STING Ligand c-di-GMP Improves Cancer Vaccination against Metastatic Breast Cancer

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Abstract

Cancer vaccination may be our best and most benign option for preventing or treating metastatic cancer. However, breakthroughs are hampered by immune suppression in the tumor microenvironment. In this study, we analyzed whether cyclic diguanylate (c-di-GMP), a ligand for stimulator of interferon genes (STING), could overcome immune suppression and improve vaccination against metastatic breast cancer. Mice with metastatic breast cancer (4T1 model) were therapeutically immunized with an attenuated Listeria monocytogenes (LM)-based vaccine, expressing tumor-associated antigen Mage-b (LM-Mb), followed by multiple low doses of c-di-GMP (0.2 μmol/L). This treatment resulted in a striking and near elimination of all metastases. Experiments revealed that c-di-GMP targets myeloid-derived suppressor cells (MDSC) and tumor cells. Low doses of c-di-GMP significantly increased the production of IL12 by MDSCs, in correlation with improved T-cell responses to Mage-b, whereas a high dose of c-di-GMP (range, 0.3–3 mmol/L) activated caspase-3 in the 4T1 tumor cells and killed the tumor cells directly. On the basis of these results, we tested one administration of high-dose c-di-GMP (3 mmol/L) followed by repeated administrations of low-dose c-di-GMP (0.2 μmol/L) in the 4T1 model, and found equal efficacy compared with the combination of LM-Mb and c-di-GMP. This finding correlated with a mechanism of improved CD8 T-cell responses to tumor-associated antigens (TAA) Mage-b and Survivin, most likely through cross-presentation of these TAA from c-di-GMP–killed 4T1 tumor cells, and through c-di-GMP–activated TAA-specific T cells. Our results demonstrate that activation of STING-dependent pathways by c-di-GMP is highly attractive for cancer immunotherapy. Cancer Immunol Res; 2(9); 901–10. ©2014 AACR.

Introduction

Cancer vaccination has shown great promise for preventing or treating metastatic cancer in mice and humans (1–3). However, immune suppression in the tumor microenvironment (TME) remains a potential limitation to immunotherapy. Myeloid-derived suppressor cells (MDSC) are one of the most important players in mediating TME-associated immune suppression because they strongly inhibit T-cell and natural killer (NK)–cell responses (4–6), with tumor-associated macrophages, Tregs, and M2 macrophages also playing a role (4–8). Adjuvants reducing immune suppression are of great value for cancer vaccination. c-di-GMP (3',5'-cyclic diguanylic acid), also known as cyclic diguanylate, could be such an adjuvant. c-di-GMP is a bacterial intracellular signaling molecule that was initially identified by the Benziman laboratory in the bacterium Acetobacter xylinum (renamed Gluonacetobacter xylitilus; ref. 9). Various in vitro and in vivo animal model studies using chemically synthesized c-di-GMP demonstrated that c-di-GMP has potent immunomodulatory effects on cellular components of both innate and adaptive immunity in infections with such bacteria as Klebsiella pneumoniae (10–12). Recently, stimulator of interferon genes (STING) has been identified as the sensor for c-di-GMP (13). STING is a transmembrane protein expressed in macrophages and dendritic cells (DCs; refs. 14–16). STING is mainly expressed in the thymus, heart, spleen, placenta, lung, and peripheral leukocytes, but is poorly expressed in the brain, skeletal muscle colon, small intestine, liver, and kidneys (14). Because of the strong immunomodulatory effects of c-di-GMP, we evaluated whether STING-dependent c-di-GMP could improve cancer vaccination through bypassing immune suppression and stimulating T-cell responses in mice with metastatic breast cancer.

As a vaccine, we used a highly attenuated Listeria monocytogenes (LM) bacterium expressing tumor-associated antigen (TAA) Mage-b (Mb), which was developed in an earlier study (3). This attenuated LM is different from wild-type LM (17, 18) in that the attenuated LM does not multiply in normal tissues and is naturally cleared by the immune system within 3 to 5 days (5, 19, 20). Mage-b is highly expressed in metastases and primary breast tumors of the 4T1 model (3), and is homologous with human melanoma-associated antigen.

