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Mast cells impair the development of protective anti-tumor immunity

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Abstract

Mast cells have emerged as critical intermediaries in the regulation of peripheral tolerance. Their presence in many precancerous lesions and tumors is associated with a poor prognosis, suggesting mast cells may promote an immunosuppressive tumor microenvironment and impede the development of protective anti-tumor immunity. The studies presented herein investigate how mast cells influence tumor-specific T cell responses. Male MB49 tumor cells, expressing HY antigens, induce anti-tumor IFN- γ + T cell responses in female mice. However, normal female mice cannot control progressive MB49 tumor growth. In contrast, mast cell-deficient c-Kit^{W^{sh}} (W^{sh}) female mice controlled tumor growth and exhibited enhanced survival. The role of mast cells in curtailing the development of protective immunity was shown by increased mortality in mast cell-reconstituted W^{sh} mice with tumors. Confirmation of enhanced immunity in female W^{sh} mice was provided by (1) higher frequency of tumor specific IFN- γ + CD8+ T cells in tumor-draining lymph nodes compared with WT females and (2) significantly increased ratios of intratumoral CD4+ and CD8+ T effector cells relative to tumor cells in W^{sh} mice compared to WT. These studies are the first to reveal that mast cells impair both regional adaptive immune responses and responses within the tumor microenvironment to diminish protective anti-tumor immunity.

Introduction

Inflammatory immune cells contribute to the development of tumors by promoting neovascularization, tissue remodeling, and inflammation [1]. Mast cells (MCs) are among the first inflammatory cells to accumulate around tumors, infiltrating pre-malignant lesions and promoting tumor growth in murine models of pancreatic cancer, neurofibromas, colorectal cancer, and squamous cell carcinoma [2–5]. MCs contribute to the growth of tumors in multiple ways. They are a rich source of pro-angiogenic molecules capable of promoting tumor vascularization and are associated with increased microvascular density in many tumors [2, 4, 6, 7]. MCs promote inflammation within the tumor microenvironment by recruiting inflammatory cells and also by switching regulatory T cells to a pro-inflammatory phenotype [3, 8–10]. Increased numbers of MCs infiltrating certain human tumors are associated with an unfavorable prognosis and decreased survival [11–14].

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Although high densities of MCs are associated with poor prognosis in human cancers, high densities of intratumoral T effector (T^{eff}) cells, especially those with Th1 and cytotoxic gene signatures (such as IFN- γ , IRF-1, and granulysin) are associated with a favorable prognosis in many human cancers [15–18]. However, the cells and molecules controlling the balance between protective and nonprotective immune responses to tumors are poorly defined. In particular, the roles of MCs, which are immunomodulatory cells having both positive and negative effects on adaptive immune responses, are not well understood [19]. In this study, we investigated how MCs influence adaptive T cell responses to tumors. We used the W^{sh} mouse strain with a profound deficiency of MCs caused by a mutation in the c-kit receptor [20, 21]. The MB49 male bladder carcinoma line was used because this tumor line recruits MCs, which promote tumor angiogenesis [6]. Thus, comparing tumor growth in C57BL/6 wildtype (WT) and W^{sh} mice provides insight on the effect of angiogenesis on tumor growth. Also MB49 cells expressing HY antigens trigger anti-tumor T cell responses in females, but not males because they are centrally tolerant of HY antigens. Thus, comparing tumor growth in males and females provides insight on the effect of rapid T cell responses on tumor growth. However, even though WT females make rapid T cell responses, such responses are not sufficient to control the growth of this tumor [22].

