sustained interaction between NKG2D and membrane-integrated form of MIC can cause tumor rejection. Using a well-characterized prostate tumor model TRAMP-C2 (TC2; ref. 27), we show for the first time that expression of the shedding-resistant but not the natural form of MICB may contribute significantly to tumor formation by transformed cells and thus inhibiting tumor shedding of MIC may have potential therapeutic implication in targeted cancer therapy.

These compelling clinical data suggest that MIC shedding from tumor cells is likely associated with tumor progression, which has prompted the hypothesis that tumor shedding of MIC is the mechanism by which MIC-positive tumors evade NK cell immunosurveillance and progress in cancer patients. However, it is impossible to test this hypothesis clinically. Taking the advantage that human MICB can be recognized by mouse NKG2D (22, 23) and that only the extracellular α1α2 domain of MIC interacts with NKG2D (24–26), here we test the hypothesis experimentally that shedding of MIC permits tumor growth and that sustained interaction between NKG2D and membrane-integrated form of MIC can cause tumor rejection. Using a well-characterized prostate tumor model TRAMP-C2 (TC2; ref. 27), we show for the first time that expression of the shedding-resistant but not the natural form of MICB prevents tumor formation by transformed cells.

Materials and Methods

Cells. TC2 cell line (gift of Dr. N.M. Greenberg, Fred Hutchinson Cancer Research Center) was maintained in DMEM as described (27). RMA-Rae-1β cells (gift of Dr. D. Raulet, Berkeley) was maintained in RPMI 1640 supplemented with 10% FCS. Eco-phoenix cells (Orbigen) were maintained in DMEM supplemented with 10% fetal bovine serum.

DNA construction, transfection, and transduction. cDNA encoding full-length human MICB (allele 0101; ref. 28) was kindly provided by Dr. A. Seinle (University of Tubingen) and subcloned into the retroviral vector pBMINZ-IRES-GFP (Orbigen). To generate recombinant soluble MICB (rsMICB), cDNA encoding the extracellular domain of MICB was amplified by PCR. To generate a putative shedding-resistant form of MICB (designated as MICB.A2), amino acids 215 to 274 of MICB were replaced with the comparable sequence of the α3 domain of HLA-A2 using recombinant PCR of the cDNA sequences (29). rsMICB-FLAG fusion peptide was generated by tagging the cDNA sequence of FLAG (DYKDDDK) to the C-terminus of the rsMICB cDNA using PCR. Error-free amplified cDNAs were identified by sequencing and subcloned into the retroviral vector pBMINZ-IRES-GFP (Orbigen). Plasmids were transfected into Eco-phoenix packaging cells to generate retrovirus. TC2 cells were transduced with respective retroviruses. Stable GFP-positive cell population was isolated by drug selection and sorted by flow cytometry.

Affinity purification of rsMICB and rsMICB-FLAG peptides. The HiTrip NHS-activated column (GE Healthcare) was conjugated with the monoclonal antibody (mAb) 6D4.6 (Santa Cruz Biotechnology) before loading with conditioned medium from TC2-rsMICB or TC2-rsMICB-FLAG cells. After washing, rsMICB or rsMICB-FLAG was eluted with 100 mmol/L sodium citrate (pH 2.5) and neutralized immediately with 1.5 mol/L Tris (pH 8.8). Immunoprecipitation and Western blotting. Supernatant was collected from TC2-rsMICB cell culture and passed through a 0.45 μm filter to remove cell debris. Cells were washed and lysed with lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 1% Triton X-100]. Clear supernatant and lysates were incubated with the mAb 6D4.6. Immunocomplexes were collected using protein A/G-agarose (Pierce). PNGase F (New England Biolabs) treatment was carried overnight at 37°C. Immunocomplexes were separated on a 4% to 15% SDS-PAGE, blotted onto nitrocellulose membrane, and probed with goat anti-MICB antibody AF1599 (R&D Systems). Immunoreactive proteins were detected by incubating the blot with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reagents (Pharmacia).

