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The expression of MHC class II molecules on murine breast tumors delays T cell exhaustion, expands the T cell repertoire and slows tumor growth

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Abstract

The expression of MHC class II molecules (MHCII) on tumor cells correlates with survival and responsiveness to immunotherapy. However, the mechanisms underlying these observations are poorly defined. Using a murine breast tumor line, we showed that MHCII-expressing tumors grew

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Animal source. BALB/c mice were purchased from Charles River Laboratories International, Inc. BALB/c.scid mice (CBySmm.CB17-Prkdc^{scid}J) were purchased from The Jackson Laboratory.

Cell line authentication. TS/A cells were obtained at passage 22 and passaged 2 times prior to freezing archival samples. TS/A cells were authenticated by assessing MHC haplotype via flow cytometry and by detection of antigens from murine leukemia virus. Transfected and control TS/A cells were also confirmed by gene expression of MHCII pathway gene products using nanostring assay and Western blot. Both cell lines tested negative for mycoplasma (and 13 other mouse pathogens) via PCR performed by Charles River Research Animal Diagnostic Services on June 2015.

more slowly than controls and recruited more functional CD4+ and CD8+ T cells. Additionally, MHCII-expressing tumors contained more TCR clonotypes expanded to a larger degree than control tumors. Functional CD8+ T cells in tumors depended on CD4+ T cells. However, both CD4+ and CD8+ T cells eventually became exhausted, even in MHCII-expressing tumors. Treatment with anti-CTLA4, but not anti-PD-1 or anti-TIM-3, promoted complete eradication of MHCII-expressing tumors. These results suggest tumor cell expression of MHCII facilitates the local activation of CD4+ T cells, indirectly helps the activation and expansion of CD8+ T cells and, in combination with the appropriate checkpoint inhibitor, promotes tumor regression.

Précis

Expression of MHCII on tumors augments T cell responses, delays T cell exhaustion and promotes tumor rejection with some checkpoint inhibitors. Hence, strategies to induce MHCII expression will likely enhance responses to immunotherapy.

Keywords

Breast cancer; MHC class II; T cell exhaustion; TCR repertoire

INTRODUCTION

The capacity of the immune system to recognize and respond to tumors is now generally accepted [1, 2], although these responses are often counteracted by a variety of local immune-inhibitory mechanisms, including signaling through T cell inhibitory receptors [3]. In fact, the therapeutic inhibition of these receptors with antibodies, known as checkpoint inhibitors, can elicit remarkable clinical responses [4, 5]. However, for many tumor types, only a minority of patients experiences a therapeutic benefit, a phenomenon ascribed to variations in the tumor microenvironment as well as the magnitude of the local immune response prior to treatment [6, 7]. Thus, it is important to understand how variations in gene expression by tumor cells and the tumor microenvironment contribute to clinical outcomes.

The expression of MHC class II (MHCII) molecules by tumor cells is one variable that strongly predicts clinical outcomes. Although the expression of MHCII is normally restricted to professional antigen-presenting cells, such as dendritic cells, macrophages and B cells, our group and others have shown that expression of MHCII on tumor cells is associated with improved patient outcomes [8–15], and more vigorous immune responses. In fact, the expression of the class II transcriptional activator (CIITA), the master regulator of MHCII expression [16], is independently associated with PFS in breast cancer [14]. These data suggest that the presentation of antigen to CD4+ T cells by tumor cells is a critical component of functional anti-tumor immune responses.

CD4+ T cells are typically not cytolytic, but instead make cytokines that promote inflammation and cellular activation. Moreover, CD4+ T cells provide “help” to CD8+ T cells by promoting dendritic cell activation and cross-presentation [17, 18]. Thus, the expression of MHCII on tumor cells likely promotes the local activation of CD4+ T cells, which indirectly help CD8+ T cells kill tumor cells [19, 20]. T cells responding to persistent

antigens, such as those expressed by tumor cells, often become exhausted [21], compromised in their ability to express cytokines or kill target cells [22], permitting tumor outgrowth despite initial immune activation. Thus, MHCII expression on tumor cells may promote successful anti-tumor immunity by enhancing CD4⁺ functionality, creating a more inflamed tumor microenvironment, and preventing the exhaustion of CD8⁺ T cells.

