Nonblocking Monoclonal Antibody Targeting Soluble MIC Revamps Endogenous Innate and Adaptive Antitumor Responses and Eliminates Primary and Metastatic Tumors

Shengjun Lu¹, Jinyu Zhang¹, Dai Liu², Guangfu Li²,³, Kevin F. Staveley-O’Carroll²,³, Zihai Li¹,³, and Jennifer D. Wu¹,³,⁴

Abstract

Purpose: The human tumor-derived soluble MHC I-chain-related molecule (sMIC) is highly immune suppressive in cancer patients and correlates with poor prognosis. However, the therapeutic effect of targeting sMIC has not been determined, due to the limitation that mice do not express homologs of human MIC. This study is to evaluate the therapeutic effect of a monoclonal antibody (mAb) targeting sMIC in a clinically relevant transgenic animal model.

Experimental Design: We treated the engineered MIC-expressing “humanized” TRAMP/MIC bitransgenic mice at advanced disease stages with a sMIC-neutralizing nonblocking anti-MIC mAb and assessed the therapeutic efficacy and associated mechanisms.

Results: A sMIC-neutralizing nonblocking anti-MIC mAb effectively induced regression of primary tumors and eliminated metastasis without inducing systemic toxicity. The therapeutic effect is conferred by revamping endogenous antitumor immune responses, exemplified by restoring natural killer (NK) cell homeostasis and function, enhancing susceptibility of MIC⁺ tumor cells to NK cell killing, reviving and sustaining antigen-specific CD8 T-cell responses, augmenting CD4 T cells to Th1 responses, priming dendritic cells for antigen presentation, and remodeling tumor microenvironment to be more immune reactive.

Conclusions: Therapy with a sMIC-neutralizing nonblocking anti-MIC mAb can effectuate antitumor immune responses against advanced MIC⁺ tumors. Our study provided strong rationale for translating sMIC-neutralizing therapeutic mAb into clinics, either alone or in combination with current ongoing standard immunotherapies.

Clin Cancer Res; 21(21); 4819-30. ©2015 AACR.

Introduction

In response to oxidative stress and oncogenic insults, human epithelial cells were induced to express families of ligands for the immune stimulatory receptor NKG2D, presumably to provoke an effective immune response, but more severely, the shedding-resolved soluble NKG2D ligands can sabotage the immune system via diversified mechanisms. These include down-regulating the expression and thus function of the receptor NKG2D on NK cell and effector T cells (10, 11), perturb NK cell homeostasis (7), promoting the expansion of arginase 1⁺ myeloid-derived suppressor cells (MDSC), and skewing macrophage to the tumor-promoting alternatively activated phenotypes (12).

In human, two families of NKG2D ligands, the MHC I-chain-related molecules A and B (MICA/B, collectively termed MIC) and the HCMV glycoprotein UL16-binding protein family molecules (ULBP), have been identified; among which the MIC family is most prevalently and restrictedly expressed by human cancer cells (15). Shedding of tumor cell surface MIC to release the soluble MIC (sMIC) into circulation is commonly found in cancer patients (16–19). Serum levels of sMIC have been well associated with progressiveness of the diseases in multiple cancer types (7, 20–23). Given the multiple immune suppressive nature of NKG2D-mediated tumor suppression in both experimental animal models and cancer patients (7, 8). In cancer patients, however, tumors evolved to escape NKG2D-mediated natural immune response by adopting a protease or exosome-mediated strategy to shed NKG2D ligands (9). As a consequence, not only tumor cells lost surface NKG2D ligands with an impaired ability to provoke an effective immune response, but more severely, the shedding-resolved soluble NKG2D ligands can sabotage the immune system via diversified mechanisms. These include down-regulating the expression and thus function of the receptor NKG2D on NK cell and effector T cells (10, 11), perturb NK cell homeostasis (7), promoting the expansion of arginase 1⁺ myeloid-derived suppressor cells (MDSC), and skewing macrophage to the tumor-promoting alternatively activated phenotypes (12).

Thus, tumor-derived soluble NKG2D ligands have been broadly accepted to be highly immune suppressive and proposed as a potential cancer immune therapeutic target (13, 14).