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doi: 10.1158/2326-6066.CIR-13-0123

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(MAGE; ref. 21). MAGE is expressed in 90% of all breast cancers (22). LM is an intracellular pathogen that delivers the vaccine antigen directly into antigen-presenting cells (APC) such as macrophages with high efficiency (23). The vaccine antigen produced by LM is processed and presented as short peptides via the MHC class I and II pathways generating both CD4 and CD8 T-cell responses (24). Killing of tumor cells occurs through CD8 T cells. Although semiprophylactic immunizations with LM-Mb (one before and two after tumor development) were highly effective against metastatic breast cancer, this effect was less abundant with a more clinically relevant immunization protocol of three exclusive therapeutic vaccinations (after tumor development; ref. 20) due to the strong immune suppression in the TME. Therefore, reducing immune suppression and improving T-cell responses to TAAs in the TME was the most important goal in this study, and c-di-GMP seemed an extremely suitable candidate.

Here, we demonstrate that c-di-GMP exhibits various mechanisms to combat metastatic breast cancer. Low doses of c-di-GMP provided strong adjuvant effects in LM-Mb vaccinations by reducing the MDSC population (highly expressing STING), by converting a subpopulation of immune-suppressing MDSCs into an immune-stimulating phenotype producing IL12, and by improving CD8 T-cell responses to TAA Mage-b delivered through LM. High doses of c-di-GMP activated caspase-3 and killed tumor cells directly. This unique combination of therapeutic low doses of c-di-GMP and LM-Mb resulted in an almost complete elimination of the metastases. Moreover, one high-dose c-di-GMP treatment followed by multiple low doses of c-di-GMP in a therapeutic setting was equally effective compared with LM-Mb + c-di-GMP, and showed improved CD8 T-cell responses to Mage-b and Survivin, most likely through cross-presentation of TAAs of c-di-GMP–killed tumor cells and through activation of the T cells by multiple low doses of c-di-GMP. These dramatic results with c-di-GMP are highly promising for human clinical application.

Materials and Methods

Mice

Normal female BALB/c mice (3 months) were obtained from Charles River and maintained in the animal husbandry facility of Albert Einstein College of Medicine according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and the Albert Einstein Institute for Animal Studies. All mice were kept under biosafety level 2 conditions, as required for LM.

Cells and cell culture

The 4T1 cell line, derived from a spontaneous mammary carcinoma in a BALB/c mouse (25), was cultured in DMEM supplemented with 10% FBS, 1 mmol/L mixed nonessential amino acids, 2 mmol/L L-glutamine, insulin (0.5 HSP units/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). This cell line was developed by Dr. Fred Miller, Karmanos Cancer Institute (Detroit, MI), and an early passage (passage 25) was kindly provided in June 2004. The 4T1 cell line is extremely metastatic in BALB/c mice (syngeneic background strain). This extreme metastatic character has been verified in BALB/c mice within the past 2 months. The cell line has a characteristic growth pattern, i.e., it forms mammosphere-like balls in cell culture. This observation has been verified within the past 2 months by inverted light microscopy. The 4T1 cell line expresses TAA Mage-b and has been verified within the past 6 months by RT-PCR. The 4T1 cell line is Mycoplasma free.

The HEK293T cell line was purchased from the ATCC (CRL-11268) in 2009. This is a human epithelial kidney cell line. This cell line is often used as a negative control for STING expression in Western blots. In this study, HEK293T was negative for STING expression by Western blotting. The HEK293T cell line is Mycoplasma free.

Plasmids, LM, and c-di-GMP

The LM-Mb strain was developed in an earlier study (3). This was constructed in the prfA-negative XFL-7 strain (referred to as LM in this study), which lacks the positive regulatory factor A that is a central mediator of virulence (26). The vaccine strain was transformed with LM plasmid pGG-34, which encodes prfA and amino acids 311–660 of murine Mage-b fused to a noncytolytic form of Listeriolysin O (LLO; refs. 3, 27). Complementation of prfA expression by the plasmid does not fully restore virulence, but enforces retention of the plasmid during infection (26, 27).

