Antibody-mediated neutralization of soluble MIC significantly enhances CTLA4 blockade therapy

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Antibody therapy targeting cytotoxic T lymphocyte–associated antigen 4 (CTLA4) elicited survival benefits in cancer patients; however, the overall response rate is limited. In addition, anti-CTLA4 antibody therapy induces a high rate of immune-related adverse events. The underlying factors that may influence anti-CTLA4 antibody therapy are not well defined. We report the impact of a cancer-derived immune modulator, the human-soluble natural killer group 2D (NKGD2) ligand sMIC (soluble major histocompatibility complex I chain–related molecule), on the therapeutic outcome of anti-CTLA4 antibody using an MIC transgenic spontaneous TRAMP (transgenic adenocarcinoma of the mouse prostate)/MIC tumor model. Unexpectedly, animals with elevated serum sMIC (sMIC<sup>+</sup>) responded poorly to anti-CTLA4 antibody therapy, with significantly shortened survival due to increased lung metastasis. These sMIC<sup>+</sup> animals also developed colitis in response to anti-CTLA4 antibody therapy. Co-administration of an sMIC-neutralizing monoclonal antibody with the anti-CTLA4 antibody alleviated treatment-induced colitis in sMIC<sup>+</sup> animals and generated a cooperative antitumor therapeutic effect by synergistically augmenting innate and adaptive antitumor immune responses. Our findings imply that a new combination therapy could improve the clinical response to anti-CTLA4 antibody therapy. Our findings also suggest that prescreening cancer patients for serum sMIC may help in selecting candidates who will elicit a better response to anti-CTLA4 antibody therapy.

INTRODUCTION

Cytotoxic T lymphocyte–associated antigen 4 (CTLA4) is a co-inhibitory receptor that controls cytotoxic effector T cell activation during initiation and maintenance of healthy adaptive immune responses (1–3). The expression of CTLA4 is often up-regulated during T cell activation upon interacting with antigen-presenting cells (APCs) (1–3). CTLA4 competes with the costimulatory T cell receptor (TCR) CD28 in binding to the B7 molecules (B7.1 and B7.2) expressed on APCs and has a higher binding affinity than CD28 (1–4). In contrast to CD28/B7 binding, which acts as a costimulatory signal to promote T cell activation and proliferation, the binding of CTLA4 to B7 transmits an inhibitory signal. CTLA4 is considered the “off switch” or “immune checkpoint” for effector T cell function. CTLA4 can also control effector T cell function through the expansion of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells, in which CTLA4 is constitutively expressed and critical for maintaining the suppressive function (5, 6). Therapy with anti-CTLA4 antibody elicited remarkable effectiveness in controlling tumor growth in mice (7–12).

Preclinical investigations have led to the development of anti-CTLA4 antibodies for cancer immunotherapy. Iplimumab, a fully human monoclonal anti-CTLA4 antibody that blocks the binding of CTLA4 to the B7 molecule, was approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced melanoma (4, 13–15). Currently, iplimumab is also undergoing clinical trials for the treatment of non–small cell lung carcinoma, small cell lung cancer, and bladder cancer (16–18). Despite the remarkable tumor control ability of anti-CTLA4 antibody demonstrated in preclinical animal models, the overall clinical response rate of anti-CTLA4 antibody as a stand-alone agent is limited to 15% in melanoma (4). Notably, therapy with anti-CTLA4 antibody has been associated with a significant risk of immune-related adverse events (IRAEs) in patients, with most of the severe cases restricted to gastrointestinal inflammation (13, 19–21). Combination therapy of anti-programmed cell death protein 1 antibody and anti-CTLA4 antibody has achieved greater overall response than monotherapy in patients with melanoma, the most immunogenic cancer; however, the response remains limited to a subgroup of patients and is accompanied by increased toxicity (22–24). It is thus imperative to understand the circumstances that may limit the clinical efficacy of anti-CTLA4 antibody therapy and the risk of toxicity to develop more effective yet safe regimens.