In human, two families of NKG2D ligands, the MHC I-chain-related molecules A and B (MICA/B, collectively termed MIC) and the HCMV glycoprotein UL16-binding protein family molecules (ULBP), have been identified; among which the MIC family is most prevalently and restrictedly expressed by human cancer cells (15). Shedding of tumor cell surface MIC to release the soluble MIC (sMIC) into circulation is commonly found in cancer patients (16–19). Serum levels of sMIC have been well associated with progressiveness of the diseases in multiple cancer types (7, 20–23). Given the multiple immune suppressive nature of NKG2D-mediated tumor suppression in both experimental animal models and cancer patients (7, 8). In cancer patients, however, tumors evolved to escape NKG2D-mediated natural immune response by adopting a protease or exosome-mediated strategy to shed NKG2D ligands (9). As a consequence, not only tumor cells lost surface NKG2D ligands with an impaired ability to provoke an effective immune response, but more severely, the shedding-resolved soluble NKG2D ligands can sabotage the immune system via diversified mechanisms. These include down-regulating the expression and thus function of the receptor NKG2D on NK cell and effector T cells (10, 11), perturb NK cell homeostasis (7), promoting the expansion of arginase 1⁺ myeloid-derived suppressor cells (MDSC), and skewing macrophage to the tumor-promoting alternatively activated phenotypes (12).

Thus, tumor-derived soluble NKG2D ligands have been broadly accepted to be highly immune suppressive and proposed as a potential cancer immune therapeutic target (13, 14).

In human, two families of NKG2D ligands, the MHC I-chain-related molecules A and B (MICA/B, collectively termed MIC) and the HCMV glycoprotein UL16-binding protein family molecules (ULBP), have been identified; among which the MIC family is most prevalently and restrictedly expressed by human cancer cells (15). Shedding of tumor cell surface MIC to release the soluble MIC (sMIC) into circulation is commonly found in cancer patients (16–19). Serum levels of sMIC have been well associated with progressiveness of the diseases in multiple cancer types (7, 20–23). Given the multiple immune suppressive nature of NKG2D-mediated tumor suppression in both experimental animal models and cancer patients (7, 8). In cancer patients, however, tumors evolved to escape NKG2D-mediated natural immune response by adopting a protease or exosome-mediated strategy to shed NKG2D ligands (9). As a consequence, not only tumor cells lost surface NKG2D ligands with an impaired ability to provoke an effective immune response, but more severely, the shedding-resolved soluble NKG2D ligands can sabotage the immune system via diversified mechanisms. These include down-regulating the expression and thus function of the receptor NKG2D on NK cell and effector T cells (10, 11), perturb NK cell homeostasis (7), promoting the expansion of arginase 1⁺ myeloid-derived suppressor cells (MDSC), and skewing macrophage to the tumor-promoting alternatively activated phenotypes (12).

Thus, tumor-derived soluble NKG2D ligands have been broadly accepted to be highly immune suppressive and proposed as a potential cancer immune therapeutic target (13, 14).

In human, two families of NKG2D ligands, the MHC I-chain-related molecules A and B (MICA/B, collectively termed MIC) and the HCMV glycoprotein UL16-binding protein family molecules (ULBP), have been identified; among which the MIC family is most prevalently and restrictedly expressed by human cancer cells (15). Shedding of tumor cell surface MIC to release the soluble MIC (sMIC) into circulation is commonly found in cancer patients (16–19). Serum levels of sMIC have been well associated with progressiveness of the diseases in multiple cancer types (7, 20–23). Given the multiple immune suppressive nature of
sMIC (7, 10, 12, 16), whether antibody neutralizing sMIC can revive host antitumor responses in MIC⁺ cancer patients is evidently an interesting therapeutic question. Because of the limitation that no MIC homolog is expressed by rodents, this critical question, however, remained unaddressed to date. Notably, although the signaling pathways of NKG2D are conserved across species, mouse NKG2D ligands differ from human MIC in their affinity, structure, regulation, and distribution in tumor tissues (15, 24, 25). These inherent differences preclude the translation of interesting biologic conclusions discovered from mouse NKG2D ligands to human cancer.

