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Sex differences in the association between tumor growth and T cell response in a melanoma mouse model

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Abstract

Epidemiological evidence suggests that females have an advantage over males in cases of melanoma incidence, progression, and survival. However, the biological mechanisms underlying these sex differences remain unclear. With the knowledge that females generally have a more robust immune system than males, we investigated sex differences in melanoma progression in a B16-F10/BL6 syngeneic mouse model. We observed significantly less tumor volume and growth rate over 14 days in female mice compared to male mice. Furthermore, higher populations of CD4⁺ and CD8⁺ T cells, which indicate adaptive immune responses, were found in the circulating blood and tumors of females and corresponded with less tumor growth, and vice versa in males. Our results highlight a mouse model that represents melanoma progression in the human population and displays a higher immune response to melanoma in females compared to males. These findings suggest that the immune system may be one of the mechanisms responsible for sex differences in melanoma.

Keywords

melanoma; sex differences; male; female; immunity

INTRODUCTION

Skin cancer is the most common form of cancer in the United States, with melanoma being the deadliest [1]. Exposure to environmental ultraviolet radiation has been associated with

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Concept and supervision by SG. Animal studies, assays, and analysis performed by PD, KP, AL, and SG. Immunology expertise, training, and antibodies from AL and HZ. Writing of the original draft by PD. Reviewing and editing by all authors.

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86% of melanoma cases [2]. Current estimates show that melanoma incidence is increasing approximately 3% every year, with males projected to account for 60% of melanoma incidences and 67% of melanoma-related deaths in 2020 [1]. This biological sex difference has been reported in epidemiological studies and cancer registries over the years, with females showing a clear advantage over males [1]. This is despite the prevalence of tanning and sun-seeking activities in females relative to males [3,4]. Despite these lifestyle differences which should disfavor females, it is remarkable that females have the advantage over males in melanoma incidence, disease progression, and survival [5,6]. Nevertheless, the mechanism(s) responsible for these sex differences are not fully understood.

The progression of melanoma from initiation to metastasis is driven mainly by proliferation events resulting from genetic mutations [7]. The genetic alterations in melanomas create antigenic epitopes such as TYRP-2 and MART-1 that are recognized by the host adaptive immune CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells [8]. In this study, we focus on the CD4 and CD8 markers because the adaptive immune response is one of the major defense mechanisms against melanoma. Interestingly, females are been shown to have higher CD4⁺ and CD8⁺ T cell populations across different species compared to males [9]. Hence, we investigated whether sex differences in the adaptive immune response would explain the disparity in melanoma progression between males and females. We used a syngeneic B16-F10/BL6 mouse model, whose tumors are similar to humans in aggressiveness and metastatic potential, to demonstrate our concept and observe immune response to the progression (growth) of melanoma.

MATERIALS AND METHODS

Animal experiments

All animal procedures were in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of Washington State University. Male and female C57BL/6 wild-type mice (8- to 10-week old) were obtained from the Jackson Laboratories. The mice were maintained under 12 h/12 h light/dark cycle and given ad libitum food and water in the animal facility at Washington State University, Spokane. For subcutaneous injection, B16-F10 melanoma cells were purchased from ATCC and cultured in RPMI-1640 + 10% FBS. For each mouse, 200,000 cells in 50% serum-free RPMI-1640 and 50% matrigel (Corning) were injected into the lower right flank region. By the visible appearance of tumors in all mice, tumor volumes were measured using a digital caliper and calculated using the formula: $V = (W^2 \times L)/2$ [10]. All mice were sacrificed when the largest tumor reached a designated endpoint of 1000 mm³. Concurrently, control mice (non-tumor bearing) were sacrificed. Blood and tumors were harvested and immediately processed for immunophenotyping.