Here, we present evidence that MC-deficient W^{sh} female mice were capable of controlling tumor growth and surviving systemic tumors significantly better than WT female mice. Enhanced resistance to tumors in W^{sh} female mice was T cell-mediated, as demonstrated by adoptive transfer of tumor immunity by T cell subsets, as well as loss of tumor resistance upon in vivo T cell depletion. Moreover, W^{sh} female mice had increased frequencies of IFN- γ -producing CD8 $^{+}$ T effector cells in tumor-draining lymph nodes (dLNs) compared with WT females. Additionally, W^{sh} female mice had significantly increased ratios of intratumoral CD4 $^{+}$ CD44 hi and CD8 $^{+}$ CD44 hi T cells relative to tumor cells compared to WT females. These studies are the first to reveal that MCs impair both regional adaptive immune responses and responses within the tumor microenvironment to diminish protective anti-tumor immunity, suggesting MCs are attractive therapeutic targets in promoting anti-tumor immunity.

Materials and methods

Mice

Male and female 6 to 8-week-old C57BL/6 mice were obtained from the National Cancer Institute (Bethesda, MD). C57BL/6 $Kit^{W-sh;W-sh}$ (W^{sh}) mice were purchased from the Jackson Laboratory and were bred in the Dartmouth Medical School specific pathogen-free animal facility. Experiments were approved by the Institutional Animal Care and Use Committee of Dartmouth College.

Antibodies, reagents and flow cytometry

Mouse monoclonal antibodies were purchased as follows: Fc γ RI (MAR-1) from eBioscience, CD44 (IM7), CD45 (30-F11), CD117 (2B8) from Biolegend, CD8 (53-6.7) and CD4 (RM4-5) from BD Bioscience, CD31 (MEC7.46) from Abcam Inc. IL-3, and stem cell factor (SCF) were purchased from Peprotech.

Immunohistochemistry

Mast cells were detected by Toluidine Blue staining of formalin-fixed samples as described [20]. Microvascular density in frozen, acetone-fixed tumor sections was determined by staining with an antibody to CD31 (PECAM) as described [46].

Cell culture and tumor challenge

MB49 was maintained in RPMI complete medium with 10 % FBS. Mice were given 2.5×10^5 MB49 cells by intradermal (i.d.) route, and tumor diameters were measured with a caliper thrice weekly. Alternatively, mice were given tumor cells (2.5×10^5) intravenously (i.v.) in the tail vein and mice were monitored for survival.

Mast cell reconstitution

Bone-marrow-derived MCs were generated by culturing bone marrow cells with IL-3 (20 ng/ml) and SCF (50 ng/ml) for 5–8 weeks [47, 48]. Purity was assessed by CD117 (c-Kit) and Fc RI staining. A total of 5×10^6 BMMCs were injected i.d., i.v., and intraperitoneally into W^{sh} recipients, which were rested 8 weeks before use.

In vivo depletion of T cell subsets

Hybridoma cell lines GK1.5 (anti-CD4) and 2.43 (anti-CD8) from ATCC were used to prepare depletion antibodies as described [49]. Antibodies were given on day –4 and 0 prior to tumor inoculation and weekly thereafter (250 μ g/mouse). A 95 % reduction of targeted cells was confirmed by flow cytometry.

Enzyme-linked immunospot assay

IFN- γ enzyme-linked immunospot assay (ELISPOT; Mabtech) was performed as described previously [49]. Briefly, magnetic-bead purified CD8 $^+$ T cells from tumor dLN were plated at 2×10^5 per well with 2×10^5 irradiated MB49 tumor cells or 2×10^5 irradiated male T-depleted spleen cells for 12–16 h. ELISPOTs were detected using BD Biosciences reagents according to manufacturer's protocol. Spots were counted using an Automated ELISPOT Reader System with KS 4.3 software (Carl Zeiss).

Isolation and characterization of tumor-infiltrating cells

Tumors were dissociated mechanically in PBS/5 mM EDTA, filtered (40 μ m mesh), cells blocked in HBSS with 10 % serum, washed, and resuspended in PBS. Amine reactive LIVE/DEAD near-IR fixable dye (Invitrogen) was included in mAb staining cocktails to stain dead cells, which were excluded from the analysis. CountBright™ beads (Invitrogen) were added to calculate absolute numbers of tumor cells (live CD45neg SSC $^+$ FSC $^+$), CD4+CD44 $^+$, and CD8+CD44 $^+$ T cells per tumor. The ratios of tumor and T cells were calculated from the total numbers of tumor cells and T effector cells in tumors.