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c-di-GMP (provided by D. Karaolis, Karagen Pharmaceuticals, Frederick, MD), used in these studies, was synthesized and prepared as previously described (28). Stock solutions of c-di-GMP were generated at 3 mmol/L in saline (equals 150 nmol in 50 μL). For in vivo experiments, 0.2 μmol/L per mouse was used, and for in vitro experiments, a range of 9.3 μmol/L to 75 μmol/L was used as indicated in the text.

Immunization and tumor challenge

Various immunization protocols have been tested in a metastatic breast tumor model 4T1, as described previously (3).

Semiprophylactic protocol A. Mice were immunized i.p. with c-di-GMP (3 mmol/L equals 150 nmol in 50 μL) on days 0, 7, and 14, and with LM-Mb (10⁷ CFU) i.p. on days 1, 8, and 15, while 4T1 tumor cells (10⁴) were injected into the mammary fat pad on day 3.

Therapeutic protocol B (high-dose LM-Mb and c-di-GMP). Mice were injected with 4T1 tumor cells (10⁴) in the mammary fat pad on day 0, immunized i.p. with c-di-GMP (3 mmol/L equals 150 nmol in 50 μL) on days 3, 10, and 17, and with LM-Mb (10⁷ CFU; i.p.) on days 4, 11, and 18.

Therapeutic protocol C (low-dose LM-Mb and c-di-GMP). 4T1 tumor cells (0.5 × 10⁷) were injected into the mammary fat pad on day 0, and c-di-GMP (0.2 μmol/L equals 0.01 nmol in 50 μL) was administrated every day i.p., starting on day 3, whereas LM-Mb (10⁷ CFU) was administrated i.p. on days 4, 7, 10, 13, and 16.

Therapeutic protocol D (high- vs. low-dose c-di-GMP). 4T1 tumor cells (0.5 × 10⁷) were injected into the mammary fat pad on day 0, and one high dose of c-di-GMP (3 mmol/L) equals 150 nmol in 50 μL was administrated on day 4, followed by i.p. low-dose c-di-GMP (0.01 nmol) every day for 16 days. A detailed schematic view of all immunization protocols is shown in Supplementary Fig. S1A–S1D. All mice were euthanized on day
19 and analyzed for the number of metastases and tumor growth. Primary tumors extend to the chest cavity lining and metastases predominantly to the mesenteric lymph nodes, and less frequently to the diaphragm, portal liver, spleen, and kidneys (20).

Isolation of MDSCs

Monocytic and granulocytic MDSCs (mMDSC and gMDSC, respectively) were isolated from spleen cell suspensions according to the manufacturer's instructions (Myeloid-Derived Suppressor Cell Isolation Kit; Miltenyi Biotec) as described previously (5). For separation of the magnetically labeled cells, the Automacs Proseparator (Miltenyi Biotech) was used. As previously described (3), the supernatant from vaccinated or control mice was used in the restimulation of the spleen 72 hours after injection (3). Furthermore, Survivin66 or control mice were transfected with pcDNA3.1-Mage-b plasmid (G. Lauvau, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY) during c-di-GMP treatment (five injections 3 days apart). All mice were euthanized 2 days after the last injection of c-di-GMP, and analyzed for tumor weight and number of metastases. After depletion, the percentage of CD8 T cells in the spleen was less than 1%. As control, isotype-matched rat antibodies against HRPN were used.