The differential expression of immune modulators by human and rodent tumors may contribute to the response disparity between clinical and preclinical anti-CTLA4 antibody therapy. The major histocompatibility complex (MHC) I chain–related family molecules, including the molecules MICA and MICB (collectively termed MIC), are an example of such immune modulators. MIC is frequently induced in human tumors upon genotoxic insults but is absent in rodents (25–31). The induction of MIC on the cancer cell surface can activate natural killer (NK) cells and costimulate cytotoxic CD8 T cells, NK-like T cells, and subsets of γδ T cells through engaging the activating immune receptor natural killer group 2D (NKGD2) (32, 33). The activation of NKGD2 has been shown to provide unique costimulation to antigen-specific CD8 T cells that is nonredundant to CD28 costimulation (34, 35). In cancer patients, MIC is often released as the highly immunosuppressive soluble form sMIC through shedding (25, 28, 29). Tumor-derived sMIC at large suppresses host immune response using multiple strategies including (but not limited to) down-regulating NKGD2 expression and destabilizing CD3ζ, in the TCR/CD3 complex on CD8 T cells (25, 38), perturbing NK cell function and peripheral maintenance (39, 40), and cultivating an immunosuppressive tumor microenvironment by driving the expansion of myeloid suppressor cells and skewing macrophage into an alternatively activated phenotype (41). Using a clinically relevant MIC-transgenic spontaneous mouse tumor model that closely recapitulates the onco-immune dynamic of human cancer (39), we have demonstrated that therapy with the nonblocking anti-sMIC monoclonal antibody (mAb) B10G5 globally abrogates the immunosuppressive effect
of sMIC, immobilizes NK and CD8 T cells to the tumor microenvironment, and overcomes immune tolerance of antigen-specific CD8 T cells (42, 43). In line with these understandings, we herein investigated the impact of tumor-derived human sMIC on the therapeutic efficacy of anti-CTLA4 antibody. We show that high levels of serum sMIC not only negatively influenced the antitumor efficacy of anti-CTLA4 antibody but also evoked colitis development during anti-CTLA4 antibody therapy. Coadministration of the sMIC-neutralizing antibody B10G5 with anti-CTLA4 antibody generated a cooperative antitumor effect and alleviated therapy-induced colitis. These results suggest a new avenue for combination immunotherapy of cancer.

RESULTS

Animals with high serum levels of sMIC respond poorly to anti-CTLA4 antibody therapy

Tumor-derived sMIC negatively affects tumor immunity through multiple mechanisms (25, 39–41). To address the impact of sMIC on the efficacy of anti-CTLA4 antibody therapy, we treated cohorts of 27- to 28-week-old TRAMP (transgenic adenocarcinoma of the mouse prostate) and TRAMP/MIC littermates with anti-CTLA4 antibody (3.0 mg/kg) or control immunoglobulin G (clgG), respectively (Fig. 1A). We previously showed that more than 40% of TRAMP/MICB mice at the indicated age had elevated serum sMIC as a result of tumor shedding (39). Consistent with other studies (44, 45), TRAMP mice presented no or marginal response to anti-CTLA4 antibody monotherapy (Fig. 1, B and D). TRAMP/MIC mice that received anti-CTLA4 antibody therapy presented reduced overall survival compared to littermates that received clgG therapy (Fig. 1B). Animals that succumbed to diseases during therapy, whether receiving clgG or anti-CTLA4 antibody, had significantly higher levels of serum sMIC before therapy than those that survived to the study end point (Fig. 1C). However, in animals that had high levels of sMIC before therapy, tumors progressed more aggressively in response to anti-CTLA4 antibody therapy. The disease progression was manifested through large primary tumor burdens and massive lung metastasis (Fig. 1, D to F). Animals that had high serum levels of sMIC and had received anti-CTLA4 antibody therapy also developed colitis (fig. S1). These data indicate that high levels of circulating sMIC adversely affect tumor response to anti-CTLA4 antibody therapy and also contribute, at least in part, to anti-CTLA4 antibody–induced autoimmune colitis.

We substantiated our observations in syngeneic TRAMP-C2 and sMICB-expressing TRAMP-C2-sMICB transplanted prostate tumor models (fig. S2), in which both tumor cell lines were subcutaneously inoculated into MICB/B6 male transgenic mice, as we have previously described (39, 42, 46). In the absence of sMIC, TRAMP-C2 tumors presented no response to anti-CTLA4 antibody therapy. Consistent with our observations in syngeneic TRAMP/MICB mice, TRAMP-C2-sMICB tumors grew more aggressively in response to anti-CTLA4 antibody therapy (fig. S2B). In animals that received anti-CTLA4 antibody therapy, TRAMP-C2-sMICB tumor–bearing mice developed more severe colon inflammation than TRAMP-C2 tumor–bearing mice (fig. S2C).

