Lineage-Specific T-Cell Responses to Cancer Mucosa Antigen Oppose Systemic Metastases without Mucosal Inflammatory Disease

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Abstract
Cancer mucosa antigens are emerging as a new category of self-antigens expressed normally in immunologically privileged mucosal compartments and universally by their derivative tumors. These antigens leverage the established immunologic partitioning of systemic and mucosal compartments, limiting tolerance opposing systemic antitumor efficacy. An unresolved issue surrounding self-antigens as immunotherapeutic targets is autoimmunity following systemic immunization. In the context of cancer mucosa antigens, immune effectors to self-antigens risk amplifying mucosal inflammatory disease promoting carcinogenesis. Here, we examined the relationship between immunotherapy for systemic colon cancer metastases targeting the intestinal cancer mucosa antigen guanylyl cyclase C (GCC) and its effect on inflammatory bowel disease and carcinogenesis in mice. Immunization with GCC-expressing viral vectors opposed nascent tumor growth in mouse models of pulmonary metastasis, reflecting systemic lineage-specific tolerance characterized by CD8⁺, but not CD4⁺, T-cell or antibody responses. Responses protecting against systemic metastases spared intestinal epithelium from autoimmunity, and systemic GCC immunity did not amplify chemically induced inflammatory bowel disease. Moreover, GCC immunization failed to promote intestinal carcinogenesis induced by germ-line mutations or chronic inflammation. The established role of CD8⁺ T cells in antitumor efficacy, but CD4⁺ T cells in autoimmunity, suggests that lineage-specific responses to GCC are particularly advantageous to protect against systemic metastases without mucosal immunization. These observations support the utility of GCC-targeted immunotherapy in patients at risk for systemic metastases, including those with inflammatory bowel disease, hereditary colorectal cancer syndromes, and sporadic colorectal cancer.

Introduction
A principal obstacle to cancer immunotherapy is the limited availability of antigens that are tumor specific, immunogenic, and universally expressed by patients (1). In the absence of such targets, anticancer immune responses are directed to tissue-specific, rather than tumor-specific, antigens. Limitations to the use of self-antigens include tolerance, which restricts antitumor immunity, and therapy-induced autoimmunity (2). These restrictions have been circumvented by using self-proteins, including cancer testis antigens, expressed in immune-privileged compartments (3). Their expression in tumors outside those compartments offers opportunities for immunologic responses essentially to tumor-specific antigens. However, this approach has been limited by the heterogeneous expression of these antigens by tumors.

This theme of immune segregation has been extended recently to the expression of mucosa-restricted antigens by tumors, exploiting the asymmetry in immunologic cross talk between mucosal and systemic compartments (4). This asymmetry offers unique benefits reflecting the nexus of immunologic privilege, limiting systemic tolerance to mucosal antigens, which promotes antitumor responses, and immunologic partitioning, which protects mucosa from systemic immune responses, limiting autoimmunity (5, 6). These observations suggest a previously unappreciated paradigm for tumors originating in mucosa, in which vaccination with cancer mucosa antigens produces effective therapeutic responses opposing systemic metastases without inducing mucosal inflammation and autoimmunity (4, 7, 8).

Guanylyl cyclase C (GCC) is a member of the guanylyl cyclase family of receptors, which convert GTP to the second messenger cyclic GMP (9). GCC is exclusively expressed by intestinal epithelial cells and uniformly overexpressed by primary and metastatic colorectal tumors (10–12). GCC is the index example of cancer mucosa antigens, reflecting expression normally restricted to mucosa but universally bridging the central immune compartment by tumor metastasis in all patients. Indeed, immunization with viral vectors containing GCC provides protection in prophylactic and therapeutic mouse models of parenchymal colon cancer metastases (4, 7, 8).