Statistical analysis

Data were expressed as the mean $\pm$ SEM and differences between groups were analyzed by two-tailed ANOVA, the unpaired t test, and the Mann–Whitney test. To detect differences in survival, log-rank analyses of Kaplan–Meier data were conducted (GraphPad Software).

Results

MCs accumulated around MB49 tumors and were associated with increased angiogenesis within tumors

MB49 tumor cells were injected intradermally (i.d.) on the right flanks. In WT mice, MCs accumulated abundantly at the growing edge of MB49 tumors at the boundary between tumor tissue and skin (Fig. 1a). To investigate angiogenesis in the tumors, sections were stained with anti-CD31 antibody to visualize the vasculature in and around the tumor mass and the staining was quantified (Fig. 1b, c). Microvascular density was similar in all groups on day 5 of tumor (not shown). However, the microvascular area of the tumor was reduced

in both female and male W^{sh} mice as compared to female and male WT mice on day 12 (Fig. 1c). Both female and male WT mice have high microvascular density at the growing edge of tumor and within the tumor mass. In contrast, microvascular density was significantly reduced in both male and female W^{sh} mice and when vasculature was observed, it was present only at the growing edge of the tumor (Fig. 1b top panels). These data confirm that the c-Kit W^{sh} strain of MC-deficient mice, similar to the WBB6F1 WW-V strain of MC-deficient mice [6], exhibit reduced angiogenesis of MB49 tumors.

Mast cell-deficient W^{sh} females, but not W^{sh} males controlled the growth of MB49 tumors

To determine whether the genetic loss of MCs influenced tumor growth, we compared the growth rates of MB49 tumors in WT and mast cell-deficient W^{sh} mice. Progressive tumor growth was observed in WT females, WT males, and W^{sh} males. In contrast, W^{sh} females controlled tumor growth significantly better than WT females ($p < 0.0001$) (Fig. 2a). Representative appearance of tumors on days 7 and 15 in WT and W^{sh} females and males are shown in Fig. 2b. On day 7, most W^{sh} female mice had developed central ulcers in their tumors, and by day 15, the tumors in most W^{sh} females regressed leaving a flat skin lesion containing few tumor cells. Some of the W^{sh} male and WT female mice also developed ulcers at the center of the tumors; however, the tumors continued to grow progressively around the apparently necrotic central core. In WT males, tumors grew progressively, forming large, raised lesions. These data indicate that W^{sh} female mice controlled MB49 tumor progression better than WT females. Moreover, MC deficiency and the consequently diminished tumor angiogenesis in W^{sh} males were not sufficient to control the progressive growth of MB49 tumors.

Reconstitution of W^{sh} female mice with MCs abolished their enhanced resistance to systemic tumors

Systemic MB49 tumors were also used to investigate the role of MCs in anti-tumor immunity. Systemic challenge with MB49, resulting in lung tumors [23], led to the death of WT female and WT male mice and all male W^{sh} mice by day 80 (Fig. 3a). In contrast, 50 % of female W^{sh} mice survived systemic tumor challenge to day 80, corresponding to the end of the monitoring period. Such enhanced survival of W^{sh} female mice with systemic MB49 tumors was abolished by reconstitution of W^{sh} females with WT bone-marrow-derived MCs (BMMCs) (Fig. 3b). All of the female W^{sh} mice reconstituted with BMMCs died of lethal systemic tumor at the same rate as WT females. Mast cell reconstitution was validated by the enumeration of MCs in peritoneal lavage of reconstituted W^{sh} mice (Fig. 3c). These results demonstrate that MCs contribute to impaired resistance to tumors in the MB49 tumor model.