MICB shedding assay and (rs)MICB ELISA. Cells were seeded at the density of 4 × 10^5 per well in a 6-well plate in complete medium overnight and replaced with 1 mL/well serum-free medium for 6 h. Supernatant was collected and filtered through 0.45 μm filter. Cells were lysed with 1 mL lysis buffer. Amount of soluble MICB in the supernatant and MICB in the cell lysates was measured using human MICB DuoSet sandwich ELISA kit (R&D Systems). For measuring mouse serum levels of soluble MICB, serum was diluted 1:2 with PBS for ELISA assay.

In vivo study. Animal studies were approved by the Institutional Animal Care and Use Committee. Six to 10 severe combined immunodeficient (SCID) male mice (6-week old Harlan-Sprague-Dawley) were used in each group. The following cells (1 × 10^6 per mouse) were subcutaneously injected into respective group of animals: TC2, TC2-MICB, TC2-MICB.A2, and TC2-rsMICB. All animals were monitored for tumor growth for up to 12 weeks. Tumor volume was estimated using the formula: V = L × W^2 / 2. Animals were euthanized when tumor volumes reached 1,000 mm^3. Tumors, spleens, and peripheral blood were terminally collected. Serum was separated by centrifugation and used for rsMICB ELISA.

In vivo NKG2D blocking or neutralization. To block NKG2D receptor, 100 μg of the functional grade of anti-NKG2D blocking antibody CX5 (eBiosciences) were injected intraperitoneally on the day before and the day after tumor implantation and thereafter every 3 days. Blocking was confirmed by flow cytometry of peripheral lymphocytes collected from orbital sinus bleeding with PE-conjugated CX5 (eBiosciences). To modify NKG2D function, animals were injected intraperitoneally with 50 ng purified rsMICB before implantation of TC2-MICB.A2 cells and thereafter twice a week for 4 weeks. Blood was collected once a week from sinus orbital bleeding and serum levels of rsMICB were measured by ELISA.

Flow cytometry. For detection of cell surface expression of NKG2D ligands, TC2 and its derivative cells were trypsinized, blocked with anti-mouse CD16/CD32 (eBiosciences), and incubated with anti-MICA/B mAb 6D4.6 or anti-MICB MAB1599 (R&D Systems) or anti-pan-RAE-1 mAb17582 (R&D Systems) followed by a PE-conjugated secondary
reagent. For detection of rsMICB expression, the BD Cytofix/Cytoperm kit (BD Sciences) was used. Briefly, TC2-rsMICB cells were cultured in the presence of BD GolgiPlug for 3 h to prevent the secretion of rsMICB before harvesting. Cells were resuspended in BD fixation/permeabilization solution for 20 min at 4°C and incubated with 6D4.6 followed with PE-conjugated secondary reagents. For mouse NKG2D binding assay, cells were incubated with 10 μg/mL of the fusion protein of recombinant soluble mouse NKG2D and human Fc (smNKG2D-Fc; R&D Systems) followed by PE-conjugated F(ab')2, goat anti-human IgG. For H-2Kb expression, cells were incubated with Alexa47-dextran anti-H-2Kb/D7 mAb (Biolegend).

Single-cell suspensions of splenocytes were prepared as described (30). Cells were stained with FITC-conjugated mAb DX5 (eBiosciences) and PE-conjugated anti-mouse NKG2D mAb CX5 (eBiosciences) or A10 (eBiosciences) and analyzed using a BD FACScan or LSRII. For ex vivo rsMICB competitive binding assay, freshly isolated splenocytes were incubated with 10 ng/mL nsmICB-FcFLAG followed with FITC-conjugated mAb M2 (Sigma-Aldrich) and PE-conjugated mAb DX5 (eBiosciences). Data were analyzed using the BD CellQuest pro (BD Biosciences) or FlowJo software (Tree Star).

**Cytotoxicity assay.** Fresh NK cells were prepared using SpinSep murine NK enrichment cocktail (STEM Cell Technology) and were >90% DX5+. LAK cells were prepared by culturing NK cells for 4 to 7 days in 1,000 units/mL recombinant human interleukin-2. Cytotoxicity was done in triplicates using the standard 4 h ³¹Cr release assay (13). Antibody blocking was done by preincubating effector cells with 30 μg/mL NKG2D blocking mAb CX5 (eBiosciences) or preincubating target cells with 100 μg/mL anti-pan RAE-1 polyclonal antibody at 37°C for 1 h (31).