Flow cytometry analysis

Immune cells from spleen, blood, lymph nodes, or tumors of treated and control mice were isolated as described previously (30). To identify MDSCs, anti–CD11b-Alexa488/PerCP-cy5.5 and anti–Gr1-PerCP-cy5.5 (clone RB6-8C5) antibodies were used. The CD11b+Gr1high population represents the gMDSC population and the CD11b+Gr1low the mMDSC population. To analyze various subsets of immune cells of 4T1 tumor–bearing mice involved in antitumor responses in spleens, tumors, and lymph nodes, anti–CD3-Alexa488, anti–CD4-PerCP-cy5.5, anti–CD8-APC, antiCD49d-PE (NK cells), anti–CD19-FITC (B cells), and anti–CD11c-FITC antibodies were used. All cell populations in the tumor were analyzed within the CD45-positive population using anti–CD45-FITC/APC antibody. For the maturation of MDSCs and DCs in the spleens of tumor-bearing mice, we used anti–CD80-APC, anti–CD86-PE, and anti-MHC class II-PE antibodies. To detect the production of intracellular cytokines, the cytokine ELISA Kit from BD Pharmingen was used according to the manufacturer's instructions, and antibodies to IFNγ and IL12 were used. Appropriate isotype controls were used for each sample. Depending on the sample size, 10,000 to 50,000 cells were acquired by scanning using a Fluorescence Activated Cell Sorter (flow cytometry; Beckton and Dickinson, Excalibur), and analyzed using FlowJo 7.6 software. Cell debris and dead cells were excluded from the analysis based on scatter signals and use of Fixable Blue or Green Live/Dead Cell Stain Kit (Invitrogen). All antibodies were purchased from BD Pharmingen or eBiosciences.

MTT test and cell death

4T1 cells or HEK293T cells (2,000 cells in 0.1 mL) were cultured with different doses of c-di-GMP as indicated in the text for 24 hours, then cell viability was analyzed by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) method at a wave length of 570 nm. Cell death in c-di-GMP–treated cultures was calculated by subtracting percent live cells from nontreated cells (100%).

Caspase-3 analysis

4T1 tumor cells were treated with different concentrations of c-di-GMP as indicated in the text, fixed in 10% neutral buffered formalin, and permeabilized with 0.1% Triton X100 for 30 minutes. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide for 10 minutes, and blocked by 5% normal donkey serum and 2% BSA for 1 hour. The cells were then stained by IHC methods, using a primary antibody to active caspase-3 (rabbit anti-mouse IgG; Cell Signaling Technology) 1:50 dilution for 1 hour at room temperature, followed by a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature (Invitrogen), and then followed with diaminobenzidine as the final chromogen. All slides were briefly counterstained with hematoxylin.

STING analysis

Expression of STING was analyzed by Western blotting. Tissues were homogenized with 1 mL of PBS containing 0.1% Triton-X100, 2 mmol/L EDTA, and protease inhibitors by using Mini Bead Beater for 2 to 5 × 30 seconds. Cultures of
4T1 tumor cells and MDSCs were centrifuged and resuspended in 400 μL of ice-cold lysis buffer (1 mol/L HEPES, 0.5 mol/L EDTA, 0.1 mol/L EGTA, 2 mol/L KCl, 0.1 mol/L DTT, cocktail of protease inhibitors 5 μg/mL, and 10% NP-40). The lysates (20 μg) were resuspended in 10% SDS-polyacrylamide gels, and proteins were electro-transferred to PVDF membranes. Membranes were probed with rabbit antibodies against STING (Cell Signaling Technology). Antibody to β-actin was used to ensure equal loading. Membranes were incubated with HRP-conjugated anti-rabbit goat IgG secondary antibodies (Cell Signaling Technology), and detection was obtained using a chemiluminescence detection kit (Thermo Scientific).

Statistical analysis
To statistically analyze the effects of LM-Mb and/or c-di-GMP on the growth of metastases and tumors and on immune responses in the mouse models, the unpaired t test and the Mann–Whitney test were used. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. Values of P < 0.05 were considered statistically significant.

Results
High doses of c-di-GMP and LM-Mb completely eliminated metastatic breast cancer without T-cell contribution in a semiprophylactic setting
Immune suppression in the TME is a major problem in cancer vaccination (5, 7), and adjuvants that can reduce or convert immune suppression are greatly needed. c-di-GMP has shown promising immune stimulatory effects on innate and adaptive immune responses in bacterial infections (10). To analyze whether c-di-GMP could also induce immune stimulatory effects on the immune system in cancer vaccination, tumor-bearing mice were immunized with LM-Mb and c-di-GMP in semiprophylactic setting (protocol A). This combination protocol completely eliminated the metastatic breast cancer (Fig. 1A and B), a result that could not be obtained with c-di-GMP or LM-Mb alone. Most interestingly, this striking result was not due to improved CD8 T-cell responses to Mage-b and LM. In contrast, it seemed that c-di-GMP did not increase but decreased Mage-b–specific (Fig. 1C) or LM-specific (Fig. 1D) CD8 T-cell responses in the spleen of vaccinated and control mice. Because c-di-GMP alone was highly effective against metastases but did not improve T-cell responses, we further investigated the mechanism of action of c-di-GMP. We also tested LM-Mb and c-di-GMP therapeutically and found that the number of metastases was reduced by 73% and tumor growth by 33% compared with the saline-only group (Supplementary Fig. S2A and S2B).