Antibody neutralizing sMIC markedly enhances therapeutic responses to anti-CTLA4 antibody

To confirm that sMIC contributes to subjects’ poor response to anti-CTLA4 antibody therapy and to determine whether neutralizing sMIC can enhance the therapeutic efficacy of anti-CTLA4 antibody or MIC+ tumors, we coadministered the anti-sMIC mAb B10G5 (3.0 mg/kg) with anti-CTLA4 antibody to a cohort of TRAMP/MICB littermates at 26 to 27 weeks old, as depicted in Fig. 1A. In our recent publications, we described that B10G5 can effectively neutralize the immunosuppressive effect of sMIC to induce the debulking of primary tumors and eliminate metastasis (42). In agreement with our previous findings, all animals responded to mAb B10G5 monotherapy (Fig. 2, A to E). Remarkably, regardless of serum levels of sMIC before therapy, all animals survived to the study end point with clearance of lung metastasis in response to anti-CTLA4 antibody therapy with the coadministration of B10G5 (Fig. 2, A and E). The coadministration of B10G5 significantly reduced the circulating level of sMIC (Fig. 2B) and generated a cooperative therapeutic effect with anti-CTLA4 antibody, eliciting a significant reduction in primary tumors at the study end point compared to monotherapies (Fig. 2C). The coadministration of B10G5 also alleviated anti-CTLA4 antibody therapy–induced colitis (fig. S3). These data demonstrate the benefit of the sMIC-neutralizing antibody B10G5 in enhancing anti-CTLA4 antibody therapy.

B10G5 neutralizing sMIC heightens CD8 T cell response to anti-CTLA4 antibody therapy

The therapeutic efficacy of anti-CTLA4 antibody is commonly determined by the activation status of effector CD8 T cells. The coadministration of B10G5 with anti-CTLA4 antibody to TRAMP/MICB mice resulted in a significant increase of CD8 T cells in tumor-draining lymph nodes (dLNs) and tumor infiltrates compared to the monotherapy with anti-CTLA4 antibody or B10G5 (Fig. 3A and fig. S5A). Consistent with our previous observation, B10G5 therapy alone increased the expression of NKG2D on CD8 T cells (42). Notably, NKG2D as a nonconventional effector T cell costimulatory molecule is expressed by all human CD8 T cells but is only expressed by activated mouse CD8 T cells (48). A cocktail therapy of anti-CTLA4 antibody and B10G5 further increased the number of NKG2D+ CD8 T cells, signifying that more CD8 T cells were activated (Fig. 3B and fig. S5B). NKG2D and CD28 can deliver nonredundant costimulatory signals to CD8 T cells (35, 49). Thus, the further increased NKG2D expression in response to cocktail therapy would presumably provide a nonconventional costimulatory signal to augment antigen-specific CD8 T cell responses to MIC+ tumors, in addition to the diminution of CTLA4 co-inhibitory signals.

The coadministration of B10G5 with anti-CTLA4 antibody therapy significantly increased the effector memory-like CD44hi CD8 T cell compartments in the spleen, dLNs, and tumor infiltrates (Fig. 3C and fig. S5C). The combination therapy also increased the intrinsic ability of CD8 T cells to produce interferon-γ (IFN-γ) in response to ex vivo phorbol 12-myristate 13-acetate (PMA) and ionomycin restimulation (Fig. 3D and fig. S5D).
We retrospectively compared the functional potential of CD8 T cells in TRAMP and TRAMP/MICB mice that had received anti-CTLA4 antibody monotherapy. CD8 T cells in all TRAMP mice uniformly exhibited a nominal response to anti-CTLA4 therapy (fig. S6). In TRAMP/MIC mice that had received anti-CTLA4 antibody therapy, the CD8 T cell response to ex vivo PMA/ionomycin restimulation varied in accordance to serum levels of sMIC; however, the percentage of CD8 T cells was not profoundly affected (fig. S7A). A severely impaired response was

![Diagram](image)

**Fig. 1.** Mice with high circulating tumor-shed soluble MIC (sMIC^hi^) had poor response to anti-CTLA4 antibody therapy. (A) Schematic depiction of therapy. (B) Kaplan-Meier survival curve. αCTLA4, anti-CTLA4. *P* < 0.05. (C) Prostate weight at necropsy. Boxed animals succumbed to disease before the study end point. (D and E) Representative micrographs (D) and overall number (E) of lung metastasis in TRAMP/MICB mice in response to anti-CTLA4 antibody therapy. Arrows, lung nodules. (F) Serum levels of sMIC in TRAMP/MICB mice before receiving therapy. Sera from TRAMP were used as negative controls. ns, not significant.
observed in mice with high levels of sMIC, whereas the responses in TRAMP/MICB mice with low levels of sMIC and in TRAMP mice remain comparable. One of the 11 TRAMP/MICB mice that developed anti-sMIC autoantibody during anti-CTLA4 antibody monotherapy demonstrated remarkable responsiveness to ex vivo restimulation (fig. S7B). Notably, the polarization of CD8 T cell functional potential was correlative with the presence and function of NK cells in the dLNs (fig. S6B). Given that sMIC perturbs NK cell homeostatic maintenance and function and that NK cell–dendritic cell (DC) cross-talk is critical in regulating T cell priming (39, 50–53), these observations further highlight a potential important role of NK cells in effectuating CTLA4 blockade therapy.