Lymphocyte isolation

Lymphocytes were isolated from peripheral blood using Lympholyte-Mammal (Cedarlane Laboratories) as previously described by us [11]. Briefly, whole blood was centrifuged for 3 minutes at 5000 rpm to separate plasma. All centrifugation steps were carried out at room temperature. The remaining blood fraction was resuspended in 2 mL of PBS prewarmed at

37°C. Density separation of lymphocytes was achieved by adding 4 mL of Lympholyte-Mammal (CedarLane labs, Cat # CL5115) to the diluted blood and centrifuging for 20 minutes at 2000 rpm. The lymphocytes at the interface between the suspension medium and Lympholyte were collected and any contaminating red blood cells were lysed using 1X RBC lysis buffer (0.15 M NH₄Cl, 0.01 M NaHCO₃, 1 mM EDTA) for 5 minutes. Afterwards, 10 mL of cold PBS was added to stop the lysis reaction. The cells were then counted and resuspended in appropriate amount of PBS for flow cytometry.

Tumor-infiltrating lymphocyte isolation

Tumor-infiltrating lymphocytes were isolated from melanoma as previously described by us [10]. Briefly, tumors were isolated and washed with ice-cold PBS + 0.1% BSA, pushed through a wire mesh strainer, resuspended in ice-cold PBS + 0.1% BSA, and centrifuged at 4°C for 1 minute at 480 rpm. The supernatant was collected and centrifuged at 4°C for 10 minutes at 1,200 rpm. The pellet was resuspended in 37.5% Percoll (GE Healthcare, Cat # 17089101), then centrifuged at 2,000 rpm for 30 minutes. The lymphocytes were collected, treated with 1X RBC lysis buffer, counted, and resuspended in appropriate amount of PBS + 0.1% BSA for flow cytometry.

Flow cytometry and analysis

The general flow cytometry method was used as previously described [11]. Isolated cells were incubated with anti-CD4 and anti-CD8 conjugated with PE-Cy7 and e610 fluorophores, respectively (eBioscience, clone #s GK1.5 and 53-6.7 respectively) and flow cytometry was performed using a Beckman Coulter Gallios model A94291. Data was analyzed using Kaluza Analysis Software v1.5. Double-stained populations were excluded in these analyses.

Statistical analysis

Tumor volumes were compared by repeated measures Two-way ANOVA and corrected for multiple comparisons using Sidak's test. Tumor growth rate was determined by nonlinear regression and one-way ANOVA comparison of the slopes. Immune differences were analyzed using Students' t test. Outlying data points were determined using the upper and lower bounds limits of data range in Excel (Microsoft) and subsequently removed from analyses. All other analyses were performed using Prism version 6.01 (GraphPad software).

RESULTS

Sex differences in the growth of melanoma

We carried out our studies in wild-type male and female mice according to the experimental timeline in Fig. 1a. B16-F10 melanoma cells were subcutaneously injected to the lower right flank region, and on day 8 all mice had visible measurable tumors. These mice were monitored daily and measurements were taken every two days. There were no signs of toxicity or discomfort observed throughout the experiment. By measurement of tumor volumes, we observed an interesting phenotype showing clear distinction in the growth of tumors. Tumors had significantly less volumes, by up to 3-fold, in the female mice compared to the male mice from day 10 through 14 (Fig. 1b). Furthermore, tumor growth

rate calculated by linear regression revealed a significantly lower tumor growth rate in females ($p < 0.0029$) compared to males (Fig. S1). This phenotype corroborates human data which shows that females had less rapid tumor growth and were at lower risk of progression compared to males [12,6]. In summary, our melanoma tumor model showed less melanoma growth in female mice compared to males, which represents melanoma progression events in the human population.