High doses of c-di-GMP activated caspase-3 in 4T1 tumor cells
Because high doses of c-di-GMP had an effect on the metastases and primary tumor in vivo, we determined whether c-di-GMP could kill tumor cells directly. We found that 75 μmol/L (equals 15 nmol in 200 μL) of c-di-GMP reduced the growth of 4T1 tumor cells in vitro by 70% (MTT test; Fig. 2A), and 750 μmol/L (equals 150 nmol in 200 μL) by 92% (Supplementary Fig. S3). To analyze the mechanism of tumor cell killing in more detail, we determined caspase-3 expression at various doses of c-di-GMP. We found that 37.5 and 75 μmol/L (equals 7.5 and 15 nmol in 200 μL) of c-di-GMP induced caspase-3 expression in 40% and 20% of the 4T1 tumor cells, respectively (Fig. 2B and C). Moreover, most of the remaining tumor cells died. Because c-di-GMP acts through interaction with its sensor STING, we analyzed 4T1 tumor cells for STING expression by Western blotting. As shown here, STING is expressed in 4T1 tumor cells and bone marrow cells (positive control), and even at much higher levels in the 4T1 metastases and primary tumor, whereas STING could not be detected in HEK293T cells (negative control; Fig. 2D). c-di-GMP had significantly less effect on the viability and cell death of STING-negative HEK293 cells than of STING-positive 4T1 at doses of 37.5 and 75 μmol/L (Supplementary Fig. S4).

Low doses of c-di-GMP improved efficacy of LM-Mb and T-cell responses to Mage-b in a therapeutic setting
On the basis of the results described above, we hypothesized that if high doses could kill tumor cells directly, it may be toxic for T cells as well. Therefore, we tested whether low doses of c-di-GMP were effective against the metastases and could improve T-cell responses. In addition, we combined this with a clinically more relevant therapeutic immunization protocol (protocol B). Various lower doses of c-di-GMP (range, 0.5–2 mmol/L) were effective against metastases and the primary tumor (Supplementary Fig. S5A and S5B), but when combined with LM-Mb, c-di-GMP did not improve T-cell responses to Mage-b. Therefore, we further decreased the dose of c-di-GMP (range, 0.2–200 μmol/L) and found that the administration of 0.2 μmol/L of c-di-GMP every day for 2 weeks was the most effective. Also, LM seemed to be more effective therapeutically at a lower dose (103 CFU) once every 3 days than a high dose (107 CFU) once per week (Supplementary Fig. S5C and S5D). On the basis of these results, we administered 0.2 μmol/L of c-di-GMP daily with 105 CFU of LM-Mb every 3 days in a therapeutic setting (Supplementary Fig. S1C) in all remaining studies. As shown in Fig. 3A and B, this combination was highly effective against the metastases but less vigorous against the primary tumor. The number of metastases in the group of LM-Mb + c-di-GMP was significantly reduced compared with all other groups, whereas the tumor weight in the group with LM-Mb + c-di-GMP was significantly reduced compared with the saline-only group. Finally, we found that therapeutic treatment of tumor-bearing mice with 0.2 μmol/L of c-di-GMP significantly improved T-cell responses to LM-Mb in vivo and in vitro after restimulation with Mage-b (Fig. 3C and D).