Fig. 2. Antibody B10G5 cotargeting sMIC remarkably enhances response to anti-CTLA4 antibody therapy. (A) Kaplan-Meier survival curve. (B) Serum levels of sMIC at necropsy. (C) Prostate weight at necropsy. (D and E) Number (D) and representative micrographs (E) of lung micrometastasis in animals of each treatment cohort.
Combination therapy of antibody neutralizing sMIC and anti-CTLA4 antibody markedly over-turns antigen-specific CD8 T cell tolerance

We have previously shown that B10G5 monotherapy overcomes antigen-specific CD8 T cell tolerance (42). To determine whether combination therapy with B10G5 and anti-CTLA4 antibody can further enhance CD8 T cell antitumor response in an antigen-specific manner, we adoptively transferred carboxyfluorescein succinimidyl ester (CFSE)–labeled SV40TAg–specific TCR-I CD8 T cells into cohorts of mice that had received therapies for 4 weeks, as indicated and schematically depicted in Fig. 1A. Notably, these TCR-I CD8 T cells bear TCRs specific for the TRAMP/MIC oncogene SV40TAg and can be detected by the SV40TAg peptide I–specific D9/I-tetramer.

Adoptively transferred antigen–specific TCR-I CD8 T cells would normally fail to sustain after initial expansion in TRAMP or TRAMP/MICB mice because of clonal deletion (42, 54). Whereas anti-CTLA4 antibody monotherapy only had a marginal effect on sustaining D9/I-tetramer+ TCR-I CD8 T cells in dLNs, tumor infiltrates, or spleen in TRAMP/MICB mice, B10G5 therapy achieved consistent sustainability of the adoptively transferred TCR-I CD8 T cells with a great percentage in the tumor infiltrates, as we have previously presented (Fig. 4A and fig. S8A) (42). Remarkably, the combination antibody therapy with B10G5 and anti-CTLA4 further significantly enhanced the sustainability of TCR-I CD8 T cells compared to B10G5 monotherapy (Fig. 4A and fig. S8A).

Adoptively transferred naïve D9/I-tetramer+ TCR-I CD8 T cells experienced initial expansion, as shown by the dilution of CFSEhi D9/I-tetramer+ CD8 T cells in all cohorts of animals (Fig. 4B and fig. S8B). However, therapy with B10G5 or B10G5 combined with anti-CTLA4 antibody provoked the continuous expansion of D9/I-tetramer+ CD8 T cells, shown as a significantly increased percentage of CFSElo D9/I-tetramer+ CD8 T cells compared to cIgG therapy (Fig. 4B and fig. S8B). The combination therapy resulted in a substantially higher percentage of CFSElo D9/I-tetramer+ CD8 T cells compared to B10G5 monotherapy (Fig. 4B and fig. S8B).

The continuous expansion of antigen–specific CD8 T cells in the tumor microenvironment and tumor–dLNs is an indication of the activation of antigen–specific CD8 T cells. Anti-CTLA4 antibody monotherapy generally did not elicit a benefit in activating D9/I-tetramer+ CD8 T cells (Fig. 4, C and D). Consistent with our previous studies (42), B10G5 monotherapy evoked a significantly higher percentage of CD44hi D9/I-tetramer+ CD8 T cells (Fig. 4C and fig. S8C) and greater intrinsic cellular response to restimulation (Fig. 4D and fig. S8D). Combination therapy of B10G5 and anti-CTLA4 antibody induced a more pronounced increase in the number of CD44hi D9/I-tetramer+ CD8 T cells (Fig. 4C and fig. S8C) and in IFN-γ production by D9/I-tetramer+ CD8 T cells in response to PMA/ionomycin restimulation (Fig. 4D and fig. S8D). Together, these data further reveal the effector mechanism whereby combination therapy of B10G5 and anti-CTLA4 antibody heightens antigen–specific CD8 T cell antitumor responses.