We have recently generated a “humanized” bitransgenic TRAMP/MIC(B) mouse model that expresses human MIC in the prostate epithelial cells of the SV40T-transgenic adenocarcinoma mouse prostate (TRAMP) directed by the prostate-specific promoter (7). The TRAMP/MIC mouse closely recapitulates the dynamic interaction of oncogenesis and NKG2D-mediated immune surveillance in human cancer patients by bridging shedding sMIC with disease progression (7). With close resemblance to MIC⁺ cancer patients (7, 17, 20, 21), high serum levels of sMIC in TRAMP/MIC mice predicted poor tumor prognosis (7). Utilizing this similarity, in this study, we evaluated the therapeutic antitumor efficacy of a sMIC-neutralizing but nonblocking anti-MIC monoclonal antibody (mAb) B10G5 in an immune tolerant spontaneous MIC⁺ tumor model.

Materials and Methods

Animals and antibody therapy

Breeding of TRAMP/MICB has been previously described (7). Animals were randomized into two cohorts receiving therapy with intraperitoneal (i.p.) injection of sMIC-specific mAb B10G5 or isotype control IgG (clgG) at the dose of 4.0 mg/kg body weight twice weekly. Generation of the B10G5 antibody were described previously (7). All animals were treated for 8 weeks before euthanization that was designated as the study endpoint. Mice received daily refreshed drinking water containing 0.8 mg/mL bromodeoxyuridine (BrdUrd) for 5 consecutive days before the study endpoint. For congenic cells transfer, splenocytes were isolated from congenic CD45.1 mice (Charles River Laboratories, Frederick Cancer Research Center, Frederick, MD) and labeled with V450 cell-trace dye according to the manufacturer’s protocol (eBioscience). V450-labeled splenocytes were resuspended in PBS and injected via tail vein into recipient TRAMP/MICB mice (CD45.2⁺) at the dose of 2 × 10⁶/mouse 5 days before endpoint. All animals were housed in specific pathogen-free facilities. All experimental procedures were approved by the Institutional Animal Care and Use Committee. The study was repeated three times unless otherwise specified.

NK and CD8 T-cell depletion

Mice were injected with antibody anti-NKp46 antibody (BioLegend) to deplete NK cells or CD8e-specific antibody (clone 53-6.7; BioXcell) to deplete CD8 T cells at the dose of 200 μg/mouse 1 day before B10G5 antibody therapy and thereafter twice weekly at the dose of 100 μg/mouse till study endpoint. Efficiency of depletion was confirmed by flow cytometry analyses in the peripheral blood.

Antigen-specific T-cell response experiment

CD8 T cells from TCR-I transgenic mice were labeled with CFSE and injected i.v. into animals (2 × 10⁶ cells/mouse) that were receiving B10G5 or control IgG therapy. Animals were sacrificed at indicated time points to assess TCR-I T cell in vivo frequency with TCR-I-specific H-2Db/TAg epitope I-tetramer (D⁷/I-tetramer; ref. 26). To assay antigen-specific CD8 T-cell response, bulked splenocytes and single-cell suspension of tumor-draining lymph nodes (dLN) and tumor digests were stimulated overnight with 0.5 μM TAg epitope 1 peptide and assaying intracellular IFNγ staining of CD8⁺ or D⁷/I-tetramer⁺ T cells.

Tissue collection

Blood was collected via tail bleeding during therapy and via cardiac puncture after euthanization. Spleens and dLN were collected for immunologic analyses. Prostate, lung, liver, kidney, pancreas, and intestines were collected, fixed in 10% neutral fixation buffer followed by paraffin embedment or directly embedded in optimal cutting temperature (OCT), for pathologic and histologic analyses. In some experiments, partial of prostate tumors was digested with collagenase for analyses of tumor-infiltrated lymphocytes.