Sex differences in adaptive immune response to melanoma

After observing that female mice have less tumor growth compared to male mice, we wanted to see if the response of the adaptive immune system could explain any of this phenotypic difference. Our B16-F10 melanoma tumor cells express melanoma-associated antigens, like Tyrp-2 and mutated p53, that are recognized by the host's CD4⁺ (helper and recruitment) and CD8⁺ (cytotoxic) T cells [13,8,14]. In addition, we had a non-tumor (control) group. All animals were sacrificed 15 days after tumor implantation (for the tumor-bearing group). Therefore, we used immunophenotyping by flow cytometry to investigate circulating CD4⁺ and CD8⁺ T cells in the blood and infiltrating lymphocytes in the tumors. In the control mice, the number of peripheral blood lymphocytes (PBL) per μL of blood in female mice was higher compared to male mice ($p = 0.0061$) (Fig. 2a). In analyzing subsets of lymphocytes, we observed significantly higher CD4⁺ T cells in females ($p = 0.028$) compared to males, whereas the CD8⁺ T cell population showed no statistical difference (Fig. 2b). In melanoma tumor-bearing mice, the number of circulating PBLs per μL of blood was increased by approximately 1.5 fold, indicating a response to the tumors compared to control mice. Again, the number of PBLs in female mice was significantly higher than male mice ($p = 0.016$) (Figs. 2a). When comparing sex differences of circulating T cells in tumor-bearing mice, we observed significantly higher CD4⁺ and CD8⁺ T cell populations in female mice compared to male mice ($p = 0.035$ and 0.020 respectively) (Fig. 2c). Furthermore, we measured tumor-infiltrating lymphocytes from harvested tumors and did not see a difference with CD4⁺ T cells but saw a significant increase in CD8⁺ T cells per mm^3 of tumor in female mice compared to male mice ($p = 0.0081$) (Fig. 2d). Additionally, we calculated lower CD4/CD8 T cell ratios in the blood and tumors of tumor-bearing female mice compared to male mice (Table 1). A lower ratio is often associated with favorable prognosis of tumor progression and survival. Collectively, we see that the response of adaptive T cells to melanoma is increased in female mice compared to male mice, corresponding to sex differences in tumor growth.

DISCUSSION

In this study, we report an interesting finding on sex differences in immune response to melanoma tumor growth in a B16-F10/BL6 mouse model. Until now, there have been no reports into the mechanistic links between sex and the disparity in melanoma incidences, growth, and survival. One assumption used to explain these differences is that behaviors that inform decisions to detect melanomas and visit to health care providers are more associated with females than males [15]. However, this hypothesis does not account for progression after disease onset which shows less aggressive metastasis in females [6]. Our mouse model omits variability caused by diagnosis-seeking behavior and still displayed a striking

phenotype of less tumor growth in females (Fig. 1b and S1). Although differences in immune function have been broadly proposed as a mechanism to explain the sex disparity in melanoma, it has not been fully investigated [16].

T cells are important players in melanoma response, and helper T cells specifically are important for protection against malignancy by activating and recruiting antigen-specific effector cells [17]. This T-cell mediated response requires CD4⁺ in the priming stage and CD8⁺ in the effector stage [8]. These T cells release functional cytokines such as interferon gamma (IFN γ) and interleukin-2 (IL-2) which are important for anti-tumor immunity [8]. Our study utilized a melanoma model that elicits responses by both CD4⁺ and CD8⁺ T cells via Tyrp-2 recognition on B16-F10 cells [8]. Our findings suggest that the sexual dimorphism in the immune system of females and males, as seen in the healthy population, could be relevant in the context of melanoma progression and outcomes [9,16]. In a healthy population, the adaptive immune system is generally stronger in females compared to males [9], a phenomenon we were able to confirm in our control mice (Fig. 2a). This difference in immune system strength was similar between the sexes in the context of melanoma (Fig. 2b–c). Previous studies using mouse models have further shown that helper T cells produce IFN γ at higher levels in female mice compared to male mice in response to parasitic infections [18]. Even in humans, females produced higher IFN γ upon stimulation of naïve CD4⁺ T cells compared to males [19]. In melanoma patients, a clinical study identified an increased number of antigen-specific helper T cells in females compared to males [20]. Taken together, the body of evidence suggests the possibility of greater anti-tumor immune activity in females compared to males. However, further work is still needed to characterize circulating and tumor-infiltrating T cells by subsets and functionality, memory versus effector T cells and cytokine activity, in our model. Nevertheless, our findings provide a framework for future mechanistic studies for understanding sex differences in immune responses against melanoma growth rate.