Here we tested how ectopic expression of MHCII on murine TS/A breast tumor cells altered tumor-specific CD4⁺ and CD8⁺ T cell responses. We found that local MHCII expression increased the magnitude and duration of both CD4⁺ and CD8⁺ T cell responses in tumors. We also observed alterations in the T cell repertoire, with more clonotypes expanded to a larger extent in MHCII-expressing tumors. Interestingly, although effector functions of T cells were significantly augmented and prolonged in MHCII-expressing tumors, both CD4⁺ and CD8⁺ T cells eventually became exhausted. The administration of anti-CTLA4, but not anti-PD-1 or anti-TIM3, further augmented immune responses and promoted the eradication of MHCII-expressing tumors. Thus, MHCII expression on tumor cells expands the T cell repertoire and delays T cell exhaustion, thereby allowing checkpoint inhibitors like anti-CTLA4 to facilitate tumor regression.

MATERIALS and METHODS

Cell culture and transfection

TS/A cells were cultured in DMEM (Corning) supplemented with 10% FBS (HyClone Laboratories), dissociated with 0.05% trypsin, 0.53 mM EDTA (Corning), and recultured in DMEM-FBS. Cells were transfected with pcDNA3.1 with or without the hCIITA cDNA (complete coding sequence, accession number AY699071) using Xfect Transfection Reagent (Clontech) and selected in 500 µg/mL geneticin (Life Technologies). MHCII-expressing clones were selected by cell sorting. Western blot for hCIITA, MHCII and CD74 was performed with antibodies against hCIITA (E-12, Santa Cruz Biotechnology), MHCII (M5/114, Millipore), and CD74 (R&D Systems).

RNA isolation and nanostring analysis

Total RNA was purified from excised tumors using the RNeasy Mini kit (Qiagen). RNA from whole blood was isolated using the Paxgene blood RNA kit (Qiagen). RNA quality was assessed using Bioanalyzer 2100 (Agilent) and final concentration determined by Qubit (Life Technologies). For nanostring analysis, 100 ng of purified RNA was added to 3 µL of Reporter CodeSet and 2 µL Capture ProbeSet using an nCounter master kit as recommended (NanoString Technologies). Samples were processed the following day using the nCounter DxPrep Station and nCounter Dx Digital Analyzer (NanoString Technologies). Data was analyzed using nSolver 2.6 software. Heat maps were created using Cluster 3.0 and Java TreeView-1.1.6r4.

Mice, tumor administration and antibody treatments

Mice were injected with 1×10^5 TS/A or TS/A-hCIITA tumor cells into the mammary fat pad. The length and width of tumors were measured by calipers and tumor volume was calculated using the formula, $0.4 \times \text{length} \times \text{width}^2$. For depletion experiments, 200 µg of

CD4-depleting antibody (GK1.5) or isotype control (LTF-2) were administered prior to tumor cell injection (day -1) and again on day 3. For antibody blockade, 200 µg of anti-PD-1 (RMP1-14), anti-CTLA4 (9H10), or anti-TIM3 (RMT3-23) were administered on days 7, 10, 13, 16 and 19. All antibodies were from BioXcell

Tumor disassociation and T cell restimulation

Tumors were excised using a scalpel and weighed using an AL54 analytical balance (Mettler Toledo). Tumors were diced and incubated in RPMI1640 media (Lonza) supplemented with 5% FBS, 1.25 mg collagenase (c7657, Sigma) and 150 U DNase (d5025, Sigma), followed by shaking at 200 rpm for 35 minutes at 37°C. Cell suspensions were filtered through 70 µm nylon cell strainers (Corning). T cells were restimulated in RPMI1640, 5% FBS, 5 ng/mL phorbol 12-myristate 13-acetate (Sigma), 65 ng/mL ionomycin (ThermoFisher Scientific), and 10 µg/mL brefeldin A (Sigma) at 37°C for 5 hours.

Flow cytometry and antibodies

Cell cycle analysis was performed by fixing single cell suspensions of TS/A and TS/A-hCIITA cells overnight with 70% ethanol at 4°C overnight and incubating them with 50 µg/ml propidium iodide (ThermoFisher Scientific) in PBS, 0.1% Triton-X100 (Sigma), and 50 µg/ml RNase at 37°C for 20 minutes in the dark. DNA content was determined using a BD FACSCalibur (BD Biosystems) flow cytometer and analyzed by ModFit LT version 3.3 (Verity Software House).

