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Murine Lung Cancer Induces Generalized T Cell Exhaustion

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Abstract

Background—Cancer is known to modulate tumor-specific immune responses by establishing a micro-environment that leads to the upregulation of T cell inhibitory receptors, resulting in the progressive loss of function and eventual death of tumor-specific T cells. However, the ability of cancer to impact the functionality of the immune system on a systemic level is much less well characterized. Because cancer is known to predispose patients to infectious complications including sepsis, we hypothesized that the presence of cancer alters pathogen-directed immune responses on a systemic level.

Materials and Methods—We assessed systemic T cell coinhibitory receptor expression, cytokine production, and apoptosis in mice with established subcutaneous lung cancer tumors and in unmanipulated mice without cancer.

Results—Results indicated that the frequencies of PD-1⁺, BTLA⁺, and 2B4⁺ cells in both the CD4⁺ and CD8⁺ T cell compartments were increased in mice with localized cancer relative to non-cancer controls, and the frequencies of both CD4⁺ and CD8⁺ T cells expressing multiple different inhibitory receptors was increased in cancer animals relative to non-cancer controls. Additionally, 2B4⁺CD8⁺ T cells in cancer mice exhibited reduced IL-2 and IFN- γ , while BTLA⁺CD8⁺ T cells in cancer mice exhibited reduced IL-2 and TNF. Conversely, CD4⁺ T cells in cancer animals demonstrated an increase in the frequency of Annexin V⁺ apoptotic cells.

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Author contributions: RM collected, analyzed, and interpreted the data and wrote the paper; CWC, JDL, LMM, and ZL collected the data; and CMC and MLF conceived of the experiments, analyzed and interpreted the data, provided critical revision of the manuscript, and obtained funding for the study.

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Conclusion—Taken together, these data suggest that the presence of cancer induces systemic T cell exhaustion and generalized immune suppression.

Introduction

The mechanisms by which tumors escape the immune system and become invasive is a major focus of cancer research. Such mechanisms include an immune suppressive microenvironment, which may contain T regulatory cells, myeloid derived suppressor cells, impaired antigen presentation and tumor-specific immune cell effector function [1-5]. Tumor micro-environments can lead to the up-regulation of inhibitory receptors such as B and T lymphocyte attenuator (BTLA), programmed death-1 (PD-1), and 2B4 (CD244) on T cells [6] resulting in the progressive loss of cell function and eventual death of tumor-specific T cells. The relative and coordinate expression levels of these and other coinhibitory receptors serve to fine-tune T cell functionality and determine the profoundness of T cell exhaustion. However, the ability of cancer to impact the functionality of the immune system on a systemic level is much less well characterized.

Previously we showed that during an acute systemic bacterial infection, the presence of pre-existing pancreatic adenocarcinoma tumors (localized to the inner thigh) resulted in increased phenotypic exhaustion and impaired differentiation of bacterial antigen-specific CD8⁺ T cells [7], suggesting that cancer may indeed function on a systemic level to impair pathogen-specific T cell responses. In addition, it is known that the presence of pre-existing malignancy is a major risk factor for increased mortality during sepsis. Specifically, pre-existing malignancy was noted to be the most common co-morbidity in human septic patients, and is associated with a mortality that is nearly 50% higher than patients without cancer [8-10]. Furthermore, septic mice with pancreatic adenocarcinoma tumors have a 24% increase in mortality following sepsis [11]. Because the integrity of the immune system is well known to play a critical role in survival during sepsis, these data suggested that the presence of malignancy may fundamentally compromise the integrity of the immune system on a systemic level and thus affect the pathophysiology of sepsis.

Given this context, we sought to determine the impact of malignancy on phenotypic and functional exhaustion within the CD4⁺ and CD8⁺ T cell compartments, with the hypothesis that localized tumors may function to modulate systemic cellular immunity. Using a murine model of lung cancer, we found that cancer fundamentally altered CD4⁺ and CD8⁺ T cell co-inhibitory receptor expression profiles and impaired T cell functionality on a systemic level.

Methods

Ethics Statement

All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine (Protocol 2001875-082815BN).