Enhanced tumor protection was T cell dependent in female W^{sh} mice

We next investigated the role of T cells in W^{sh} female mice with systemic tumors. In contrast to untreated W^{sh} female mice, all W^{sh} female mice depleted of CD4+ or CD8+ T cells succumbed to systemic tumors (Fig. 4a, b). Thus, the enhanced survival of W^{sh} female mice during systemic tumor challenge required both CD4+ and CD8+ T cells. Additionally, T cell-mediated tumor immunity established in a mast cell-deficient environment was transferable to naive WT female mice. “Immune” T cells were isolated from female W^{sh} mice 30–50 days after their complete rejection of tumors and were transferred into naive female WT mice. In parallel, T cells were isolated from naive W^{sh} female mice and transferred to naive female WT mice. T cell recipients and male and female controls were then challenged with MB49 tumors. WT recipients of “immune” T cells from W^{sh} mice rejected tumor faster than naive W^{sh} mice rejected primary tumor (Fig. 4c, d). Moreover, naive WT mice that received T cells from naive W^{sh} mice grew tumor with the same

kinetics as naïve WT mice (Fig. 4c, d). Thus, immune T cells generated in a mast cell-deficient environment were protective upon transfer into a mast cell-sufficient environment.

W^{sh} female mice have increased frequencies of IFN- γ -secreting anti-tumor CD8⁺ T cells in tumor dLNs

The improved survival and enhanced tumor regression of W^{sh} female mice prompted us to compare their anti-tumor T cell responses with those of WT female mice. At 2 weeks after MB49 tumor inoculation, we purified CD8⁺ T cells from the tumor dLNs to assess IFN- γ production in response to male spleens and tumor antigens using a sensitive ELISPOT assay. CD8⁺ T cells from the tumor dLN of W^{sh} female mice had significantly higher frequencies of IFN- γ + cells in response to irradiated male spleen cells (Fig. 5a) as well as to irradiated tumor cells compared with WT cells (Fig. 5b). Also, CD8⁺ T cells from the tumor dLNs of WT and W^{sh} males exhibited equally low IFN- γ responses to tumors, consistent with their failure to control tumor growth. Thus, in female W^{sh} mice lacking MCs, there were more IFN- γ -producing CD8⁺ T cells responding to both male antigens and tumor antigens as compared to WT female mice.

Mast cell-deficient female mice have significantly increased ratios of CD4⁺ and CD8⁺ T effector cells relative to tumor cells in the tumor microenvironment

Since the majority of W^{sh} female mice rejected their tumors, we hypothesized that these mice may have increased infiltration of effector T cells within the tumor. We next measured the numbers of tumor cells and tumor-infiltrating T effector cells on days 7 and 12 after MB49 tumors were given. Tumors were excised and dissociated to single cell suspensions, stained with antibodies, and analyzed by flow cytometry with absolute count beads to determine the cellular composition. The time points were selected because all tumors were of similar size on day 7, but on day 12, the tumors on W^{sh} females had regressed. On day 7, the total numbers of tumor cells, CD8⁺CD44^{hi} and CD4⁺CD44^{hi} T cells, were similar in tumors of all groups. On day 12 after tumor implantation, the W^{sh} female mice had the lowest total numbers of tumor cells as well as the lowest total numbers of intratumoral CD8⁺CD44^{hi} and CD4⁺CD44^{hi} T cells compared with all other groups (Fig. 6a, b, c). In contrast, WT male mice with progressively growing tumors had significantly more total numbers of both tumor cells and intratumoral effector T cells compared with the other groups, indicating that the largest tumors contained the highest total numbers of T effector cells. We next compared the ratios of intratumoral T cells relative to tumor cells. Tumors from the W^{sh} female mice contained significantly higher ratios—on average 6–10- times more of CD8⁺CD44^{hi} T cells and CD4⁺CD44^{hi} T cells, respectively, relative to tumor cells compared with tumors from WT female mice and other groups (Fig. 6d, e). Although increased absolute numbers of CD4⁺ and CD8⁺ T effector cells were present in WT males with progressively growing tumors, tumor cells outnumbered T effector cells by 100 to 1 in nine of twelve B6 male tumors investigated. Therefore, the ratio of T effector cells to tumor cells was low in B6 males. Hence, MCs greatly diminished the ratios of intratumoral T cells relative to tumor cells.