Immunophenotyping was performed by blocking cell suspensions in PBS with 2% donor calf serum and 10 µg/ml FcBlock (2.4G2 -BioXCell) for 10 min on ice, followed by staining with fluorochrome-conjugated antibodies for 30 minutes. Stained cells were fixed in 10% neutral buffered formalin (Sigma) and permeabilized with 0.1% IGEPAL CO-630 (Sigma) in staining solution for 45 minutes. All staining was performed in the dark at 4°C. Antibodies against I-A/I-E (M5/114.15.2) and CD4 (GK1.5) were obtained from BioLegend. Antibodies against IFNγ (XMG1.2) and CD8 (53-6.7) were obtained from BD Biosciences. Antibodies against CD3 (17A2), KLRG1 (2F1), Granzyme B (NGZB), CD45.2 (104) and PD-1 (J43), were obtained from eBioscience. The MuLV class I tetramer-H2L^d (SPSYVYHQF) was obtained from the NIH Tetramer Core Facility at Emory University. The LIVE/DEAD red fixable dye was obtained from Life Technologies. Samples were run on a BD FACSCanto II system (BD Biosciences) and data was analyzed using FlowJo version 9.9.

T cell repertoire sequencing and analysis

TCR sequencing was performed using the Mouse TCR beta, Illumina, V-C genes kit from iRepertoire, Huntsville, AL. In brief, 500 ng of RNA was reverse transcribed using a One-Step reverse transcription and amplification kit (Qiagen). The PCR product was purified using Ampure XP magnetic beads (Agencourt), and amplified again (TopTaq PCR Kit, Qiagen), to add adaptor sequences. Libraries were purified with Ampure XP magnetic beads and sequenced using Illumina MiSeq 150 nt paired-end read-length. The TCR CDR3 sequences were extracted from the raw sequencing data by iRepertoire. Briefly, raw paired-end fastq files were first demultiplexed based on barcode and merged reads were mapped

using a Smith-Waterman algorithm to germline V, D, J and C reference sequences from the IMGT web site (<http://www.imgt.org>). To define the CDR3 region, the position of CDR3 boundaries of reference sequences from the IMGT database were migrated onto reads through mapping results and the resulting CDR3 regions were extracted and translated into amino acids. Reading frames not containing a stop codon were filtered and error-corrected using iRepertoire proprietary SMART algorithm.

Statistical Analysis

Hypothesis of difference testing between pooled replicate group means at each time point was done using multiple, unpaired independent t-tests with the Holm-Sidak method and assuming $\alpha=0.05$. Statistical analyses were calculated using GraphPad Prism version 7.0a and all reported p-values are two-tailed. The TCR high-throughput sequencing data were analyzed in R environment using tcR package and common R routines. Diversity was measured using the Inverse Simpson Index. CDR3 sequence similarity between repertoires was assessed using overlap index and weighted Horn's index available in the tcR package.

RESULTS

Transfection with hCIITA promotes MHCII expression on TS/A breast cancer cells

To test the role of MHCII on anti-tumor immunity, we transfected the murine breast cancer cell line, TS/A, with the human class II transcriptional activator (hCIITA) or with empty vector. By Western blot, we found that TS/A cells transfected with hCIITA expressed hCIITA as well as murine CD74 (invariant chain) and MHCII (Supplemental Figure 1a), whereas cells transfected with empty vector did not. We also found that hCIITA-transfected cells expressed high levels of murine MHCII on the cell surface (Supplemental Figure 1b). Importantly, we found that the cell cycling times of hCIITA-expressing and control TS/A cells were nearly equivalent (Supplemental Figure 1c).