B10G5 neutralizing sMIC and anti-CTLA4 antibody cooperatively heighten DC costimulatory potential

We investigated the mechanisms whereby the cocktail therapy with B10G5 and anti-CTLA4 antibody would synergistically bolster antigen–specific CD8 T cell responses. We previously showed that B10G5 therapy alone can enhance DC activation in tumor–dLNs and increase the expression of the DC costimulatory molecules CD80 and CD86 (42). With the antibody cocktail therapy, a further significant increase occurs in the DC surface costimulatory molecules CD80 and CD86, along with the DC activation molecule CD40 (Fig. 5, A and B). Such an effect was not observed in non–tumor–dLNs (fig. S9). Given that CD80 and CD86 can engage both the costimulatory molecule CD28 and the co-inhibitory molecule CTLA4, these data indicate that B10G5 neutralizing sMIC in combination with CTLA4 blockade would allow more robust CD28–mediated costimulatory signal delivered to antigen–specific CD8 T cells that may in part confer the synergistic effect on sustaining antigen–specific CD8 T cell activity.

Cotargeting sMIC increases TCR clonality and repertoire complexity

In general, better clinical outcome in response to immunotherapy is associated with higher clonal expansion of CD8 T cells in patients (55–58).
In metastatic melanoma and castration-resistant prostate cancer patients, improved survival in response to anti-CTLA4 antibody therapy has been shown to be associated with TCR clonal-type stability as well as repertoire complexity (55, 57). Surveying TCRβ3 sequences within the TRAMP/MIC prostate tumor tissues using in situ immunoSEQ technology revealed that B10G5 cotargeting sMIC remarkably increased not only TCR content in tumor tissues but also TCR clonality (Fig. 6, A and B). Immunohistochemistry (IHC) assessing CD8 T cell infiltration in tumors further confirmed the relevance of an increase in tumor TCR content to B10G5 cotargeting sMIC (Fig. 6C). The data indicate that cotargeting sMIC facilitates the recruitment of T cells into the tumors and the subsequent expansion, which presumably leads to the enhanced therapeutic effect of CTLA4 blockade.

**B10G5 therapy stabilizes CD3ζ in CD8 T cells**

We sought to further understand the mechanisms whereby anti-sMIC therapy increases antigen-specific CD8 T cell maintenance during anti-CTLA4 antibody therapy. In vitro studies have shown that sMIC not only down-modulates NKG2D expression but also impairs the TCR/CD3 complex signaling of CD8 T cells by destabilizing its key downstream signaling molecule CD3ζ (38). We thus analyzed the intracellular levels of CD3ζ in splenic CD8 T cells from the cohorts of experimental animals by flow cytometry. Unstimulated splenic CD8 T cells from TRAMP/MIC mice with high levels of circulating sMIC (sMICh) had diminished intracellular CD3ζ with anti-CTLA4 antibody therapy compared to those with low levels of circulating sMIC (sMICl) (Fig. 7A). All TRAMP/MICB mice that received B10G5 and anti-CTLA4 antibody therapy had an increased intracellular level of CD3ζ in CD8 T cells (Fig. 7A). A pronounced increase in the level of CD3ζ was also observed in prostate tissues with B10G5 cotargeting sMIC (Fig. 7B). These data suggest that neutralizing sMIC to stabilize CD3ζ and thus sustain TCR activation would allow clonal expansion of antigen-specific T cells and increases in TCR clonality in tumor infiltrates.

**DISCUSSION**

Using a clinically relevant TRAMP/MICB double transgenic mouse model that closely recapitulates the onco-immunology dynamics of human cancer (39), we demonstrate that the antibody cocktail cotargeting the soluble NKG2D ligand sMIC not only remarkably augmented the therapeutic efficacy of immune checkpoint blockade anti-CTLA4 antibody but also generated a cooperative therapeutic effect with anti-CTLA4 therapy. We show that a cocktail therapy of antibody neutralizing the immunosuppressive effect of sMIC and CTLA4 blockade cooperatively primed DC activation and up-regulated the expression of CD80/CD86 costimulatory molecule on DCs, overcame CD8 T cell tolerance, enhanced TCR/CD3 signaling capacity in CD8 T cells, and increased T cell clonality or repertoire complexity in tumor infiltrates. Given the clinical observations that melanoma patients who developed anti-sMIC autoantibody during anti-CTLA4 therapy displayed better prognosis (47), this is the first proof-of-concept preclinical study to demonstrate that antibody cotargeting soluble NKG2D sMIC and anti-CTLA4 antibody therapy can generate a cooperative therapeutic effect. Our study launched a new viable avenue of combination immune therapy of cancer.