**Statistical analysis.** Data were analyzed using JMP software. Significance between two animal groups was determined by Student's t test. P < 0.05 was considered significant.

**Results**

**Putative cleavage region of MIC(B) in TC2 tumor cells.** TC2 is a mouse prostate tumor cell line generated from the TRAMP mouse (27), which does not express any homologous molecules to human MIC (28). TC2 cells were transduced with retroviruses that carry cDNAs of human MICB and GFP. Transduced cells stably expressing high levels of MICB (designated as TC2-MICB cells) were generated by puromycin selection and multiple rounds of flow cytometry cell sorting for GFP-positive cells.

To generate a shedding-resistant form of MICB, we first performed experiments to predict putative cleavage region of MICB by tumor cells. Soluble MICB resulted from TC2 shedding (designated as ssMICB) was immunoprecipitated from supernatant of TC2-MICB cells with a mouse mAb 6D4.6 specific to the α1α2 ectodomain of MICA/B (10). The full-length MICB was immunoprecipitated from cell lysates with the same antibody. Immunocomplexes were separated and immunoblotted with a goat polyclonal antibody AF1599 specific to the ectodomain of MICB. After N-glycosidase (PNGase F) treatment, ssMICB yield two bands of molecular mass ~31 to 33 kDa (Fig. 1A). The molecular mass is consistent with other studies showing soluble MICB and soluble MICA released by human tumor cells (32, 33). When samples were treated with dithiotreitolcyclobenzene, a disulfide isomerase inhibitor, only a single band of soluble MICB was revealed (data not shown), suggesting that the two bands of soluble MICB released by TC2 cells are the reduced and nonreduced forms of ssMICB. Similar observation of soluble MICA shed by human tumor cell lines was shown by Kaiser et al. (33). The deglycosylated full-length MICB is shown to be ~41 kDa in the cell lysates (Fig. 1A), consistent with other studies (34). Although the precise cleavage site cannot be determined, these data suggest that MICB was cleaved at the α3 domain proximal to the transmembrane region to generate ssMICB (Fig. 1B). Similar cleavage region is also predicted for human tumor cell lines to generate soluble MICA (33).

**Generation of tumor cell lines expressing the putative shedding-resistant MICB A2 and rsMICB.** To study the effect of MIC shedding on tumor formation and growth in vivo, we generated two forms of MICB, the recombinant secretable form of MICB (rsMICB) and a putative shedding-resistant form of MICB (MICB A2). MICB was generated by deletion of the transmembrane and cytoplasmic domains. MICB A2 was generated by replacing part of the α3 domain of MICB (amino acids 215-274) with the corresponding residues from HLA-A2 (Fig. 2A). Because NKG2D only interacts with the α1α2 domain of MIC (24), MICB A2 would presumably continue to recognize NKG2D. rsMICB and MICB A2 were overexpressed in TC2 cells using the GFP retroviral system described above. Positive-expressing clones were selected by puromycin and repeated sorting by flow cytometry for GFP-positive cells. The expression level of cellular ssMICB and surface MICB A2 in TC2 cells was confirmed by flow cytometry with the anti-MIC mAb 6D4.6 (Fig. 2B).

**Partial replacing the α3 domain of MICB protects from tumor cell shedding.** An ELISA assay was used to assess the degree of shedding ofMICB and MICB A2 in TC2 cell lines. Both the capture and the detection antibodies are specific to the extracellular domain of MICB and can also detect MICB A2 by Western blotting (data not shown). With a given number of cells, the amount of cleaved soluble MIC in the culture supernatant and the amount of MIC in the lysates were
measured by a sandwich ELISA assay (Fig. 2C). The degree of MIC shedding was estimated by the molar percentage of soluble MICB released into the supernatant. Approximately 30% of MICB was cleaved into the medium in TC2 cells, whereas no cleaved form of MICB.A2 was detectable in the culture supernatant (Fig. 2C). This indicates that MICB.A2 cannot be cleaved into soluble forms by tumor cells and is shedding-resistant.