The mucosal immune system discriminates between normal antigens and autoantigens, to which a response is inappropriate, and invading microorganisms, to which a response is protective. Occasionally, protective tolerance is lost, producing inflammatory bowel disease, reflecting barrier disruption and exposure of mucosal antigens to dendritic cells in the context of costimulatory signals provided by bacterial pathogens, resulting in pathologic T-cell responses (13–16). Of significance, patients with inflammatory bowel disease have a 10% to 50% risk of developing colorectal cancer (17), reflecting tumor promotion by chronic inflammation (18). Indeed, intestinal inflammation predisposes mice to tumor induction by carcinogens or germ-line mutations (19, 20).

These foregoing observations bring in specific relief issues of safety of immunotherapy targeting cancer mucosa antigens and the risk of autoimmune disease and inflammation-associated cancer. These issues are underscored by the potential application of such vaccines to prophylaxis of metastases in patients at...
greatest risk for metastatic tumors, particularly patients with inflammatory bowel disease and hereditary colon cancer syndromes (21). In those clinical populations, the convergence of ongoing barrier disruption with induction of mucosally targeted immune effectors could exacerbate intestinal inflammation leading to carcinogenesis. These considerations highlight the potential risk for GCC-targeted immunity to amplify mucosal inflammation and intestinal tumorigenesis. Here, we explored the relationship between the therapeutic efficacy of GCC immunization to protect against metastatic colorectal cancer and its effect on autoimmune and tumorigenesis in mouse models of inflammatory bowel disease and intestinal carcinogenesis.

Materials and Methods

Mice. C57BL/6 and BALB/c mice were obtained from the National Cancer Institute Animal Production Program. GCC-deficient (GCC−/−) and wild-type (GCC+/+) C57BL/6 littermate mice were described previously (8, 22). APCmin/+ mice were acquired from The Jackson Laboratory. Animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Recombinant viruses. GCC and control adenovirus (AV), rabies virus (RV), and vaccinia virus (VV) were described previously (8). NP/SIINFEKL−derived CT26 colorectal cancer cells were from American Type Culture Collection. The GCC-expressing CT26 cell line was described previously (8).

ELISA. GCC and AV-specific ELISAs were described previously (8). Briefly, immunosorbsent plates (Nunc) were coated with purified GCC-6×His at 10 μg/mL or with irrelevant adenoval particles at 1 × 105 IFU/mL. Coated plates were incubated with serum collected from immunized mice, and specific antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin (The Jackson Laboratory) and ABTS substrate (Pierce).
Dextran sulfate sodium colitis. Six-week-old female C57BL/6 mice were immunized with GCC-AV or Control-AV, RV, and VV at 28-d intervals, a heterologous prime-boost regimen (PBB) that maximizes GCC-specific antitumor responses (8). Mice were treated 4 d after the final immunization with 4% dextran sulfate sodium (DSS; Sigma-Aldrich) ad libitum in the drinking water for 7 d, and body weights were monitored daily beginning at DSS administration (24, 25). Some mice were euthanized on day 9 following the first DSS administration, and tissues collected for assessment of colitis.

Colitis assessment. Intestinal contents were scored for stool consistency (normal, 0; slightly loose feces, 1; loose feces, 2; watery diarrhea, 3) and visible fecal blood (normal, 0; slightly bloody, 1; bloody, 2; blood in whole colon, 3; ref. 25). Subsequently, intestines were formalin fixed, paraffin embedded, stained with H&E (H&E), and scored by a blinded pathologist (R.B.). The histologic score represents the arithmetic sum of the epithelial damage score (normal, 0; loss of goblet cells, 1; loss of goblet cells in large areas, 2; loss of crypts, 3; loss of crypts in large area, 4) and inflammation score (no infiltrate, 0; infiltrate around crypt base, 1; infiltrate reaching muscularis mucosa, 2; extensive infiltration reaching the muscularis mucosa, 3; visible foci of edema (normal, 0; slightly bloody, 1; bloody, 2; blood in whole colon, 3; ref. 25). Subsequently, intestines were formalin fixed, paraffin embedded, stained with H&E (H&E), and scored by a blinded pathologist (R.B.). The histologic score represents the arithmetic sum of the epithelial damage score (normal, 0; loss of goblet cells, 1; loss of goblet cells in large areas, 2; loss of crypts, 3; loss of crypts in large area, 4) and inflammation score (no infiltrate, 0; infiltrate around crypt base, 1; infiltrate reaching muscularis mucosa, 2; extensive infiltration reaching the muscularis mucosa, 3; visible foci of edema (normal, 0; slightly bloody, 1; bloody, 2; blood in whole colon, 3; ref. 25).