c-di-GMP targets MDSCs
MDSCs represent a major population in the TME that strongly suppresses T-cell activation (4–6). Here, we tested whether low doses of c-di-GMP had an effect on MDSCs. We found a significant reduction in the number of MDSCs (35%) in blood compared with that in the saline group when combined with LM-Mb, but separately this effect was less abundant and not significant (Fig. 4A). Because T-cell responses were improved by the administration of low doses of c-di-GMP, we analyzed the production of IL12 by MDSCs. IL12 is known for stimulating naive and mature T cells (31). Low concentrations
of c-di-GMP (range, 0.05–10 μmol/L) increased the IL12 secretion in vitro by the granulocytic MDSCs (CD11b⁺Gr1high) and even more by monocytic MDSCs (CD11b⁺Gr1low) isolated from 4T1 tumor-bearing mice (Fig. 4B and C). Because c-di-GMP interacts with STING, we also analyzed STING expression. As shown in Fig. 4D, both mMDSCs and gMDSCs expressed STING, but the expression was more abundant in mMDSCs.

**One high dose followed by multiple low doses of c-di-GMP is highly effective against metastases in a therapeutic setting**

Our results show that high doses of c-di-GMP killed tumor cells directly and that low doses of c-di-GMP activated T-cell responses in vivo. Here, we combined one high dose of c-di-GMP (3 mmol/L equals 150 nmol in 50 μL) with multiple injections of low-dose c-di-GMP (0.01 nmol) every day for 2 weeks, and found similar efficacy in reducing the number of metastases, tumor weight (Fig. 5A and B), and tumor size (Supplementary Fig. S6) compared with the combination of low doses of LM-Mb and c-di-GMP (Fig. 3A and B). Compared with the saline group, the c-di-GMP–treated mice had significantly improved CD8 T-cell responses to Mage-b in vivo (Fig. 5C) and in vitro (Fig. 5D). We then analyzed T-cell responses to TAAs in more detail and restimulated spleen cells from c-di-GMP–treated and control mice with immunodominant Survivin66–74 peptide (GWEPDDNPI), matching the H2-D
haplotype of BALB/c mice, in the absence or presence of anti-MHC class I antibodies. Survivin is strongly expressed by 4T1 tumor cells (32). We found a significant increase in the number of IFNγ-producing CD8 T cells to TAA Survivin compared with that of the saline group, which was almost completely reduced when blocked with anti-MHC class I antibodies (Fig. 5E). Most importantly, we demonstrated a significant increase in the growth of tumor and metastases in mice that received c-di-GMP plus anti-CD8 antibodies compared with mice that received c-di-GMP alone, but not compared with the saline and isotype control mice (Fig. 5F and G).

Discussion

In the study presented here, we report the role of STING-dependent pathways in cancer immunotherapy. For this purpose, we used LM-Mb as the vaccine and c-di-GMP as the STING ligand in a metastatic breast cancer model, 4T1. In the past, we have shown that LM-based vaccination is highly effective in stimulating innate and adaptive immune responses in tumor-bearing mice when used in a semiprophylactic setting (one immunization before and two after tumor development; ref. 20). However, when used in a therapeutic setting (after tumor development), which is clinically more relevant, LM-based vaccination alone was not able to overcome immune suppression in the TME (19, 20), and clearly needs help to bypass this problem. Here, we demonstrate that STING ligand c-di-GMP overcomes immune suppression and stimulates TAA-specific CD8 T-cell responses.

To analyze the potential pathways of c-di-GMP in cancer vaccination, we tested various immunization protocols of LM-Mb and c-di-GMP in relation to efficacy and Mage-b–specific CD8 T-cell responses in 4T1 tumor–bearing mice. First, we tested LM-Mb and c-di-GMP in a semiprophylactic setting. The results were spectacular. For the first time, we obtained a complete eradication of the metastatic breast cancer. This was not possible with either LM-Mb or c-di-GMP alone. Despite the complete elimination of the metastatic breast cancer, we found that c-di-GMP did not stimulate but inhibited Mage-b–specific and LM-specific CD8 T-cell responses. Because c-di-GMP alone was also highly effective against metastases and even against primary tumors, we concluded that c-di-GMP itself had an effect on the tumor cells. Indeed, we found that c-di-GMP strongly
reduced the viability of 4T1 tumor cells and induced activation of caspase-3 in vitro at a dose of 75 μmol/L. In support of our results, Karaolis and colleagues found that c-di-GMP had an effect on growth factor–stimulated colon cancer cells in vitro (28). On the basis of these results, we hypothesized that if c-di-GMP is toxic for tumor cells, it may also be toxic for T cells and lower doses may stimulate the T cells. Therefore, we analyzed the effect of various low doses of c-di-GMP on vaccine efficacy and on Mage-b–specific CD8 T cells. We found that 0.2 μmol/L of c-di-GMP, administered daily, significantly improved CD8 T-cell responses to Mage-b in vivo and in vitro. Because c-di-GMP is secreted by various types of bacteria such as *A. xylinum*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (33), and because c-di-GMP serves as a danger signal for the early detection of microbes (16), it may not be surprising that extremely low doses can be sensed by the immune system.