Discussion

The role of MCs in promoting malignancy is multifaceted, as they orchestrate the character of the tumor microenvironment promoting inflammation, angiogenesis, and tissue remodeling [24]. In addition, we now provide evidence that MCs impair the development of protective anti-tumor immunity resulting in diminished host survival. MCs in female mice impair effective anti-tumor IFN- γ -producing CD8⁺ effector T cells capable of controlling progressive tumor growth. We found that adoptive transfers of tumor specific effector W^{sh} T cells were protective in MC-sufficient hosts suggesting that MCs likely influence the

inductive, rather than effector, phase of anti-tumor immunity. Reconstitution of W^{sh} female mice with BMMCs abolished their resistance to tumors, implicating MCs in impairing anti-tumor immunity.

Insights into the relative importance of angiogenesis in the tumor microenvironment and adaptive immunity to host survival were provided by comparing tumor growth in male and female, WT and mast cell-deficient hosts. MC-deficient male and female W^{sh} mice both exhibited reduced tumor angiogenesis, confirming the role of MCs in tumor vascularization. The progressive growth of MB49 tumors in W^{sh} male mice indicates that the absence of MCs and their pro-angiogenic factors will not sufficiently impair tumor growth to promote enhanced host survival. Thus, the reduced angiogenesis in the W^{sh} tumor microenvironment had no overall impact on the growth of tumors in W^{sh} male mice. This is consistent with the minimal impact of antiangiogenic therapeutics as monotherapies in oncology indications [25]. These findings are in striking contrast to tumor growth and host survival in female W^{sh} mice, where there was greatly impaired tumor growth and 50 % or more of W^{sh} female mice survived systemic tumors up to 80 days. In females, but not males, MB49 tumors induce anti-HY T cell responses. However, the adaptive immune response in WT female mice in the absence of tumor microenvironment disruption also was largely incapable of interfering with tumor growth. Our preliminary studies underway show that blockade of VEGF in female mice and not in male mice will similarly promote tumor rejection in female mice. Enhanced anti-tumor immunity to an immunogenic tumor with anti-VEGF has also been previously reported [26]. Taken together, these findings suggest that impaired angiogenesis synergizes with an adaptive T cell response to enhance protective antitumor immunity.

Mast cells are a source of a number of pro-angiogenic molecules including VEGF, bFGF, and IL-8 [27, 28]. Proangiogenic molecules not only play a role in the development of the tumor microenvironment, but also are powerful immunoregulatory molecules that control the development of tumor-specific immunity. For example, blockade of VEGF-induced angiogenesis has been shown to enhance infiltration of tumors with T effector cells during adoptive immunotherapy and tumor vaccination [29, 30]. Consistent with this, we found that in the absence of MCs, angiogenesis was reduced and tumors had increased ratios of CD4+ and CD8+ T effector cells relative to tumor cells. A systemic impact of MCs on T cell responses may manifest through the ability of MCs to influence dendritic cells (DCs). MCs influence the early stages of DC migration and function [31, 32], and this may be the critical way in which MCs contribute to the development of aberrant tumor immunity. Mast cell mediators such as TNF- α , leukotrienes, histamine, and GM-CSF can dramatically modulate DC maturation and induce DC migration [31, 33, 34]. Many of the mast cell-mediated DC modifications reported must consequently be reflected in the development of particular T cell responses. In models of allergy for example, MCs induce a Th2 profile by the production of prostaglandin E2 and histamine, mast cell mediators that induce DCs to produce CCL17/22 [35]. The aforementioned mediators are Th2-cell recruitment factors and are known to suppress the frequency of Th1 allergen-specific cells both in vivo and in vitro [35–37]. In allergy, this augments inflammation and the pathology of the disease; yet in a different setting, such as that of the tumor microenvironment may contribute to impaired antitumor response.