Tumorigenesis. Four-week-old male and female APCmin/+ mice were immunized with AV, RV, and VV as above, and tumorigenesis was quantified at 14 wk of age. For inflammation-associated tumorigenesis, 6-wk-old female C57BL/6 mice were immunized as above with AV, RV, and VV. A single dose of axozymethane (Sigma-Aldrich) 15 mg/kg was administered i.p. 3 d before the final immunization, and 4% DSS administration was begun 7 d later. Following 7 d of DSS, water was returned to the mice for 14 d, followed by two more cycles of 3% DSS (20). Tumorigenesis was quantified 10 d after the final cycle of DSS. Tumors were enumerated, and their sizes quantified under a dissecting microscope. Tumor burden in APCmin/+ mice was determined by calculating the sum of the (diameter)\(^2\) of individual tumors for the small and large intestines in each mouse (22).

Intestinal tissues were processed for H&E staining, and tumors from axozymethane-DSS treated mice were confirmed by histology and graded (A,B.).
revealed by specific recognition of GCC-expressing, but not \( \beta \)-galactosidase-expressing, cells on GCC-AV immunization (Fig. 2A) and specific recognition of \( \beta \)-galactosidase-expressing, but not GCC-expressing, cells on LacZ-AV immunization (Fig. 2B). Responses to GCC-AV immunization were attenuated in GCC\(^{+/+}\) compared with GCC\(^{-/-}\) mice (Fig. 2C), reflecting partial CD8\(^{+}\) T-cell tolerance in GCC\(^{+/+}\) mice. Tolerance was GCC specific because CD8\(^{+}\) T-cell responses to the ovalbumin\(^{257-264}\) epitope SIINFEKL were equivalent in GCC\(^{+/+}\) and GCC\(^{-/-}\) mice (Fig. 2D).

GCC immunization induces antitumor immunity opposing parenchymal metastases. Mice were immunized with GCC-AV or Control-AV, followed 7 days later by i.v. challenge with GCC-expressing CT26 colon cancer cells. Tumor burden revealed by \([^{18}F]\)fluorodeoxyglucose PET/microCT was nearly eliminated in GCC-expressing, cells on GCC-AV immunization (Fig. 2A). Indeed, the number of tumors was reduced \( \sim 75\% \) in GCC-immunized compared with control immunized mice (Fig. 3A). Instead, mice immunized with Control-AV or GCC-AV and then challenged with \( 5 \times 10^5 \) CT26-GCC cells by tail vein injection 7 d later. A, on day 14 after challenge, metastases were visualized by PET/microCT. Images indicate merged PET and microCT images and lungs are outlined for clarity. Images are representative of \( n = 5-7 \) mice per immunization. B, lungs were removed and stained with India ink to visualize lung nodules. Lung nodules were enumerated on visual inspection. Columns, mean of \( n = 12 \) mice per immunization; bars, 95% confidence intervals (***, \( p < 0.001 \), two-sided Student’s \( t \) test).