Flow cytometry

Single-cell suspension from splenocytes, dLN, or tumor infiltrates was prepared as described previously (7). Combination of the following antibody was used for cell surface or intracellular staining to define populations of NK, CD8, and subsets of CD4 T cells: CD3e (clone 145-2c11), CD8a (clone 53-6.7), CD4 (clone GK1.5), NK1.1 (clone PK136), NKG2D (clone CX5), CD45.1 (clone A20), and T-bet (clone eBio4B10). For ex vivo restimulation, single-cell suspension of freshly isolated splenocytes or LN were cultured in complete RPMI-1640 medium containing 50 ng/mL phorbol 12—myristate 13—acetate (PMA) and 500 ng/mL ionomycin for 4 hours and analyzed by intracellular staining with antibodies specific to IFNγ (XMG1.2). For NK cell renewal, intracellular BrdUrd staining was performed using anti-BrdUrd antibody (clone Bu20a). All antibodies and the corresponding isotype controls were fluorochrome conjugated and were purchased from eBioscience or BD Biosciences. Multicolored flow cytometry analyses were performed on an LSRII (BD). Data were analyzed with the FlowJo software (TreeStar).
Histology and immunohistochemistry staining
Prostate, lung, and other organs were sectioned and stained with H&E for histologic evaluation. For immunohistochemistry (IHC) staining to detect specific antigens, the following antibodies were used: anti-SV40T (Santa Cruz Biotechnology), anti-Ki67 (Neomarker), anti-cleaved Caspase-3 (Cell Signaling Technology; clone 5A1E), anti-CD8 (BD Biosciences), anti-NK1.1 (eBiosciences; PK136), and anti-synaptophysin (aBCAM). The IHC staining protocol has been previously described (7, 17). All tissues were counter stained with hematoxylin for visualization of nucleus.

Serum sMIC, total IgG, and cytokine detection
Serum levels of sMICB and total IgG were assessed using respective Sandwich ELISA kit (R&D Systems). Serum levels of cytokines were assayed by Eve Technologies Corporation using the Luminex Technology.

Statistical analysis
All results are expressed as the mean ± SEM. Mouse and sample group sizes were n > 5, unless otherwise indicated. Data were analyzed using unpaired t test, and differences were determined significant at P < 0.05. The Kaplan–Meier survival curves were generated using the GraphPad Prism software.

Results
Functional characterization of the anti-MIC mAb B10G5
The B10G5 mAb not only neutralizes free sMIC but also recognizes tumor cell surface membrane-bound MIC (Supplementary Fig. S1A and S1B), owing to sMIC sharing the same sequence and structure as the ectodomain of cell-surface MIC (16). Because B10G5 and the receptor NKG2D recognizes different epitopes of MIC (data not shown), B10G5 does not block the sensitivity of NKG2D-mediated NK cell cytolytic activity against MIC+ cells (Supplementary Fig. S1C). Conversely, B10G5 enhances the sensitivity of MIC+-tumor cells to NK cell cytotoxicity (Supplementary Fig. S1C), presumably through antibody-mediated cell cytotoxicity (ADCC) and/or enhanced immune synapse formation through simultaneously engaging NKG2D and Fc receptor on NK cells.

B10G5 antibody therapy effectively induced regression of primary tumors and eliminated metastasis
We have previously shown that TRAMP/MIC mice have greater than 40% incidence of developing highly invasive PD carcinoma and distant metastasis by 24-weeks of age (7). We also have previously determined that the accelerated tumor progression in TRAMP/MICB mice in comparison with TRAMP littermate is associated with elevated serum sMIC and its immune suppressive effects as in cancer patients with advanced diseases (7, 20, 21, 27). With the specific aim to treat advanced cancer, we treated random cohorts of 27–to 28-week-old TRAMP/MIC mice with the B10G5 mAb or control mouse IgG (clgG) twice weekly via i.p. route at the dose of 4 mg/kg body weight (Fig 1A). Following an 8-week therapy, all mice that received B10G5 survived, whereas approximately 50% of mice in the control group, succumbed to the disease during the same time frame with the remaining of mice showing severe symptoms of illness (Fig 1B–E and data not shown). Mice that received the 8-week B10G5 therapy had significantly smaller prostate tumor mass in comparison with those having received clgG or to those at the same disease stage when treatment began (27–28 week old; Fig 1C and D). No metastasis was found in the B10G5-treated mice. Conversely, over 90% of the mice receiving clgG developed metastasis (Fig 1E).