The anti-CTLA4 antibody ipilimumab was approved by the FDA for the treatment of unresectable or metastatic melanoma; however, ipilimumab was discontinued for adverse reactions in 10% of patients in a clinical trial for melanoma (www.cancer.gov/about-cancer/treatment/drugs/ida-ipilimumab). In clinical trials with castration-resistant prostate cancer patients, there were ipilimumab toxicity–related deaths, although some subgroups of patients benefited from ipilimumab (18, 59–62). Of all the treatment toxicity–related clinical manifestations, the IRAE of colitis was the most frequent and most severe event that had even led to death (13, 20). Currently, there is no biomarker to predict the patient population that will elicit colitis-related adverse reactions to ipilimumab therapy. Our unexpected findings in TRAMP/MIC mice suggest that high levels of tumor-derived sMIC may be one of the co-founders and also indicators for anti-CTLA4 antibody therapy–induced autoimmune
colitis in MIC+ cancer patients. We acknowledge that our current findings cannot be generalized to MIC− cancer patients. However, because MIC is broadly expressed by nearly all human tumors, prescreening cancer patients for serum sMIC may be necessary to eliminate the potential intestinal inflammation–related IRAEs of anti-CTLA4 antibody therapy. Although not addressed here, large-scale retrospective clinical investigations on anti-CTLA4 antibody therapy are warranted to support this concept.

Our data demonstrate that multiple pathways may be involved in the cooperative therapeutic effects of antibody targeting sMIC and anti-CTLA4 antibody. We previously showed that monotherapy with the sMIC-neutralizing antibody B10G5 up-regulates CD80 and CD86 expression on DCs in tumor-dLNs (42). Therefore, neutralizing sMIC in combination with anti-CTLA4 antibody would provide superior availability of CD80 and CD86 to CD28 compared to respective monotherapy. This mechanism was supported by our data demonstrating that combination therapy resulted in a marked increase in the expression of CD80 and CD86 on DCs from tumor-dLNs and tumor parenchyma. Given that NKG2D and CD28 are known to provide nonredundant costimulatory signals to CD8 T cells (35) and that neutralizing sMIC has been shown to restore surface NKG2D expression on CD8 T cells (42), increased dual costimulation by NKG2D and CD28 would also contribute to the heightened effects of combination therapy. Last, colleagues and our current study consistently showed that sMIC destabilizes the critical TCR signaling molecule CD3ζ on CD8 T cells during prolonged stimulation (38), which would impair antigen-specific CD8

**Fig. 5.** B10G5 and anti-CTLA4 antibody therapy cooperatively increases DC activation (CD40) and the expression of costimulatory molecules CD80 and CD86 in tumor sites. (A) Representative histograms from flow cytometry analyses of CD40, CD80, and CD86 expression on DCs from tumor-dLNs and tumor beds. Gray-filled profiles, control isotype staining; open dark profiles, antibody to specific DC surface molecules. (B) Summary data of mean fluorescence intensity (MFI) of CD40, CD80, and CD86 on DCs.
T cell function and, in turn, restrict its clonal expansion. Thus, stabilizing the TCR/CD3 complex and restoring antigen-specific TCR signaling in CD8 T cells by B10G5 also contribute to the enhanced response to anti-CTLA4 antibody therapy. Our data of increased TCR repertoire clonality and CD3ε expression in the tumor tissue in response to B10G5 therapy corroborated this underlying mechanism.

Notably, animals with high levels of circulating sMIC developed severe colon inflammation or colitis in response to anti-CTLA4 antibody therapy; an antibody neutralizing sMIC alleviated this adverse autoimmune effect. This biology was never observed or reported in other preclinical mouse models with anti-CTLA4 antibody therapy. The NKG2D signaling pathway has been associated with inflammatory bowel disease in humans (63–65); however, this finding is not conclusive in mice because of the differences in the models studied, some of which do not naturally express NKG2D ligands (66, 67).

We also observed progressive lung metastasis in sMIChi TRAMP/MICB mice in response to anti-CTLA4 antibody monotherapy. One limitation to the current study is that we were not be able to determine whether increased lung metastasis and development of colitis were related as a cause and an effect or whether both were the collateral or concurrent events of a common cause. Studies have shown that anti-CTLA4 antibody therapy selectively propagates specific species of gut microbiota (68). Studies have also shown that gut microbiota could regulate NKG2D expression in the gut and that NKG2D signaling in IFN-γ-producing intestinal CD4 T cells plays a role in the pathogenesis of colitis (66, 69, 70). Together, it is conceivable to propose that anti-CTLA4 antibody therapy–induced colitis in sMIChi mice may result from the interplay of microbiota composition and NKG2D signaling in pathogenic CD4 T cells in the gut. Moreover, gut microbiota has also been shown to regulate the therapeutic efficacy of CTLA4 blockade (68). Thus, whether the development of colitis and increased lung metastasis are related and how they interplay could be complicated biological events. These questions are under active investigation.