Mice

Adult male 6-week old C57BL/6 were obtained from The Jackson Lab (Bar Harbor, ME). This study was conducted prior to the NIH mandate that both genders be examined during animal experimentation. After allowing the mice to acclimate for one week, they were randomized to cancer and no cancer groups. Animals were sacrificed at 3 weeks following tumor injection, at which point all had palpable tumors, using asphyxiation by CO₂. All animals were housed in the animal facility and had access to chow and water.

Cancer Model

A syngeneic mouse lung cancer line, Lewis lung carcinoma 1 (LLC1) was used to induce tumors (ATCC). Cells were maintained in 1640 RPMI culture medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin/streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). All mice in cancer group received a subcutaneous injection of 250,000 LLC1 cells suspended in 0.1mL of phosphate buffered saline at the inner right thigh. Mice were then housed in the animal facility with free access to water and chow for three weeks to allow for tumor growth, and all mice with palpable tumors were used for experimentation. Tumor sizes ranged between 1.5-1.7 cm. During the three weeks of tumor growth, mice were assessed using Emory University's IACUC tumor burden scoring guidelines. At no time did any animals meet criteria for euthanasia. Mice in the no cancer group were unmanipulated.

Phenotypic flow cytometric analysis

Three weeks after injection of cancer cells in CA group, spleens were collected from WT and CA mice and single cell suspensions were prepared for flow cytometric analysis. Cells were stained with anti-CD4-PO (Invitrogen), anti-CD8-PB and anti-2B4-APC (eBioscience), anti-PD-1-FITC and anti-BTLA-PE (BD Pharmingen), and Fc Block (Biolegend). TruCount Beads from BD Pharmingen were prepared using manufacturer's instructions and used to determine absolute cell numbers where shown.

Intracellular cytokine staining

For intracellular cytokine staining, $2-3 \times 10^6$ splenocytes were plated in a 96-well plate. Cells were suspended and incubated in culture medium consisting of RPMI 1640 containing 10% FBS (Mediatech, Herndon, VA), 2mM L-glutamine, 0.01 M HEPES buffer, 100mg/ml gentamicin (Mediatech), and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). To measure production of IFN- γ , TNF, and IL-2, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA, 30ng/mL) and ionomycin (400ng/mL) with 10 μ g/mL of Brefeldin A. Following incubation and stimulation, cells were stained with anti-CD4-PO (Invitrogen), anti-CD8-PB and anti-2B4-APC (eBioscience), anti-IL-2-FITC, anti-BTLA-PE, anti-TNF-PECy7, anti-IFN- γ -Alexa700 (BD Pharmingen). Cells were processed using BD Biosciences intracellular staining kit. An LSR II flow cytometer (BD Biosciences) was used to run all samples and FlowJo 9.5 software (Tree Star, San Carlos, CA) was used to analyze all data.

Staining for apoptosis and cell death

To stain for apoptotic and death cells, 1×10^6 splenocytes were processed using an Annexin V and 7-AAD kit from Biolegend using manufacturer's instructions. Cells were also stained with anti-CD4-PO (Invitrogen), anti-CD8-PB and anti-2B4-APC (eBioscience).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0 software (San Diego, CA) and presented as mean $\pm$ SEM. Two-way comparisons were performed using a Mann-Whitney non-parametric test or two-way ANOVA followed by Tukey post-test, and a p value of <0.05 was considered to be statistically significant.

Results

Cancer does not alter CD4⁺ or CD8⁺ T cell numbers in the spleen

To determine the impact of LLC1 on T cell immunity, groups of animals were injected with 2.5×10^5 LLC1 cells in the inner thigh, and tumors were allowed to grow for 3 weeks and control animals were unmanipulated. 3 weeks post-LLC1 injection, animals were sacrificed and splenic T cells were analyzed by flow cytometry. While mice with lung cancer tumors appeared to have decreased frequencies of CD4⁺ T cells, the absolute numbers CD4⁺ and CD8⁺ T cells were not different in cancer mice as compared to controls (Figure 1A and 1B).