Expression of MICB.A2 in TC2 cells stimulates mouse NK cell cytolytic activity. Human MICB can be recognized by mouse NKG2D and activate mouse NK cells (22, 23). To test whether overexpressing MICB.A2 can also activate mouse NK cells, we first addressed the physical interaction of MICB.A2 with soluble mouse NKG2D-Fc by flow cytometry analyses. As measured by mean fluorescence intensity (Fig. 3A), smNKG2D-Fc was more prominently bound by TC2-MICB and TC2-MICB.A2 cells and only weakly bound by TC2 and TC2-rsMICB cells. Accordingly, in vitro cytotoxicity assay revealed marked increase in sensitivity of TC2 cells to interleukin-2-activated mouse NK (LAK) cells when MICB or MICB.A2 was overexpressed (Fig. 3B; P < 0.01). The increased susceptibility of TC2-MICB and TC2-MICB.A2 cells to LAK cells can be inhibited by preincubation of LAK cells with the NKG2D-specific blocking antibody CX5 (ref. 35; Fig. 3B), suggesting a NKG2D-dependent LAK cell-killing effect. Thus, the shedding-resistant MICB.A2 maintained the functional property of MICB to be recognized by mouse NKG2D.

Although not expressing any MIC homologue, TC2 cells express some levels of endogenous NKG2D ligand RAE-1 variants but not H60 (22, 36). However, the level of endogenous NKG2D ligands is not sufficient to stimulate LAK cell in vitro cytotoxicity (Fig. 3B). To address whether the increased

**Fig. 2.** Construction and expression of the shedding-resistant noncleavable and soluble recombinant forms of MICB (rsMICB) in TC2 cell lines. **A,** generation of the noncleavable form MICB.A2 by replacing amino acids 215 to 274 of the MICB α3 domain with the corresponding sequence of HLA-A2. rsMICB was generated by deletion of the entire transmembrane and cytoplasmic region of MICB. **B,** flow cytometry showing expression levels of MICB, MICB.A2, and rsMICB in TC2 cell lines. cDNAs of MICB, MICB.A2, or rsMICB were inserted into a IRES-GFP retroviral vector pBMNZ. TC2 cells were transduced with respective retrovirus. GFP-positive cells were sorted by flow cytometry. For detection of MICB and MICB.A2 expression, cells were directly incubated with anti-MIC 6D4.6 antibody followed a PE-conjugated secondary reagent. For detection of the secretable rsMICB expression, TC2-rsMICB cells were cultured in the presence of BD GolgiPlug for 3 h to prevent the secretion of rsMICB before harvesting. Cells were resuspended in BD Fixation/Permeabilization solution for 20 min at 4 °C and incubated with 6D4.6 followed with a PE-conjugated secondary reagent. C, MICB.A2 is shedding-resistant. Top, amount of shed soluble MICB in the culture supernatant and MICB in the cell lysates. Cells (4 × 10⁵ per well) were plated on a 6-well plate overnight. Medium was removed and replaced with 1 mL serum-free medium. Six hours later, medium was collected and filtered. Cells were lysed with 1 mL lysis buffer, and 50 μL culture supernatant and cell lysates were used for (s)MICB ELISA assay. Bars, SE. Bottom, degree of shedding as calculated by molar of soluble MICB in the supernatant versus total molar of soluble MICB and MICB (a sum of supernatant and cell lysates). Final results were normalized by cell numbers at the time of the assay. Results of three independent experiments. *, P < 0.001.
sensitivity of TC2-MICB and TC2-MICB.A2 cells to LAK cell killing is possibly due to increased expression of RAE-1, we analyzed endogenous RAE-1 expression on these cell lines by flow cytometry with a rat anti-pan RAE-1 mAb. A consistency of RAE-1 expression among TC2, TC2-MICB, and TC2-MICB.A2 cell lines is shown in Fig. 3C. Furthermore, preincubation of target cells with an anti-pan RAE-1 blocking antibody (30) did not significantly reduce the susceptibility of TC2-MICB or TC2-MICB.A2 cells to LAK cells, whereas the sensitivity of the control RMA-Rae-1.5 cells to LAK cells was significantly reduced (Fig. 3B). These suggest that the increased killing of TC2-MICB or TC2-MICB.A2 cells by LAK cells is not due to increased RAE-1 expression.