One mechanism of the STING-dependent pathways is the production of cytokine IFNβ in CD11c+ cells and macrophages (14). Recently, it has been shown that IFNβ is involved in the intratumoral accumulation of CD8α+ DCs, which is required for T-cell stimulation (34). DCs highly
immune cells in 4T1 tumor we analyzed the effect of c-di-GMP on various subsets of nodes due to c-di-GMP treatment and increase in the CD8 T-cell population in the lymph inent effect was the decrease in the CD4 T-cell population IL12 is known to stimulate na with LM-Mb vaccinations in tumor-bearing mice. Because improved T-cell responses to TAA Mage-b when combined GMP induced the production of IL12 by MDSCs and demonstrated that STING is highly expressed on MDSCs. Because MDSCs play an important role in immune suppression, we also analyzed the effect of c-di-GMP on MDSCs. First, we demonstrated that STING is highly expressed on MDSCs. Moreover, we found that a low dose of STING ligand c-di-GMP induced the production of IL12 by MDSCs and improved T-cell responses to TAA Mage-b when combined with LM-Mb vaccinations in tumor-bearing mice. Because IL12 is known to stimulate naive and mature T cells (31), it is possible that c-di-GMP in the 4T1 model has activated CD8 T cells through the generation of IL12 by MDSCs. We also found that c-di-GMP–treated MDSCs could reduce immune suppression and partly restore CD8 T-cell responses to CD3/CD28 stimulation compared with nontreated MDSCs (Supplementary Fig. S9). Interestingly, the combination of c-di-GMP and LM-Mb significantly reduced the MDSC population in blood, but less so with either c-di-GMP or LM-Mb alone. Reduction in the MDSC population may also contribute to the reduction in immune suppression and consequently to reduced growth of tumor and metastases. Because MDSCs and immune suppression are present in almost all cancers (6), c-di-GMP might be used as an adjuvant against other cancers and with other cancer vaccines as well.

The exact mechanism of how the MDSC population in blood was decreased by LM-Mb and c-di-GMP immunizations is not yet clear, but various mechanisms are possible. One option is that the combination of LM-Mb and c-di-GMP may have destroyed the tumor cells in an early stage of treatment and prevented tumor growth and thus the recruitment of MDSCs. Another option could be that because LM-Mb infects MDSCs (5) and c-di-GMP activates T cells, the LM-Mb–infected MDSCs were eliminated by the LM-activated and Mage-b–activated T cells. Therefore, both reduced migration of MDSCs toward smaller tumors in treated compared with nontreated mice, and elimination of LM-Mb–infected MDSCs by c-di-GMP–activated T cells may contribute to the therapeutic efficacy, but more detailed analysis is required.