Cancer increases co-inhibitory receptor expression on CD4⁺ and CD8⁺ T cells

Splenocytes were stained for co-inhibitory receptors 2B4, BTLA, and PD-1 in LLC1 tumor-containing animals and unmanipulated controls. Compared to mice without cancer, mice with cancer had increased frequencies of 2B4⁺, BTLA⁺, and PD-1⁺ CD4⁺ T cells. Furthermore, the absolute numbers of CD4⁺ T cells expressing PD-1, were significantly increased in cancer mice relative to non-cancer controls, while absolute numbers of 2B4⁺ and BTLA⁺ CD4⁺ T cells trended toward being increased (Figure 2A). Importantly, the CD8⁺ T cell compartment in cancer animals exhibited both increased frequencies and absolute numbers of 2B4⁺, BTLA⁺, and PD-1⁺ T cells relative to non-cancer controls (Figure 2B).

Cancer modulates inhibitory receptor profiles on both CD4⁺ and CD8⁺ T cells

Lymphocytes in cancer microenvironments tend to exhibit an exhausted phenotype, which manifests in part in the co-expression of several distinct coinhibitory receptors, including PD-1, BTLA, and 2B4. The expression of multiple distinct coinhibitory receptors have been shown to function in aggregate to contribute to increased functional exhaustion in antigen-specific CD8⁺ T cells [6, 12]. To further investigate the systemic change in T cell inhibitory receptor expression profiles, splenic CD4⁺ and CD8⁺ T cells were categorized based on the number of different coinhibitory receptors they expressed. Interestingly, a significantly higher percentage of T cells in the mice with LLC cancer expressed one, two, or three coinhibitory receptors as compared to control mice (Figure 3A). Figure 3B demonstrates that in both the CD4⁺ and CD8⁺ T cell compartments, a greater proportion of the cells isolated

from cancer mice exhibited expression of one, two, or three coinhibitory receptors as compared to cells isolated from control animals.

Cancer impairs cytokine production by splenic CD4⁺ and CD8⁺ T cells

To determine the impact of LLC1 tumors on T cell functionality, splenocytes from LLC1-injected animals and unmanipulated controls were stimulated ex-vivo with PMA and ionomycin and stained for IL-2, TNF, and IFN- γ to determine the ability of total CD4⁺ and CD8⁺ T cells as well as sub-populations expressing 2B4 and BTLA to produce cytokines (Figure 4).

Total CD4⁺ T cells in the cancer group did not exhibit any differences in IL-2, TNF, or IFN- γ production compared to CD4⁺ T cells in mice without cancer. In order to determine whether co-inhibitory receptor expression induces T cell exhaustion and alters cytokine production, CD4⁺ T cells expressing 2B4 and BTLA were assayed for intracellular cytokine production. We found that CD4⁺ 2B4⁺ produced similar levels of IL-2, TNF, and IFN- γ , as compared to CD4⁺ 2B4⁺ cells isolated from mice with no cancer. Interestingly, CD4⁺ T cells expressing BTLA produced less IL-2 compared to the same population of cells in mice without cancer (Figure 4A).

With regard cytokine production within the CD8⁺ T cell compartment, we observed that the presence of malignancy impaired IL-2 production by total CD8⁺ T cells in the cancer group compared to the no cancer mice. In addition, 2B4 expression on CD8⁺ T cells in cancer mice further impaired cytokine production, in that we observed even more profound reduction in IL-2 and IFN- γ production and a trend toward impaired TNF reduction. Interestingly, while BTLA expression on CD8⁺ T cells in cancer mice impaired IL-2 and TNF production, it had no effect on IFN- γ production (Figure 4B)

Cancer results in increased CD4⁺ and 2B4⁺ T cell apoptosis

To investigate the impact of LLC1 tumors on systemic T cell apoptosis, splenocytes from animals 3 weeks after tumor cell injections were stained with Annexin V, 7-AAD, and anti-CD4, anti-CD8, anti-2B4, anti-PD-1, and anti-BTLA. Annexin V positivity was then used to identify apoptotic cells. In the CD4⁺ but not CD8⁺ T cell compartments, the presence of malignancy was associated with increased T cell apoptosis. Furthermore, 2B4 expression on CD4⁺ and CD8⁺ T cells was also associated with increased apoptosis in mice with lung cancer tumors compared control animals. PD-1⁺ and BTLA⁺ T cells in both the CD4⁺ and CD8⁺ compartments in cancer mice exhibited similar frequencies of Annexin V⁺ cells as those populations isolated from control mice.