Mast cells have been considered intermediaries in immune suppression in several immune contexts. They express MHC-II and MHC-I, and recent reports have shown MCs to be bona fide antigen-presenting cells in that they express other co-stimulatory molecules such as OX40L, CD30 ligand (CD30L), Fas, glucocorticoid-induced TNF receptor (GITR) as well as CD80, CD86, PD-L1, and PD-L2 [38, 39]. MCs expressing MHC class II are capable of expanding antigen-specific T regulatory cells [40]. MCs have the ability to skew naïve T

cells into a Th2 phenotype by inducing production of IL-4, IL-10, and IL-13 and suppressing production of IFN- γ [41, 42]. Th2-mediated immunity in turn has been associated with the inhibition of anti-tumor immunity, both by promoting angiogenesis and by suppressing cell-mediated immunity and effective tumor clearance [43]. One example of mast cell-induced downregulation of Ag-specific T cell proliferation is the IL-10-mediated suppression that is seen in the context of mosquito bites [44]. It is, therefore, plausible to hypothesize that MCs are directly contributing to the immune suppression seen in our model, albeit not necessarily in a contact-dependent manner. MCs also produce IL-10 and TGF- β and may as such be able to suppress T cell proliferation and even mediate in the generation of adaptive Tregs. It will be crucial to assess whether MCs and mast cell-derived VEGF, IL-10, or/and TGF- β could affect the number, phenotype, or function of Tregs in the tumor microenvironment.

Mast cells may impair the development of protective anti-tumor immunity by multiple mechanisms including direct MC interactions with T cells, MCs influencing DCs cytokines and functions, and by alterations in the tumor microenvironment such as increased tumor angiogenesis [45]. Studies are currently underway to understand the precise mechanisms by which MCs influence the adaptive immune responses to tumors.

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Figure 1.

Mast cells accumulate around MB49 tumors and contribute to increased microvascular density in tumors of WT mice. (a) Toluidine blue staining of MB49 tumor sections show mast cells surrounding the tumor. Two different sections from MB49 tumors (day 12) in WT female mice are shown. One representative normal skin section stained with Toluidine blue is shown. (b) Representative images of CD31 staining on tumor sections (day 12) from C57BL/6 female mice and Wsh female mice. The dotted lines on the top images show the boundary between tumor and normal tissues. (c) Quantification of immunohistochemical staining for CD31 on sections of C57BL/6 female and male and Wsh female and male i.d.

tumor sections (n = 4 per group). Microvascular density was calculated by dividing number of pixels staining with CD31 over number of pixels in total skin section examined

Figure 2.

Mast cell-deficient Wsh female mice show superior regression of MB49 tumors. a MB49 tumor growth curves in C57BL/6 female and male mice and the genetically mast cell deficient W^{sh} male and female mice. Mice were inoculated intradermally (i.d.) with 2.5×10^5 cells per mouse and perpendicular diameters of tumors were measured; shown are the mean and SEM for 6–8 mice per group. The graph shown is representative of 10 experiments. Tumor growth in female B6 compared with female W^{sh} mice was significantly different (2-way ANOVA). b Top panels are images of representative day 7 tumors. Bottom panels are images of representative day 15 tumors in WT males and female and W^{sh} males and females.

Figure 3.