Therapy eliminates invasive prostate tumor cells and primes tumor microenvironment to be more immune reactive
Histologic examination revealed that animals having received B10G5 therapy exhibited a mixture of normal prostate gland and organ-defined well-differentiated prostate carcinoma, whereas mice having received clgG therapy exhibited high frequency of invasive prostate carcinoma (Fig 2A). B10G5 therapy resulted in markedly reduced proliferation (Ki67+) and increased apoptosis (C-caspase+) of carcinoma cells (Fig 2A). In the primary tumor of majority TRAMP/MIC mice receiving clgG or no therapy, a distinct population of disseminated tumor cells that gained neuroendocrine differentiation marker synaptophysin was abundantly present in the stroma (SYN; Fig 2A). Clinically, these tumor cell types were considered to be therapy-resistant and confer a poor prognosis (28, 29). B10G5 therapy effectively eliminated the "neuroendocrine-differentiated" tumor cells.

In most solid tumor of human cancers, tumor infiltration of NK cells correlated with better clinical outcomes or better response to therapy (30–34), suggesting an important role of NK cell in controlling cancer progression. Resembling human cancer, we have recently shown that NK cell is rarely found in poorly differentiated prostate carcinoma (7). B10G5 therapy evidently enriched NK cell infiltration in the prostate tumor parenchyma (Nkp46; Fig 2B). Moreover, CD8 T-cell infiltration to tumor parenchyma was also enriched by B10G5 therapy (CD8; Fig 2C).

We have recently shown that sMIC can facilitate the expansion of MDSC and skew macrophage into the arginase 1+ alternatively activated phenotypes (12). Consistently, TRAMP/MIC had rich infiltration of arginase 1+ cells in the prostate infiltrates, which were eliminated significantly by B10G5 therapy (arginase I; Fig 2B). As arginase I can be produced by MDSC (generally defined as CD11b+/Gr-1+) and/or the alternatively activated tumor-associated macrophage (generally defined as CD206+/CD11c--; refs. 35–38), we further defined these immune suppressive subsets in tumor infiltrates by flow cytometry analyses. The numbers of CD11b+/Gr-1+ MDSC and CD206+/CD11c- macrophages are both significantly decreased in response to B10G5 therapy (Fig 2C and D). Together, these data demonstrate that neutralizing sMIC primed tumor microenvironment to be more immune active.
B10G5 therapy restores NK cell homeostasis and enhances NK and NKT cell function

Elevated serum sMIC was shown to impair peripheral NK cell function in cancer patients at large through downregulating NKG2D expression and reducing the number of NK cells (7, 17, 18, 39). With the clinically relevant TRAMP/MIC mouse model, we demonstrated that high levels of sMIC ablates the ability of NK cells to self-renew (7). B10G5 therapy remarkably restored NK cell pool in the periphery and the ability of NK cell homeostatic to self-renew as evidenced by BrdUrd uptake after a...
consecutive 5-day BrdUrd pulsing (Fig. 3A–D). Moreover, B10G5 therapy markedly enhanced NK cell function, illustrated by increased production of IFNγ in response to mitogen stimulation and cytolytic ability against NKG2D ligand-positive target cells (Fig. 3E–G). Together, these data conclude that targeting serum sMIC significantly restores NK cell homeostatic maintenance and function in MIC+ cancer host. The conclusion was further supported by adoptively transfer experiments with V450-labeled CD45.1 congenic NK cells to B10G5 or cIgG-treated TRAMP/MIC mice (Supplementary Fig. S3). Furthermore, although B10G5 therapy did not significantly affect the pool of NKT cells (data not shown); however, therapy enhanced Th1-like functional potential of NKT cells as represented by significantly increased IFNγ production in response to PMA/I stimulation (Fig. 3H and I).

B10G5 therapy potentiated CD8 and CD4 T cells antitumor responses

Tumor-derived sMIC has been shown to downregulate NKG2D expression on CD8 T cells in cancer patients (16). NKG2D, as a T-cell costimulatory receptor, is expressed by all human CD8 T cells but only by activated mouse CD8 T cells (4). B10G5 therapy in TRAMP/MIC mice significantly increased the population and number of NKG2D+ CD8 T cells in the periphery (Fig 4A and B), indicating activation of CD8 T cells. When stimulated ex vivo with mitogen, CD8 T cells from mice receiving B10G5 therapy had significantly higher frequency and magnitude of IFNγ production (Fig. 4C and D). Moreover, B10G5 therapy significantly increased the number of CD8 T cells with CD44hi memory phenotype in the spleen, tumor-dLN, and tumor infiltrates (Fig. 4E and F). Given that anergy and lack of trafficking to tumor sites are the major mechanisms of cytotoxic T dysfunction in most cancer types, these data suggest that targeting sMIC can effectively revive cytotoxic CD8 T-cell antitumor response in cancer host.