Discussion

This study aimed to characterize and quantify T cell exhaustion in a murine model of lung cancer to determine how malignancy affects the immune system on a systemic level. Data previously published by our lab showed T cell exhaustion following systemic bacterial infection in a model of murine pancreatic adenocarcinoma. Here our results were surprising in that lung cancer tumors alone were sufficient to induce T cell exhaustion and T cell apoptosis.

Co-inhibitory receptors are best characterized during states of chronic antigen stimulation as seen during chronic viral infections or states of chronic inflammation [6, 12]. Malignant tumors are known to alter the local microenvironment to evade tumor-specific immune responses by down-regulating HLA antigen expression, upregulation of immune inhibitory cells (e.g., Tregs and myeloid derived suppressor cells), and upregulation of co-inhibitory receptors on cells tumor-specific effector cells [1-3, 5, 13-16]. The vast majority of research in this arena focuses on the identification of pathways by which tumors escape the immune system, grow and invade, and may be targeted to increase tumor-specific immunity [17-20]. The effects of tumors on the systemic immune system, including the response to pathogen infection, are relatively unknown. Our data suggest that by increasing the frequencies of exhausted and apoptotic T cells, malignancy may contribute to immune incompetence following infection. As our understanding of the role of the immune system in sepsis continues to evolve, it becomes even more important to understand how different malignancies may alter systemic immune responses.

In our model of malignancy, while lung cancer tumors did not alter absolute numbers of CD4⁺ and CD8⁺ T cells in the T cell compartment, cancer did increase co-inhibitory receptor expression. The co-inhibitory receptors BTLA and PD-1 play important mechanistic roles in the pathophysiology of both cancer progression and sepsis. In multiple models of malignancy, BTLA and PD-1 expression have been shown to be associated with reduced tumor-specific T cell effector function [21-23]. Furthermore, there is growing evidence that expression of these receptors on T cells, in particular, plays an important mechanistic role [24-26]. Blockade of both BTLA and PD-1 inhibitory pathways improved viral-specific immune responses, attenuated sepsis mortality, and were shown to reverse T cell exhaustion [27-33]. Similarly, 2B4, a member of the CD2 immunoglobulin family, is also shown to play a co-inhibitory role on tumor-specific and antigen-specific immune cells, particularly T cells [34-36].

To determine whether co-inhibitory receptor expression induced T cell exhaustion, we assessed IL-2, TNF, and IFN- γ production by total CD4⁺ and CD8⁺ T cells and by those expressing 2B4 and BTLA. It is known that T cells progressively lose cytokine producing ability as they upregulate co-inhibitory receptor expression [6, 12]. In our model, CD8⁺ T cells demonstrated more significant exhaustion than CD4⁺ T cells. While total CD8⁺ T cells in cancer mice produced less IL-2 than those in control animals, BTLA-expressing CD8⁺ T cells produced less IL-2 and TNF, and those expressing 2B4 had impaired IL-2 and IFN- γ production with a trend toward less TNF production as well. Wherry et al. describe a progressive diminution in cytokine-producing ability beginning with loss of IL-2, followed by TNF and IFN- γ [6]. Based on the observed reduction in IFN- γ , we hypothesize that CD8⁺ T cells expressing 2B4 may be more exhausted than those expressing BTLA.

In the last phases of exhaustion, T cells lose all effector function and become apoptotic [6]. In our study, LLC1 tumors were sufficient to increase total CD4⁺ T cell apoptosis, and cancer mice had a higher frequency of apoptotic T cells expressing 2B4 compared to mice without tumors. This further strengthens our hypothesis that 2B4 expression may be indicative of later stages of T cell exhaustion. One potential limitation of the current study is

that exclusively male mice were used, thus it is possible that our results may not be generalizable to female gender.