TC2 cells express a very low level of H-2K\textsuperscript{b}/Db (37), which is a potential ligand for inhibitory Ly49 receptor families. We analyzed H-2K\textsuperscript{b}/Db expression on these cell lines by flow cytometry. Consistent levels of H-2K\textsuperscript{b}/Db expression were found in TC2 and cell lines expressing MICB or MICB.A2 (Fig. 3C), suggesting that the increased sensitivity of TC2-MICB and TC2-MICB.A2 cells to LAK cells was not attributed to a reduced level of H-2K\textsuperscript{b}/Db expression.

Shedding-resistant MICB.A2 but not the natural MICB prevents TC2 tumor formation in vivo. In three independent experiments, when SCID animals were implanted with TC2-rsMICB, TC2-MICB, or TC2-MICB.A2 cells, none of animals that were implanted with the TC2-MICB.A2 cells developed tumors with a 12-week follow-up observation period, whereas all the animals that were implanted with TC2-rsMICB or TC2-MICB cells developed tumors within 3 weeks (Fig. 4A and B). In addition, no significant difference in tumor growth was observed among TC2, TC2-rsMICB, and TC2-MICB originated tumors (Fig. 4A). To address whether the failure to reject TC2-MICB tumors is due to the large dose ($1 \times 10^8$) of tumor cells injected, we repeated the experiment with TC2-MICB and TC2-MICB.A2 cells using smaller numbers of inoculated cells. A 10-fold ($1 \times 10^5$) and a 100-fold ($1 \times 10^4$) decrease in the number of inoculated tumor cells did not change the outcome (Fig. 4C). We also examined MICB expression in the TC2-MICB-originated tumor cells extracted from SCID animals by flow cytometry. All the extracted tumor cells expressed the similar levels of MICB before implantation (Fig. 4D). This suggests that tumor growth in animals that were implanted with TC2-MICB cells is not due to NK cells selectively eliminating MICB-positive cells.

Shedding of MICB by TC2 cells allows TC2-MICB tumor growth in mice. In 4 h in vitro cytotoxicity assays, both TC2-MICB and TC2-MICB.A2 cells were sensitive to LAK cells (Fig. 3B). However, NK tumor immunity was effective only in animals when the noncleavable MICB.A2 was expressed on tumor cells. We propose that the discrepancy of in vivo and in vitro observation is attributed to tumor cell shedding of MICB in vivo, which accumulatively compromises NK cell function in animals implanted with MICB-expressing tumor cells. To test this hypothesis, we measured serum levels of soluble MICB in all the animals 4 weeks after tumor implantation using a sandwich ELISA assay. A significant level of soluble MICB was detected in the sera of animals that were implanted with tumor cells expressing rsMICB and MICB, whereas no soluble MICB was detectable in animals implanted with tumor cells expressing MICB.A2 (Fig. 5A). To address why TC2-MICB cells were sensitive to LAK cell in vitro, LAK cells were incubated with the supernatant of TC2-MICB cells for various periods and used as effector cells to kill target TC2-MICB.A2 cells. Only after 8 h incubation, LAK cell-killing ability was significantly affected. Therefore, in the 4 h in vitro cytotoxicity assay, the killing ability of LAK cells was not significantly affected by soluble MICB.
resulted from target TC2-MICB cells (data not shown). We further examined NK cell tumor-killing ability from these animals. For this purpose, freshly isolated splenic NK cells were used as effector cells for in vitro cytotoxicity assay. NK cells from mice bearing MICB- and rsMICB-expressing tumors had a significant reduction in cytotoxicity against TC2-MICB.A2 target cells in comparison with those from TC2 tumor-bearing or tumor-free animals ($P < 0.01$; Fig. 5B). The cytotoxicity of these NK cells was inhibited by preincubating with a NKG2D-specific inhibitory mAb CX5 (Fig. 5B), suggesting a NKG2D-dependent effect. Together, these results suggest that persistent presence of soluble MICB in vivo due to tumor cell shedding of MICB compromised NKG2D-mediated NK cell lytic activity and thus permitted the growth of MICB-expressing tumor cells. Persistent presence of soluble MICB blocks the NKG2D-mediated NK cell recognition of target cells. When animals were treated with the CX5 antibody to block NKG2D receptor, injection of TC2-MICB.A2 cells gave rise to tumor formation in all the SCID animals (Fig. 6A). This suggests that the inhibition of TC2-MICB.A2 tumor formation in SCID animals is NKG2D-dependent. To test the effect of presence of soluble MICB on tumor formation of MICB.A2-expressing cells, we injected animals with purified rsMICB (50 ng) before and after implanting TC2-MICB.A2 cells. Under this experimental