Enhanced survival of W^{sh} females with systemic MB49 tumors was abolished by their reconstitution with bone-marrow derived mast cells. **a** MB49 tumor was injected systemically through the tail vein, and mice were monitored for survival. Survival curve is a composite of four independent experiments. **b** Systemic reconstitution of W^{sh} female mice with C57BL/6 bone-marrow-derived mast cells (BMMC). Cells were inoculated i.v., i.d., intraperitoneally, and subcutaneously to ensure mast cell reconstitution in all anatomical locations. Survival graphs show data from three independent experiments. **c** FACS plots showing representative samples from C57BL/6, W^{sh} , and W^{sh} reconstituted with WT bone-

marrow mast cells. Cells are from an intraperitoneal lavage stained for Fc RI and CD117 (ckit), to identify mast cells.

Figure 4.

Enhanced resistance of W^{sh} female mice to tumors is T cell mediated and can be adoptively transferred into mast cell-sufficient mice. Groups of mice were given MB49 tumors i.v. and monitored for survival. a and b Enhanced survival of W^{sh} female mice with systemic MB49 tumors was abolished by antibody-mediated depletion of CD8+ (-CD8 W^{sh} F) or CD4+ T cells (-CD4 W^{sh} F). Survival curves of W^{sh} mice depleted of CD4+ T cells and CD8+ T cells were significantly different from survival of intact W^{sh} mice *p<0.04. c and d MB49 tumor was injected into groups of mice by i.d. route. Growth rates (perpendicular diameters) of i.d. MB49 tumor are shown with each point representing the mean and SEM of 6–8 mice. c Growth rate of MB49 tumor in W^{sh} females and B6 female and male controls shown for comparison to those in d. d Adoptive transfer of combined CD4+ and CD8+ T cells isolated from spleen and dLNs of W^{sh} female mice that had previously completely rejected i.d. MB49 (immune T cells), or from naive W^{sh} female mice into naive C57BL/6 female mice. Growth of MB49 tumor in recipients of immune T cells was significantly reduced compared with growth rate of MB49 tumors in naive W^{sh} mice (p<0.001). Data show composite results from 3 independent experiments. p values were calculated by the 2-way ANOVA.

Figure 5.

Increased IFN- responses in tumor dLNs of Wsh female tumor-bearing mice. The frequencies of tumor-responsive CD8+ T cells were measured in an ELISPOT assay. Tumor-draining inguinal lymph nodes from four mice were pooled and CD8+ T cells were enriched to 90–95 % purity and plated into triplicate wells for each condition. Enriched CD8+ T cells were plated at 2×10^5 cells per well with T-depleted male spleens as antigen-presenting cells (APCs) or b irradiated MB49 tumor cells. Bars show the mean and SEM of ELISPOTS per 2×10^5 input CD8+ T cells with 3–10 points per group measured in four independent experiments. On the X-axis, the following abbreviations were used: B6 F, WT females; W^{sh} F, W^{sh} females; B6M, WT males; W^{sh} M, W^{sh} males. Significance was determined by unpaired *t* test.

Figure 6.

Wsh female mice have significantly increased ratios of intratumoral CD4⁺ and CD8⁺ T effector cells relative to tumor cells compared with other groups. Mice were given MB49 tumors by i.d. route. Tumors were excised and analyzed by flow cytometry on days 7 and 12. Absolute numbers of a CD4⁺CD44^{hi} T cells b CD8⁺CD44^{hi} T cells c and tumor cells in tumors from WT and W^{sh} female and male mice. d Ratios of intratumoral CD4⁺CD44^{hi} T cells per total numbers of tumor cells are shown for each group. Wsh female compared with WT females $p = 0.0024^{**}$. e Ratios of intratumoral CD8⁺CD44^{hi} T cells per total tumor cells are shown for each group. Ratios for W^{sh} females compared with WT females, $p = 0.006^{**}$. Points represent the mean and SEM of 8–9 mice/group on day 7 and 10–16 mice/group on day 12, data points were obtained in two independent replicate experiments. The Mann–Whitney test was used to determine significance.