NKG2D is rarely expressed by CD4 T cells. However, B10G5 therapy potentiated CD4 T cells to Th1 responses in the tumor dLNs, shown by the significant increases in IFNγ+ CD4 T cells in response to mitogenic stimulation (Fig 4G and H) and increases in T-bet expression (Supplementary Fig. S4). Furthermore, therapy also resulted in significant increase in CD4 T cells with CD44hi memory phenotype (Fig 4I).

Therapy breaks CD8 T-cell tumor antigenic tolerance and augments antigen-specific responses

Male TRAMP mice express the SV40-T-Antigen (TAg) oncoprotein specifically in the prostate to drive spontaneous
prostate tumor development through inactivation of tumor suppressor genes p53 and Rb pathways (40). Akin to the biology in cancer patients, the TAGs in male TRAMP mice serve as a self-antigen and a tumor antigen, leading to tolerance of TAG and clonal deletion of TAG-specific T cells (41). Adoptively transferred naïve CD8 T cells that bear TAG-specific
Figure 4.
B10G5 therapy in TRAMP/MIC mice potentiates CD8 and CD4 T cell antitumor function. A and B, representative flow cytometry plots (A) and summary statistics (B) demonstrating that B10G5 therapy significantly increases the expression of the costimulatory NKG2D on CD8 T cells, an indication of CD8 T-cell activation in mouse. C and D, representative flow cytometry plots (C) and summary statistics (D) demonstrating B10G5 therapy potentiates the CD8 T-cell IFNγ production in response to ex vivo restimulation by PMA and ionomycin. E and F, representative flow cytometry CD8 T cells with CD44hi memory-phenotype. G and H, representative plot of flow cytometry and summary data demonstrating Th1 polarization of CD4 T cells in tumor-dLN. I, summary data demonstrating that B10G5 therapy increases CD44hi memory phenotype of CD4 T cells in tumor-dLN. Data are representatives of three independent studies. n > 5 for animals in each group. *, P < 0.05; **, P < 0.01.
TCR became rapidly tolerant in TRAMP mice after initiation expansion (42). We sought to address whether anti-sMIC antibody B10G5 therapy can enhance antigen-specific CD8 T-cell responses in TRAMP/MIC mice. Because of the clonal deletion of TAg-specific CD8 T cells (41), we thus adoptively transferred CFSE-labeled naïve CD8 T cells from the TCR-I mice that express a transgenic TCR recognizing H-2D<sup>b</sup>-restricted TAg epitope I (residues 206-215; ref. 26). As examined at day 9 after transfer, a significantly higher number of H-2D<sup>b</sup>/epitope I-specific tetramer (D<sup>b</sup>/I-tetramer) positive CD8 T cells was detected in the spleen, tumor-dLN, and tumor infiltrates of mice receiving B10G5 therapy than those receiving cIgG therapy (Fig. 5A). Notably, endogenous D<sup>b</sup>/I-tetramer<sup>+</sup> CD8 T cells were not detectable in TRAMP/MIC mice with or without antibody treatment (Supplementary Fig. S5). The D<sup>b</sup>/I-tetramer<sup>+</sup> CD8 T cells are thus originated from adoptively transferred TCR-I cells. Consistent with findings in other studies (26, 43), adoptively transferred naïve TCR-I CD8 T cells (identified by D<sup>b</sup>/I-tetramer<sup>+</sup>) underwent initial rapid expansion in all mice.
Targeting Soluble MIC for Cancer Immunotherapy

Intriguingly, with depletion of NK cells, B10G5 therapy failed to evoke CD4 T cells to generate optimal Th1 immune responses and maintaining CD44hi memory phenotypes (Fig. 6D–F and Supplementary Fig. S9). Depletion of CD8 T cells had nominal effect on CD4 T cells (data not shown). These data not only suggest that both NK and CD8 T cells are required to generate optimal antitumor responses, but also suggest that functional NK cells are essential for generating optimal CD4 T-cell responses.