![Fig. 4](image_url)

Expression of MICB.A2 but not the cleavable MICB prevents tumor formation in vivo. Six animals were used in each group. Tumor growth was monitored twice weekly. Tumor volume was estimated by the formula: $V = L^2 \times W / 2$. A, tumor growth of various MICB-expressing TC2 cells in SCID mice. B, rate of tumor formation of various MICB-expressing TC2 cells in SCID mice. Cells ($1 \times 10^6$) were injected subcutaneously into each animal in A and B. C, tumor growth of TC2-MICB cells when injected at lower doses ($1 \times 10^5$ and $1 \times 10^6$ cells per animal). D, representative flow cytometry histograms showing MICB expression in tumor cells extracted from animals inoculated with TC2-MICB cells compared with prior inoculation. The MICB-specific antibody MAB1599 (R&D Systems) was used as primary antibody. Filled histogram, MICB expression in TC2 cells; open histogram, MICB expression in TC2-MICB cells or tumor cells. Results of three independent experiments.
condition, implantation of TC2-MICB.A2 cells gave 100% tumor formation (Fig. 6A). Tumor cells extracted from these animals were shown to be GFP-positive and express MICB.A2 by flow cytometry analyses (data not shown). NK cells isolated from these animals showed very little cytolytic activity against TC2-MICB.A2 target cells (data not shown). These data suggest that persistent presence of soluble MICB compromises the cytotoxicity of NK cells against TC2-MICB.A2 cells.

We sought the mechanisms by which tumor shedding-derived soluble MICB would diminish NK cell activity. Soluble MICB may down-modulate surface NKG2D expression on NK cells (16) or block the recognition of NK cells to target cells by physical occupancy of the NKG2D receptor. To distinguish these two mechanisms, we first analyzed NKG2D expression on splenic NK cells freshly isolated from animals injected with various TC2 tumor cells using flow cytometry analyses with a nonblocking NKG2D antibody A10 (29). There was no significant difference in surface NKG2D expression on NK cells from mice bearing TC2-MICB and TC2-rsMICB tumors compared with those from animals bearing TC2 tumors or tumor-free animals (Fig. 6B), suggesting that the suppressive effect of soluble MICB on NK cell activity was not through down-modulation of surface NKG2D receptor. We further examined the occupancy of NKG2D receptor on NK cells by tumor-derived soluble MICB using competitive binding assay. Freshly isolated splenocytes were incubated with purified rsMICB-FLAG, and NK cell-binding ability to rsMICB-FLAG was measured by flow cytometry using the anti-FLAG mAb M2.

The shedding of MICB can lower NK cell activity. This study has provided conclusive evidence supporting the hypothesis that shedding of MIC by transformed cells can promote tumor growth. In this study, we generated a shedding-resistant NKG2D ligand MICB.A2 by partially modifying the a3 domain of MICB and showed that overexpressing MICB.A2 prevented tumor formation by the mouse prostate tumor cell line TC2. We also showed that, when soluble MICB was persistently present, expression of the shedding-resistant MICB.A2 on the tumor cell surface did not prevent or delay tumor formation in vivo. Our study signifies the effect of MIC shedding on tumor formation and the magnitude of sustained MIC-induced NKG2D immunity in preventing early tumor development.