A possible confounder in this study is the endocrine system, and more specifically sex steroids, which can influence immune responses by binding to specific-hormone receptors on immune cells [21]. In particular, estrogen is widely hypothesized to be a key player in the sex disparity in melanoma [22]. The influence of estrogen could be direct, as estrogen receptor alpha (ER α) is differentially expressed by T cells, allowing for direct classical regulation by estrogen [23]; or indirect through non-classical signaling that can occur via protein–protein interactions between estrogen receptors and estrogen receptor element-independent transcription factors like NF κ B [24]. Estrogen has been shown to enhance IFN γ secretion, by increasing *Ifng* transcription or upregulating the Th-1 transcription factor, T-bet, which promotes T cell responses and cell-mediated immunity [25,26]. Furthermore, exogenous estrogen has been demonstrated to enhance the expansion of regulatory T cell populations in vivo, introducing additional environmental confounders [27]. These links suggest a secondary regulation of the immune system, and potentially the immune response, through sex steroids like estrogen. It is important to uncouple the mechanisms involved with sex differences in immune response against melanoma, but it will require further investigation as to whether these differences are independent of hormonal signaling.

Finally, the sex differences in immune response hold implications for immunotherapy, which is widely used in melanoma treatment. A common therapeutic strategy is using anti-PD-1/PD-L1 antibodies as checkpoint inhibitors to bolster the host's immune response [28]. Interestingly, there were sex differences observed in the use of PD-L1 blockade in B16 melanoma tumor-bearing mice where the females responded better to treatment by showing greater decrease in regulatory T cells which increased cytotoxic T cells. Consequently, this resulted in reduced tumor growth in females compared to males [29]. Despite similar expression levels, the difference in PD-L1 signaling between males and females remains unclear. The identification of sex-dependent differences in immune regulation and response will be important in identifying novel treatment strategies against melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlight

Females have less melanoma progression compared to males. Using a B16-F10/BL6 mouse tumor model, this study identified differential immune responses of CD4 and CD8 T cells to melanoma as potential mechanisms that underlie this sex disparity in melanoma growth rate.

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Fig. 1. Sex differences in melanoma growth.

(a) Description of timeline. Male and female wild-type C57BL/6 mice were injected (subcutaneously) with 200,000 B16-F10 melanoma cells. Tumor growth was measured from day 8 through 14. When any tumor sizes approached a maximum of 1000 mm³, all animals were sacrificed, and tissue samples were collected for further processing. **(b)** Tumor volumes were measured on day 8 through 14 for male and female mice. Statistical comparison was performed using repeated measures Two-way ANOVA with Sidak's correction. n=6 mice for each group. *=p<0.05, ***=p<0.001, ****=p<0.0001.

Fig. 2. Sex differences in immune response to melanoma.

Melanoma tumor-bearing mice were sacrificed on Day 15 after tumor implantation, alongside control mice with no tumors. Lymphocytes were isolated from blood (a–c) and tumors (d), counted and immunophenotyped for CD4⁺ and CD8⁺ T cell markers and analyzed by flow cytometry. Lymphocytes from blood were represented per μL of blood and from tumors were represented per mm^3 of tumor volume. Statistical analysis was done using student's t test between sex. $n=5-6$ for tumor bearing mice and $n=3$ for control mice.

*= $p<0.05$, **= $p<0.01$. Error bars = S.E.M.

Table 1:

CD4/CD8 T cell ratios in tumor-bearing mice

Tissue/Sex	Male	Female
Blood	2.09	1.45
Tumor	0.69	0.36