Discussion

Tumor-derived sMIC, whether through protease-mediated shedding or exosome secretion, has been shown to be highly immune suppressive in cancer patients through multiple mechanisms (9, 10, 46). However, whether a therapy targeting sMIC can revitalize host endogenous immune response remains untested. Using a clinically relevant double transgenic TRAMP/MIC mouse model that closely recapitulates the dynamics of serum sMIC and tumor progression, here we demonstrate that therapy with a nonblocking anti-MIC mAb to neutralize serum sMIC effectively induce regression of advanced primary tumors and eliminated metastasis through revamping host endogenous innate and adoptive antitumor immune responses and remodeling tumor microenvironment. Therapy effectuated endogenous antitumor responses by recuperating the ability of NK cells to self-renew and to be tumor-destructive, enhancing antigen-specific CD8 T-cell antitumor responses, and potentiating CD4 T cells to Th1 responses. Not only so, therapy also remodels tumor microenvironment demonstrated by enhanced tumor infiltration of NK cells and cytotoxic CD8 T cells and eliminating arginase I+ immune suppressive myeloid cells in tumor parenchyma. Remarkably, therapy elicited a systemic cytokine “storm” including major antitumor cytokines, without inducing systemic autoimmune cytotoxicity. Our study has provided the compelling first-in-field preclinical evidence that therapy with a sMIC-neutralizing nonblocking anti-MIC mAb alone can obliterate immune suppressive effective of tumor-derived sMIC and revive endogenous antitumor immune responses. Our findings define a new effective and feasible translational approach for cancer immunotherapy.

During oncogenesis, whether due to mutations in tumor suppressor genes or oncogenes, cells undergo aberrant DNA replication which initiates DNA repair responses as the cellular check point. This DNA repair response also triggers systemic checkpoint by upregulating NKG2D ligand, predominantly MIC, expression to alert the immune system to eliminate abnormal cells (1, 3). However, this oncoimmunologic coevolutionary process eventually renders tumor immune escape of NKG2D-mediated immune surveillance and allows tumors to progress. Proteolytic proteases or exosome-mediated tumor-shedding of sMIC accounted for one of the major mechanisms for MIC+ tumor evasion of NKG2D immune surveillance (9, 10, 46). Earlier studies demonstrated that sMIC induced global NKG2D downmodulation on all subsets of antitumor effector cells, such as CD8, NK, NKT, and γδ T cells, in cancer patients (10, 11, 46). Recent studies have further demonstrated that sMIC can produce more profound immune suppressive effect by perturbing NK cell homeostatic maintenance and facilitating the expansion of arginase I+ myeloid suppressor cells (7, 12). These studies endorsed the viability of blocking sMIC pathways in cancer immunotherapy.
How to feasibly target the immune suppressive effect of sMIC to translate into clinics is a critical question. Strategies have been proposed to blocking MIC shedding through targeting enzymes that regulates MIC shedding, such as the disulfide isomerase ERp5 (47, 48), ADAM 10/17 (49), and MMPs (50). These strategies are likely work well in early stage of diseases to retain maximal surface MIC expression and to preserve NKG2D-mediated antitumor immunity. In advanced cancer patients, however, providing that tumor cells lost surface MIC expression at large as a result of shedding and that endogenous immune responses have been severely sabotaged by high serum levels of sMIC through multiple mechanisms, strategy to prevent MIC shedding is likely to elicit nominal therapeutic effect. Under this scenario, neutralizing serum sMIC with mAb would be the logical approach in advanced cancer patients to alleviate the immune suppressive effect. Jinushi and colleagues (22, 45) reported that patients who developed high serum levels of anti-MIC autoantibody during clinical trial with CTLA-4 blockade and/or vaccine therapy had sustained clinical response. These clinical findings strongly support the translational potential of our study and our unique sMIC-neutralizing nonblocking anti-MIC mAb.