Although the mechanisms of MIC shedding is still under investigation (19, 33, 38), clinical evidence has shown that shedding of MIC is common in MIC-positive cancers, such as prostate, colon, breast adenocarcinomas, and melanomas (10–13). In these patients, the function of NK and/or CD8 T cells was compromised due to soluble MIC-induced internalization of the NKG2D receptor (13, 16–18). Thus, it was hypothesized that MIC shedding in tumors can promote tumor immune evasion and progress to advanced disease. Recent studies have shown that MIC expression is not restricted in tumor cells and that MIC can be induced in cells in response to DNA damage (39), a prior event to transformation. Therefore, the current study indicates that, in the event of malignant transformation, inhibiting shedding of MIC from MIC-positive transformed cells can prevent the initiation of tumor formation.

We chose to overexpress human MICB rather than mouse NKG2D ligands in this study for the following rationales. Firstly, MIC has been shown to be shed by tumor cells in cancer patients; thus, the study is clinically relevant. Secondly, MICB has been shown to interact with mouse NKG2D, and MICB-positive cells are sensitive to mouse NK cells (22, 23). We also have shown that MICB was shed by the mouse prostate cell line TC2 in the same pattern as MIC shedding in prostate cancer patients. Lastly, although mouse NKG2D ligands are functionally similar to human MIC in NK cell activation, these molecules are structurally different and may have different physiologic roles. Mouse NKG2D ligands lack the a3 domain and are mostly GPI-linked proteins (9); in addition, little is known about what controls the expression of mouse NKG2D ligands in vivo and whether they would shed in a similar fashion to MIC in human tumor cells. Lastly, different from human NKG2D ligands, studies have shown that naturally expressed mouse NKG2D ligands on tumor cells may not cause tumor rejection largely due to insufficient levels of the ligand expression (2) or low affinity of binding to NKG2D (40). In

Fig. 5. Shedding of MICB by TC2-MICB cells compromises NK cell activity in vivo. A, serum levels of soluble MICB in all the tumor-bearing animals. B, reduced NKG2D-dependent NK cell cytotoxicity of splenic NK cells from animals bearing TC2-rsMICB and TC2-MICB tumors. Freshly isolated NK cells were used as effectors; TC2-MICB.A2 cells were used as target cells. **, P < 0.01, compared with TC2 or TC2-MICB.A2. Results of three independent experiments.

Discussion

This study has provided conclusive evidence supporting the hypothesis that shedding of MIC by transformed cells can promote tumor growth. In this study, we generated a shedding-resistant NKG2D ligand MICB.A2 by partially modifying the a3 domain of MICB and showed that overexpressing MICB.A2 prevented tumor formation by the mouse prostate tumor cell line TC2. We also showed that, when soluble MICB was persistently present, expression of the shedding-resistant MICB.A2 on the tumor cell surface did not prevent or delay tumor formation in vivo. Our study signifies the effect of MIC shedding on tumor formation and the magnitude of sustained MIC-induced NKG2D immunity in preventing early tumor development.

Although the mechanisms of MIC shedding is still under investigation (19, 33, 38), clinical evidence has shown that shedding of MIC is common in MIC-positive cancers, such as prostate, colon, breast adenocarcinomas, and melanomas (10–13). In these patients, the function of NK and/or CD8 T cells was compromised due to soluble MIC-induced internalization of the NKG2D receptor (13, 16–18). Thus, it was hypothesized that MIC shedding in tumors can promote tumor immune evasion and progress to advanced disease. Recent studies have shown that MIC expression is not restricted in tumor cells and that MIC can be induced in cells in response to DNA damage (39), a prior event to transformation. Therefore, the current study indicates that, in the event of malignant transformation, inhibiting shedding of MIC from MIC-positive transformed cells can prevent the initiation of tumor formation.