On the basis of our results with high and low doses of c-di-GMP, we hypothesized that one administration of high-dose c-di-GMP could induce immunogenic tumor cell death, resulting in cross-presentation of TAAas from c-di-GMP–killed tumor cells by APCs to the immune system, and that repeated low doses of c-di-GMP could activate the TAA-specific T cells. We demonstrate that one high dose followed by multiple low doses of c-di-GMP, without LM-Mb vaccination, induced CD8 T-cell responses to Mage-b in vivo and in vitro, and that the treatment was equally effective against metastases compared with the combination of c-di-GMP and LM-Mb. We found a significant increase in CD8 T-cell responses to Survivin in spleen cultures from mice that received one high dose followed by multiple low doses of c-di-GMP compared with that from saline-treated control mice [4T1 tumor cells highly express Survivin (32), and the cultures were restimulated with immunodominant peptide Survivin(66–74)]. These CD8 T-cell responses to Survivin were strongly reduced by blocking the interaction of the Survivin peptide/MHC class I complex in the restimulation assay with anti-MHC class I antibodies, indicating that c-di-GMP induced CD8 T-cell responses

Figure 4. Therapeutic immunizations with low doses of c-di-GMP and LM-Mb reduced the percentage of MDSCs and c-di-GMP induced IL12 production by MDSCs. BALB/c mice were immunized with low doses of c-di-GMP and LM-Mb and challenged with 4T1 tumor cells according to immunization protocol C. Nineteen days after tumor challenge, mice were euthanized and the percentage of MDSCs (CD11b+/Gr1+) was determined in blood in flow cytometry (A). An example of gating MDSC in blood is shown in Supplementary Fig. S10. In addition, gMDSCs (B) and mMDSCs (C) were isolated from 4T1 tumor–bearing mice, incubated with various concentrations of c-di-GMP in vitro, and then analyzed for IL12 secretion in the culture supernatant by ELISA (range, 0.05–10 µmol/L). Also, the expression of STING was analyzed in MDSCs by Western blotting (D). The results shown here are the averages of three independent experiments (n = 5 mice per group). In A, all groups were compared with LM-Mb + c-di-GMP. Error bars, SEM. Mann–Whitney test. **, P < 0.01. Values of P < 0.05 were considered statistically significant. Also, the expression of STING was analyzed in MDSCs by Western blotting. Bone marrow (BM) cells and HEK293 cells were used as positive and negative controls, respectively.
Immunotherapy and in combination with agents capable of activating the TAA-specific response against metastatic breast cancer through multiple pathways. We believe that results from this novel study provide a rationale for the development of new directions in cancer immunotherapy and in combination with agents capable of inducing immunogenic tumor cell death such as chemotherapy, radiotherapy, and other therapies.

Disclosure of Potential Conflicts of Interests
D.K.R. Karaolis is the inventor of patents for use of cyclic dinucleotides to modulate the immune system. No potential conflicts of interest were disclosed by the other authors.

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Figure 5. One therapeutic high dose followed by multiple low doses of c-di-GMP almost completely eliminated the metastases and improved T-cell responses to TAAs. BALB/c mice received one high dose (3 mmol/L) of c-di-GMP followed by multiple low doses (0.2 μmol/L) of c-di-GMP every day according to immunization protocol D. Nineteen days after tumor challenge, mice were euthanized and analyzed for the number of metastases (A) and tumor weight (B). In the same experiments, CD8 T-cell responses to Mage-b were analyzed in the spleen (pooled) in vitro by ELISPOT (C), or in blood (pooled) in vivo without any restimulation by flow cytometry (D). The results shown here are the averages of three independent experiments. In each experiment, n = 5 mice per group. Unpaired t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Values of P < 0.05 were considered statistically significant. Spleen cells of c-di-GMP–treated and saline control mice were restimulated with Survivin66-74 peptide (GWEPDDNPRI) in the absence or presence of anti-MHC class I antibodies (E). Tumor-bearing mice were treated with c-di-GMP plus anti-CD8 antibodies (H35), and the number of metastases (F) and tumor weight (G) were compared with treatments with c-di-GMP alone, saline, or isotype controls. In this experiment, n = 7 mice per group. Error bars in all graphs, SEM. Mann–Whitney test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Values of P < 0.05 were considered statistically significant. Error bars in all graphs, SEM.
Grant Support
This work was supported by NIH grant (R01 AG023906-01), NCI grant (R21 AI090652-01), The Paul F Glenn Center for the Biology of Human Aging Research 34118A, and National Science Foundation (NSF) 1307218 (to H.O. Smit).

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Published OnlineFirst June 9, 2014; DOI: 10.1158/2326-6066.CIR-13-0123

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