In consistent with the current understanding of the mechanisms, whereby sMIC impairs host antitumor response, we demonstrated that our antibody neutralizing serum circulating sMIC restored NK cell homeostatic maintenance and function, restored NKG2D expression on CD8 T cells, and eliminated arginase I+ myeloid suppressor cells systemically and in tumor microenvironment. Our data suggest that the immune suppression of sMIC in cancer patients could be reversed once sMIC is removed. Beyond overcoming the multiple immune suppressive effects of sMIC, very intriguingly, B10G5 therapy also enhanced antigen-specific CD8 T-cell responses and potentiated CD4 T cells to Th1 responses. More importantly, depletion of NK cells during therapy had more profound effect on CD4 T cells than on CD8 T cells. These findings suggest that the therapeutic effect on CD8 T cells is likely to be direct, whereas the effect on CD4 T cells is mediated through functional NK cells. The direct effect of anti-MIC antibody on CD8 T-cell responses may, in part, be due to enhanced DC presentation of antigens on MIC. Beyond overcoming the multiple immune suppressive effects of sMIC, Jinushi and colleagues (22, 44, 45) reported that patients who developed high serum levels of anti-MIC autoantibody during clinical trial with CTLA-4 blockade and/or vaccine therapy had sustained clinical response. These clinical findings strongly support the translational potential of our study and our unique sMIC-neutralizing nonblocking anti-MIC mAb.
potential complex formation of sMIC/B10G5 and subsequent immune stimulatory effect; (v) enhanced NK-DC cross-talk to better prime the adoptive immune responses. The impact of each mechanism on the therapeutic outcome warrants future investigation.

It is intriguing that antibody targeting sMIC therapy elicit a systemic cytokine and chemokine “storm,” but with no detectable autoimmune toxicity. Notably, therapy not only induced high serum levels of antitumor cytokines, such as IL2, IFNγ, IL9, IL12, II17, but also induced cytokines that regulates immune tolerance, such as IL4, IL10, and IL13. The balanced induction of immune active and immune tolerance cytokines may explain the absence of systemic autoimmune cytotoxicity. Given that MIC is a tumor-specific target, the absence of systemic autoimmune immunity may be reasonably expected.

In conclusion, with a “humanized” genetic engineered mouse model that recapitulate the dynamic interaction of human NKG2D ligand MIC with tumor progression, we demonstrate that an antibody neutralizing sMIC effectively induced regression of primary tumors and eliminated metastasis in a mouse model that recapitulate the dynamic interaction of human NKG2D ligand MIC with tumor progression, we demonstrate that an antibody neutralizing sMIC effectively induced regression of primary tumors and eliminated metastasis in advanced MIC± malignancy. We further demonstrated that the mechanism of tumor suppression and clearance was conferred through restoring NK cell homeostatic maintenance and function, overcoming CD8 T-cell tolerance to tumor antigen, and heightening CD4 T cells to Th1 responses, and priming DCs for enhanced antigen presentation and tumor microenvironment to be more immune reactive. Furthermore, we demonstrate a critical role of NK cells in potentiating adaptive immune responses against tumors. Collectively, our study provided the first-in-field preclinical evidence demonstrating that an antibody neutralizing sMIC can reset and revamp endogenous antitumor responses to effectuate elimination of MIC± malignancies. Our findings are highly translatable to clinics to treat MIC± cancers with antibodies to neutralize sMIC. Conceptually, given the global overturning of endogenous antitumor responses induced by the sMIC-neutralizing antibody, sMIC may be considered as a tumor-specific immune checkpoint molecule.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Lu, D. Liu, J.D. Wu
Development of methodology: S. Lu, G. Li, J.D. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Lu, J. Zhang, D. Liu, J.D. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Lu, J. Zhang, G. Li, K.F. Staveley-O’Carroll, Z. Li, J.D. Wu
Writing, review, and/or revision of the manuscript: S. Lu, G. Li, K.F. Staveley-O’Carroll, J.D. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Lu, D. Liu, G. Li, K.F. Staveley-O’Carroll, J.D. Wu
Study supervision: K.F. Staveley-O’Carroll, J.D. Wu
Other (oversaw collaboration): J.D. Wu

Grant Support
This work was supported by NIH-NCl grant 1R01CA149405 and A. David Mazzone—Prostate Cancer Foundation Challenge Award (to J. Wu) and, in part, supported by the Flow Cytometry Core Facility Shared Resource, Hollings Cancer Center, Medical University of South Carolina (P30 CA138313)

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 5, 2015; revised June 4, 2015; accepted June 12, 2015; published OnlineFirst June 23, 2015.

References
Lu et al.