We chose to overexpress human MICB rather than mouse NKG2D ligands in this study for the following rationales. Firstly, MIC has been shown to be shed by tumor cells in cancer patients; thus, the study is clinically relevant. Secondly, MICB has been shown to interact with mouse NKG2D, and MICB-positive cells are sensitive to mouse NK cells (22, 23). We also have shown that MICB was shed by the mouse prostate cell line TC2 in the same pattern as MIC shedding in prostate cancer patients. Lastly, although mouse NKG2D ligands are functionally similar to human MIC in NK cell activation, these molecules are structurally different and may have different physiologic roles. Mouse NKG2D ligands lack the a3 domain and are mostly GPI-linked proteins (9); in addition, little is known about what controls the expression of mouse NKG2D ligands in vivo and whether they would shed in a similar fashion to MIC in human tumor cells. Lastly, different from human NKG2D ligands, studies have shown that naturally expressed mouse NKG2D ligands on tumor cells may not cause tumor rejection largely due to insufficient levels of the ligand expression (2) or low affinity of binding to NKG2D (40). In

4 Wu, unpublished data.
this study, although TC2 cells express some levels of mouse NKG2D ligand RAE-1 variants, TC2 tumors were palpable in SCID mice within 1 week after implantation and grew aggressively (Fig. 4), suggesting that the levels of activating RAE-1 variants expressed by TC2 cells are too low to induce antitumor immunity. This was also supported by the low binding ability of soluble mouse NKG2D to TC2 cells (Fig. 3A). Therefore, it could be challenging to define an optimal level of mouse NKG2D ligand expression for tumor rejection. Together, the choice of MICB makes the in vivo study described here more clinically relevant to human cancers.

In activated mouse NK cells, due to alternative DNA splicing, two isoforms of NKG2D couple with two intracellular adaptors, DAP10 and DAP12, which trigger phosphatidylinositol 3-kinase and Syk family protein tyrosine kinase, respectively (40, 41). In human, NKG2D only associates with DAP10. However, in mouse NK cells lacking DAP12 or Syk family kinases, DAP10-phosphatidylinositol 3-kinase pathway alone is sufficient to initiate ligand-induced NKG2D-mediated killing of target cells (41). Thus, regardless that signaling via mouse NKG2D is more complex than human NKG2D, effect of NKG2D ligand shedding on tumor formation as found in the current study would be significant in both species.

Most of the in vitro evidence suggests that engagement of tumor cell surface MIC to NKG2D can activate NK cell immunity against tumor cells. Thus, expression of MIC on tumor cells is proposed to activate host protective antitumor immunoresponse. However, most of the epithelial originated human cancer cells were found to have MIC expressed on the surface, suggesting the ineptness of MIC-induced NK cell immunity. Consistent with clinical observations, we also show that overexpressing the natural cleavable form of MICB in TC2 cells has no significant effect on tumor growth in vivo. Although overexpressing the noncleavable shedding-resistant MICB.A2 can cause TC2 tumor rejection, this effect can be inhibited by the persistent presence of soluble MICB (Fig. 6A). Together, our data suggest that the role of MIC in host tumor immunosurveillance is determined by whether MIC is all or partially membrane-bound. If all the MIC molecules sustain to be membrane-bound and noncleavable, expression of MIC activates NK cell-mediated host immunity. In contrast, if a portion of the MIC molecules is cleaved and becomes soluble, tumor cells cannot be targeted by NK cells due to soluble MIC-mediated masking and possible down-regulation of the receptor NKG2D regardless of abundant MIC remaining on the tumor cell surface as observed in many cancer patients (10–13).

In summary, our data provide the first in vivo conclusive evidence of the effect of MIC shedding on tumor growth and the importance of sustained MIC ligand-NKG2D receptor interaction in control of tumor growth. In addition, our results show no significant difference in tumor growth among animals whether the natural form of MICB or soluble recombinant MICB was expressed. This observation implies that wild-type MIC expression in established tumors may have very little effect on inducing host NK cell activation due to shedding of MIC by tumor cells and the consequent dampening of host immunity. Together, our results suggest that strategies to sustain the recognition of NKG2D receptor and tumor MIC ligand may have potential anticancer therapeutic implications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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