MIC is a human molecule that is not expressed by rodents. Because of this limitation, preclinical studies on CTLA4 blockade therapy to date have not had the opportunity to consider the immune modulatory impact of sMIC. Using an “MIC humanized” double transgenic model, we demonstrated that antibody neutralizing sMIC not only enhances the therapeutic efficacy of anti-CTLA4 antibody but also generates a cooperative therapeutic effect with anti-CTLA4 antibody. Given that sMIC was prevalent in a broad range of malignancies and that anti-CTLA4 antibody is currently in active clinical trial for cancers other than melanoma, our study revealed a new avenue to enhance the therapeutic effect of anti-CTLA4 antibody and concurrently eliminate therapy-induced autoimmune colitis. This concept was supported by a published case report in a melanoma patient (47) and our current case study.

**Fig. 6.** B10G5 cotargeting sMIC during CTLA4 blockade therapy increases T cell content and TCR clonality in tumors. DNA were isolated from paraffin-embedded tumor sections and subjected to immunoSEQ assay, as previously described (Adaptive Biotechnologies). Tumor in situ TCR abundance (A) and clonality (B) were assayed by surveying TCRβ expression and analyzed with the Shannon diversity index using immunoSEQ Analyzer software. (C) Representative IHC confirming increased CD8 T cell infiltration in tumors with B10G5 cotargeting sMIC.
study in a metastatic prostate cancer patient that reveals the association of better clinical prognosis with the development of anti-sMIC autoantibody during early response to ipilimumab. The impact of sMIC on colon inflammation during anti-CTLA4 antibody therapy is an unexpected aspect of our findings. Although the underlying mechanisms may be complex and are not currently understood, our findings nevertheless also suggest a potential clinical biomarker to predict responsiveness or toxicity of anti-CTLA4 antibody therapy in cancer patients. Large-scale clinical investigations are warranted.

**MATERIALS AND METHODS**

**Antibodies and flow cytometry**

Single-cell suspensions from spleens, dLNs, non-dLNs, or tumor tissues were prepared as previously described (42). Combinations of the following fluorochrome-conjugated antibody were used for cell surface or intracellular staining to define populations of NK, CD8, and subsets of CD4 T cells: CD3ε (clone 145-2C11), CD8α (clone 53-6.7), CD4 (clone GK1.5), NK1.1 (clone PK136), NKG2D (clone CX5), CD44 (clone eBio4B10), CD11c (clone N418), MHCII (clone M5/114.15.2), CD80 (clone 16-10A1), CD86 (clone PO3), CD40 (clone 1C10), and CD3ζ (clone 6B10.2, BioLegend). For ex vivo restimulation, freshly isolated single-cell suspension was cultured in complete RPMI 1640 medium containing PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hours before it was analyzed for IFN-γ production by intracellular staining with an antibody specific to IFN-γ (XMG1.2). Multicolored flow cytometry analyses were performed on LSR II (BD). Data were analyzed with FlowJo software (Tree Star).

**Animals and antibody therapy**

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina (MUSC). Mice were maintained at the MUSC Hollings Cancer Center animal facility under specific pathogen-free conditions. Male TRAMP mice at the age of 27 to 28 weeks were randomized into two treatment cohorts: (i) cIgG or (ii) anti-CTLA4 antibody (clone 9H10, BioXCell). In parallel, age-matched TRAMP/MIC double transgenic mice were randomized into four treatment cohorts: (i) anti-sMIC mAb B10G5 [which was previously described (39)], (ii) anti-CTLA4 antibody (clone 9H10), (iii) a cocktail of B10G5 and anti-CTLA4 antibody, and (iv) a cocktail of cIgGs. All antibodies were administered at a dose of 3 mg/kg body weight through intraperitoneal injection twice per week. All animals received the treatment for 8 weeks before they were euthanized at the study end point, unless they succumbed to diseases or adverse effects at an earlier time point.