**Figure 3.** GCC-specific immunotherapy against colon cancer metastases in lung. BALB/c mice were immunized with Control-AV or GCC-AV and then challenged with \( 5 \times 10^5 \) CT26-GCC cells by tail vein injection 7 d later. A, on day 14 after challenge, metastases were visualized by PET/microCT. Images indicate merged PET and microCT images and lungs are outlined for clarity. Images are representative of \( n = 5-7 \) mice per immunization. B, lungs were removed and stained with India ink to visualize lung nodules. Lung nodules were enumerated on visual inspection. Columns, mean of \( n = 12 \) mice per immunization; bars, 95% confidence intervals (***, \( p < 0.001 \), two-sided Student’s \( t \) test).
also had no effect on disease severity (Supplementary Figs. S1–S3). Importantly, this first examination of systemic immunity to intestinal antigens in the context of fulminant inflammatory bowel disease suggests that autoimmunity and acute inflammation are not contraindications to immunotherapy directed to cancer mucosa antigens.

**GCC immunization does not promote colon tumorigenesis.**

The adenomatous polyposis coli (*APC*) gene is mutated early in >80% of sporadic human colorectal cancers and its germ-line mutation underlies the inherited intestinal neoplastic syndrome familial adenomatous polyposis, establishing *APC* mutation as an early event in human colorectal tumorigenesis (26). Moreover, mice heterozygous for mutant *APC* (*APC*<sup>min/+</sup>) develop intestinal polyps and are the most frequently used model of colorectal tumorigenesis (27). Here, tumorigenesis in *APC*<sup>min/+</sup> mice was examined following control or GCC-based immunization. Again, the heterologous prime-boost regimen was used to maximize GCC-specific immune responses. *APC*<sup>min/+</sup> mice were immunized with control or GCC-AV, RV, and VV at 4, 8, and 12 weeks of age, respectively, and tumor burden was quantified 2 weeks later. Unlike humans, *APC*<sup>min/+</sup> mice develop tumors in small and large intestine (27). Tumorigenesis in small (Fig. 5A and B) and large (Fig. 5C) intestines in control and GCC-immunized mice were comparable, reflected by histopathology (Fig. 5A) and tumor burden (Fig. 5B and C). Histology confirmed that tumors were adenomatous polyps in both control and GCC-immunized *APC*<sup>min/+</sup> mice and did not progress to invasive carcinoma (Fig. 5A).

These observations were extended to colon cancer associated with inflammatory bowel disease, using the axozymethane-DSS model of colitis-associated colorectal cancer. Axozymethane is a procarcinogen, which when metabolized forms O<sup>6</sup>-methylguanine and induces the formation of distal colon tumors in rodents (28). Moreover, repeated exposure of axozymethane-treated mice to DSS induces chronic inflammation, mimicking human inflammatory bowel disease, and dramatically enhances axozymethane-induced tumorigenesis (29). Here, mice were immunized with control or GCC-AV, RV, and VV at 6, 10, and 14 weeks of age, respectively. A single dose of 15 mg/kg axozymethane was administered 3 days before the final immunization. The first cycle of 4% DSS was initiated 7 days after axozymethane treatment and was followed by two more cycles of 3% DSS with 2 weeks between cycles (20). This regimen produced tumors in 100% of control and GCC-immunized mice (data not shown), specifically restricted to the distal colon (Fig. 5D). As observed in *APC*<sup>min/+</sup> mice, tumor number (Fig. 5E) and tumor size (Fig. 5F) were identical in control and GCC-immunized mice. Histologic analysis of tissue sections revealed similar incidence of carcinoma *in situ* in control (19.6%) and GCC-immunized (25%) mice (*P* = 0.5184, Fisher’s exact test; Table 1).