In sum, here we examined T cell exhaustion as a result of lung cancer malignancy. This study lays important groundwork for future studies involving the effect of this pre-existing T cell exhaustion on subsequent infection and sepsis, as different models of malignancy appear to have very different immunosuppressive properties. While inhibition of lymphocyte apoptosis is normally beneficial in sepsis, one may speculate that in the presence of cancer, this protective benefit further exacerbates immune dysregulation. Furthermore, our data suggests that 2B4 may be an important marker for the severity of T cell exhaustion.

In summary, our data interrogate the development of systemic T cell exhaustion in a model of lung cancer and highlight the importance of identifying the unique immune suppressive properties of individual malignancies.

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Figure 1. Cancer does not alter CD4⁺ or CD8⁺ T cell numbers in the spleen

Unmanipulated B6 mice and those injected with LL1 cells were sacrificed after 3 weeks and splenocytes were collected for analysis. A) Representative flow cytometric analysis of CD3⁺ T cells. B) Gating on CD3⁺ cells, the frequency and absolute numbers of CD4⁺ and CD8⁺ T cells were calculated. **p<0.01, n=9-10.

Figure 2. Cancer increases co-inhibitory receptor expression on CD4⁺ and CD8⁺ T cells
 In control and cancer mice, 3 weeks after tumor cell injections, splenocytes were stained with anti-2B4, anti-BTLA, and anti-PD-1. A) Frequency of 2B4 expression on CD4⁺ T cells in no cancer vs. cancer was $1.5 \pm 0.2\%$ vs. $4.8 \pm 1.3\%$, respectively; BTLA: $2.4 \pm 0.3\%$ vs. $6.8 \pm 1.4\%$; PD-1: $7.2 \pm 0.4\%$ vs. $19.0 \pm 1.8\%$. B) On CD8⁺ T cells the frequency of 2B4 expression was $1.9 \pm 0.2\%$ vs. $5.5 \pm 0.8\%$; BTLA: $2.1 \pm 0.3\%$ vs. $7.9 \pm 1.6\%$; PD-1: $4.6 \pm 0.7\%$ vs. $10.3 \pm 1.5\%$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 13-16$.

Figure 3. Cancer modulates inhibitory receptor profiles on both CD4⁺ and CD8⁺ T cells
 CD4⁺ and CD8⁺ T cells co-expressing different inhibitory receptors (PD-1, BTLA and 2B4) were further divided into different groups. A) Frequency of CD4⁺ and CD8⁺ T cells expressing one, two and three inhibitory receptors T cells in no cancer vs. cancer were shown. B) The proportion of the CD4⁺ and CD8⁺ T cell compartments that are expressing one, two, or three inhibitory receptors is shown.

Figure 4. Cancer impairs cytokine production by splenic CD4⁺ and CD8⁺ T cells

Intracellular cytokine production was assessed in control and cancer mice splenocytes after 3 weeks of tumor growth. CD4⁺ and CD8⁺ T cells were stimulated for production of IL-2, TNF, and IFN- γ . The histograms show cytokine production by total CD4⁺ and CD8⁺ T cells. Cytokine production by CD4⁺ T cells (A) and CD8⁺ T cells (B) expressing 2B4 and BTLA is also shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 11-13$.

Figure 5. Cancer results in increased CD4⁺ and 2B4⁺ T cell apoptosis

After allowing 3 weeks for tumor growth, splenocytes in unmanipulated control mice and cancer mice were stained with Annexin-V and 7-AAD to determine frequency of apoptotic cells, and those staining positive for 7-AAD were excluded from analysis. (A, B) The histograms show apoptotic total CD4⁺ and CD8⁺ T cells in no cancer (black filled histogram) and cancer mice (blue unfilled histogram). The bar graphs on the bottom show frequency of apoptotic CD4⁺ (C) and CD8⁺ (D) T cells in PD-1⁺, BTLA⁺ and 2B4⁺ populations. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 8-20$.