**Antigen-specific T cell tolerance experiment**

The experimental procedure was described previously (42). Briefly, CD8 T cells from TCR-I transgenic mice were labeled with CFSE and injected intravenously into animals (2 × 10^6 cells per mouse) in the respective experimental groups. Animals were sacrificed at the indicated time points to assess TCR-I T cell in vivo frequency with TCR-I–specific H-2D^b/TAg epitope I–tetramer (D^b/I-tetramer) (71). To assay antigen-specific CD8 T cell responses, a bulked single-cell suspension prepared from the spleen, lymph nodes, and tumor digests was stimulated overnight with 0.5 μM TAg epitope I peptide (SAINNYAQKL) and assayed for intracellular IFN-γ expression of CD8+ or D^b/I-tetramer+ T cells by flow cytometry.

**Tissue collection**

Blood was collected via tail bleeding during therapy and via cardiac puncture after euthanasia. Splenocytes, tumor-dLNs, and non–tumor-dLNs were collected for immunological analyses. Prostates, lungs, livers, kidneys, pancreata, and colons were collected and fixed in 10% neutral fixation buffer, followed by paraffin or Tissue-Tek OCT (optimum cutting temperature) compound embedding for pathological and histological examinations. In some experiments, portions of prostate tumors were used for parathion in single-cell suspensions.

**Histology and IHC staining**

All collected tissues were sectioned and stained with hematoxylin and eosin for histological evaluation. For IHC staining to detect specific antigens, the following antibodies were used: anti-Ki67 (Neomarkers), anti-cleaved caspase-3 (clone SA1E, Cell Signaling), anti-CD8 (BD Biosciences), anti-NK1.1 (PK136, eBioscience), anti-SV40Tag (Santa Cruz), anti–arginase I (Santa Cruz), and anti-p63 (Neomarkers). The IHC staining protocol has been previously described (39, 42). All sections were counterstained with hematoxylin for nucleus visualization.
Serum sMIC and cytokine detection

Serum levels of sMIC were assessed using respective sandwich enzyme-linked immunosorbent assay kits (R&D Systems). Serum levels of cytokines were assayed by the Eve Technologies Corporation using the Luminex technology.

High-throughput TCR sequencing in tumor tissues

A high-throughput TCR sequence survey from paraffin-embedded tissue was performed according to protocol described elsewhere (56). Briefly, sections from paraffin blocks of the prostate were collected for DNA extraction using the QIAamp DNA FFPE Tissue Kit (QIAGEN Inc.), per the manufacturer’s instructions. The TCRβ CD3 (CDR3β) region was amplified, sequenced, and quantified from a standardized 1200 ng of DNA using the immunoSEQ assay as previously described (Adaptive Biotechnologies) (56, 72). TCR sequence and clonality were determined by the Shannon diversity index using immunoSEQ Analyzer software (Adaptive Biotechnologies) (56).

Statistical analysis

All results are means ± SEM, unless specified otherwise. Mouse and sample group sizes were n > 5, unless indicated otherwise. Data were analyzed using the analysis of variance unpaired t test. Differences between means were considered significant at P < 0.05. Kaplan-Meier survival curves were generated and analyzed using GraphPad Prism software.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1602133/DC1

fig. S1. Induction of subclinical colitis in sMIC+ TRAMP/MIC mice in response to anti-CTLA4 therapy.

fig. S2. Recapitulation of the negative effect of sMIC on anti-CTLA4 therapy in a transplantable tumor model.

fig. S3. Antibody neutralizing sMIC eliminates colitis in TRAMP/MIC mice that received anti-CTLA4 therapy.

fig. S4. Detection of anti-sMIC autoantibody in the sera of a small cohort of prostate cancer patients who have metastatic disease and enrolled in a clinical trial (NCT01499978) of ipilimumab in combination with hormone suppression at the Knight Cancer Institute.

fig. S5. Representative graphs of flow cytometry analyses demonstrate that combination therapy of anti-sMIC and anti-CTLA4 antibody remarkably increases CD8 T cell population, activation, and functional potential in tumor-draining lymph nodes and tumor beds.

fig. S6. Marginal response of CD8 T cells to anti-CTLA4 therapy in TRAMP/MIC mice compared to MIC-negative TRAMP mice.

fig. S7. Circulating sMIC or anti-sMIC autoantibody affects response to anti-CTLA4 therapy in TRAMP/MIC mice compared to MIC-negative TRAMP mice.

fig. S8. Representative graphs of flow cytometry analyses demonstrate that combination therapy of anti-sMIC antibody and anti-CTLA4 antibody cooperatively enhances antigen-specific CD8 T cell anti-tumor responses.

fig. S9. Therapy has no effect on the activation or costimulatory molecule on DCs in the spleen or non-tumor-dLNs.

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Antibody-mediated neutralization of soluble MIC significantly enhances CTLA4 blockade therapy
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