**Discussion**

Cancer mucosa antigens are emerging as a new category of self-antigens expressed normally in immunologically privileged mucosal compartments and by tumors originating therein (4, 7, 8). Universal expression of mucosa-restricted antigens by derivative tumors offers a unique solution to the application of self-antigens from immunologically privileged sites to tumor immunotherapy. This approach leverages the established immunologic partitioning of systemic and mucosal compartments (5, 6). Expression confined
to mucosa restricts antigen access to the systemic compartment, limiting tolerance opposing autoimmunity. Conversely, asymmetry in signaling across compartments, wherein systemic immune responses rarely extend to mucosa, limits the risk of autoimmune disease following systemic immunization. In that context, immunization with the intestinal cancer mucosa antigen GCC produced lineage-specific immune cell responses in the systemic compartment, comprising CD8+ T cells, but not CD4+ T or B cells. Lineage-specific tolerance reflects thymic and/or peripheral mechanisms, rather than antigenicity, because GCC−/− mice responded to GCC in all arms of the adaptive immune system, whereas in GCC−/+ mice, GCC elicited only CD8+ T-cell responses. Incomplete systemic tolerance to GCC presumably reflects anatomic, functional, and immunologic compartmentalization wherein sequestration of mucosal antigens provides insufficient antigen for complete systemic tolerance (30,31). Importantly, CD8+ T-cell responses to GCC alone were sufficient for immunoprophylaxis, and a single immunization with GCC-AV dramatically reduced pulmonary colon cancer metastases.

There remains an unresolved issue about autoimmunity surrounding the use of self-antigens generally and cancer mucosa antigens specifically as immunotherapeutic targets (32–34). Immune effectors to self-antigens could amplify autoimmune disease and chronic inflammation, which, in turn, promote carcinogenesis (35). With respect to intestinal cancer mucosa antigens, immune effectors targeting mucosal antigens could potentiate inflammatory bowel disease and/or tumorogenesis, reflecting disruption of normal mucosal barriers creating novel effector access to compartmentalized antigens, amplifying chronic inflammation. Those considerations notwithstanding, immunization of mice with GCC activated systemic CD8+ T-cell responses that, while mediating effective parenchymal antitumoral colon cancer immunity, spared GCC-expressing intestinal epithelium from autoimmune disease. Further, systemic GCC immunity induced by a heterologous prime-boost regimen producing maximum effector responses (8) did not influence fulminant intestinal inflammation associated with chemically induced colitis. Moreover, GCC immunity failed to promote intestinal carcino genesis, reflecting germ-line mutations in a key tumor suppressor, or chronic inflammation. These studies, which are the first to examine the effect of systemic immunity to an intestine-specific self-antigen on inflammatory bowel disease or inflammation-associated colon tumorogenesis, support the safety of GCC immunization in patients at risk for inflammatory bowel disease or hereditary colorectal cancer. Indeed, GCC immunization has substantial translational potential, reflecting the universal expression of GCC in metastatic human colorectal cancer (10–12). Whereas GCC immunogenicity has not yet been explored in patients, studies here reveal uncoupling of systemic antitumor immunity and intestinal autoimmunity through immune compartmentalization and support examination of GCC immunotherapy for metastatic colorectal cancer.

Preservation of immune compartmentalization and tissue integrity in the face of mucosa-targeted immune effector cells...
and fulminant barrier disruption likely reflects the convergence of parallel homeostatic mechanisms. Compartmentalization is supported by tissue-restricted lymphocyte recirculation mediated by chemokine and adhesion molecules, particularly the interactions of addressins, integrins, and selectins, which define tissue-specific lymphocyte migration (7, 36–38). The local lymphoid microenvironment imprints new circulation patterns during activation, and effector T cells from mesenteric lymph nodes home to the gut wall, lamina propria, Peyer’s patches, and mesenteric lymph nodes, whereas those from peripheral lymph nodes migrate to the spleen and peripheral lymph nodes (38). Moreover, the lineage specificity of effector responses to GCC may be particularly advantageous because autoreactive CD4+ T cells are more efficient mediators of autoimmunity than CD8+ T cells. Thus, CD4+ T cells targeted to a gastric self antigen (39) or commensal bacterial antigen (40) induce autoimmunity in mice. In addition, experimental autoimmune diseases such as autoimmune encephalomyelitis (41), thyroiditis (42), colitis (43), mice. In addition, experimental autoimmune diseases such as autoimmune encephalomyelitis (41), thyroiditis (42), colitis (43), or oophritis (44) are CD4+ T-cell mediated. By contrast, CD8+ autoimmune diseases (46).

The present results have substantial implications for the control of metastatic colorectal cancer in patients. The majority of colorectal cancer cases are sporadic, reflecting environmental and genetic risk factors (21). However, of the ~1 million patients developing colorectal cancer worldwide, ~2% are associated with inflammatory bowel disease and ~5% to 10% reflect the inherited syndromes hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis (21). These diseases are associated with a cancer penetrance of ~20% in inflammatory bowel disease and 70% to 100% in hereditary cancer syndromes (21). Indeed, prophylactic colectomy is routinely used in those patients at greatest risk for developing colorectal cancer. Therefore, prophylactic GCC-specific immunization may be a useful adjunct for cancer control in these populations. Whereas immunization will not prevent the development of primary tumors (Fig. 5), GCC-based immunity could protect against systemic metastases. Importantly, GCC-targeted immunity provided effective antitumor therapy without exacerbating intestinal inflammation or primary carcinogenesis, suggesting that cancer mucosa antigen immunotherapy is not contraindicated in patients with inflammatory bowel disease or inherited colorectal cancer syndromes.

Interestingly, results here contrast with prior studies examining immunotherapy targeting self-antigens in mouse models of intestinal carcinogenesis (47–49). Whereas mechanisms underlying these differences remain to be defined, immune responses in earlier studies were characterized by antibody production, in addition to T-cell induction. Antibodies have established efficacy in animal models and humans, which may extend to primary colorectal tumorigenesis. Indeed, cetuximab [Erbitux; anti–epidermal growth factor receptor (EGFR) monoclonal antibody], bevacizumab (Avastin; anti–vascular endothelial growth factor monoclonal antibody), and trastuzumab (Herceptin; anti-EGFR monoclonal antibody) provide clinically important antitumor efficacy against various tumors. Moreover, antitumor effects induced by immunization against 5T4, a human oncofetal antigen, seem to be mediated exclusively by antibodies (50). In that context, GCC-specific immunization does not induce IgG responses in GCC+/+ mice, the absence of which may restrict efficacy against primary tumors. Studies using passive transfer of serum from immunized GCC−/− mice to APCmin+/+ GCC−/− mice may reveal primary antitumor efficacy that is absent in the current immunization paradigm, which will inform the development of the next generation of immunization regimens that activate both CD8+ T-cell and antibody responses.

GCC-targeted immunity, which protects against parenchymal colon cancer metastases, did not exacerbate inflammatory bowel disease or promote inflammation-induced or genetically induced intestinal carcinogenesis. These observations support the utility of GCC-targeted immunotherapy for cancer prevention and control in patients at risk for developing systemic metastases, including those with inflammatory bowel disease, hereditary colorectal cancer syndromes, and sporadic colorectal cancer. Moreover, GCC-based vaccines could be applied to patients with esophageal and gastric cancer, reflecting the role of intestinal metaplasia and the associated novel ectopic expression of that antigen in those malignancies (11). Beyond the gastrointestinal tract, the present observations suggest the utility and safety of exploiting immunologic compartmentalization to achieve antitumor therapy in tumors originating from other mucosae, including oral, respiratory, mammary, and urogenital, for the treatment of cancers of the head and neck, lung, breast, vagina, and bladder, respectively. Indeed, the established principles of immune compartmentalization in the context of the present results with GCC underscore the importance of defining the generalizability of cancer mucosa antigens as targets for immunotherapy of mucosa-derived tumors (4, 7, 8).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/3/08; revised 1/3/09; accepted 2/4/09; published OnlineFirst 4/7/09.

Grant support: NIH grants CA75123 and CA95026 and Targeted Diagnostic and Therapeutics, Inc. (S.A. Waldman) and Measey Foundation Fellowship (A.E. Snook). S.A. Waldman is the Samuel M.V. Hamilton Endowed Professor.

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