Surface expression of MHCII impairs tumor growth *in vivo*

We next injected TS/A and TS/A-hCIITA cells into the mammary fat pads of BALB/c mice and used flow cytometry to assess the expression of MHCII on CD45^{neg} cells from disassociated tumors 14 days later. Similar to our findings *in vitro*, we observed that the majority of CD45^{neg} cells from TS/A-hCIITA tumors expressed high levels of MHCII (Figure 1a), whereas CD45^{neg} cells from control tumors did not. However, in contrast to their similar growth kinetics *in vitro*, we found that TS/A-hCIITA tumors grew more slowly than control TS/A tumors, as measured by tumor volume (Figure 1b) and excised tumor mass (Figure 1c). To test whether this difference in tumor growth was dependent on adaptive immunity, we injected tumor cells into BALB/c.scid mice and found that the growth of TS/A-CIITA tumors was indistinguishable from the growth of control TS/A tumors over the same period (Figure 1d). These results suggested that the observed difference in growth of MHCII-positive and MHCII-negative tumors in wild type mice is mediated by adaptive immunity.

To test changes in gene expression, we excised whole tumors, prepared mRNA and analyzed the expression of 48 genes using a NanoString assay. We found that counts of mRNAs

encoding MHCII α and β chains were more numerous in TS/A-hCIITA tumors relative to control tumors (Figure 1e), whereas counts of mRNAs encoding MHC class I molecules were largely similar. We also observed that IFN γ , granzyme B and perforin were upregulated in TS/A-hCIITA tumors relative to control tumors (Figure 1e). Interestingly, we found that the master transcription factor for Tregs, FoxP3, was expressed similarly in TS/A-hCIITA and control tumors. Together, these data demonstrated that MHCII expression on TS/A tumors promoted adaptive immune responses, altered the tumor microenvironment, and impaired tumor growth.

We next tested whether the local expression of MHCII promoted a systemic immune response by first injecting mice with TS/A or TS/A-hCIITA tumors in the left mammary pad, and 7 days later injecting a second tumor into the right mammary pad (Figure 1f). We found that primary tumors grew at rates similar to that seen above (Figure 1g). However, secondary tumors grew more slowly in all groups (Figure 1h), with the expression of MHCII on either the primary or secondary tumors conferring a reduction of tumor growth and, when both primary and secondary tumors express MHCII, 5/5 mice completely rejected the secondary tumor (Figure 1h). Thus, tumor expression of MHCII benefits anti-tumor immunity both locally and systemically.

MHCII-expressing tumors have increased CD4⁺ T cell activation

To test whether MHCII expression bolstered the local CD4⁺ T cell response, we harvested TS/A-hCIITA and control tumors at the indicated times, restimulated the TILs for 5 hours and measured the expression of IFN γ , IL-17 and IL-4. We found very little IL-17 or IL-4 made by T cells from either tumor type (Supplemental Figure 2). In contrast, CD4⁺ T cells in control tumors made some IFN γ , whereas CD4⁺ T cells from MHCII-expressing tumors produced strikingly greater amounts of IFN γ (Figure 2a), even after normalizing the numbers of T cells to tumor mass (Figure 2b). Interestingly, CD4⁺ T cells in TS/A-hCIITA tumors also produced substantially more granzyme B (Supplemental Figure 3), raising the possibility that CD4⁺ T cells may be directly cytolytic to tumor cells. Nevertheless, CD4⁺ T cells in both groups experienced a progressive loss in the ability to produce IFN γ and granzyme B (Figure 2a and Supplemental Figure 3), suggesting that they eventually became exhausted.

Since CD4⁺ T cell stimulation without appropriate costimulation can lead to the differentiation of Tregs, we next enumerated Tregs. We found that the frequency of Tregs was similar in both tumor types (Figure 2d), but that there was a significant reduction of total Tregs per gram in MHCII-expressing tumors (Figure 2e). Moreover, Tregs infiltrating MHCII-expressing tumors expressed lower levels of PD1, suggesting a decrement in suppressive capacity (Figure 2f). Importantly, the CD8:Treg ratio was increased in MHCII-expressing tumors (Figure 2g). These data indicate that tumor expression of MHCII promotes CD4⁺ T cell effector function without promoting Treg accumulation.

Tumor expression of MHCII enhances the CD8⁺ T cell response

Help from CD4⁺ T cells enhances CD8 T cell responses [23, 24]. Therefore, we next enumerated CD8⁺ TILs in control and MHCII-expressing tumors. Given that TS/A tumor

cells harbor an immunogenic murine leukemia virus (MuLV) [25], we initially enumerated MuLV-specific CD8⁺ T cells. We found similar frequencies of total and tumor-specific CD8⁺ T cells in both TS/A-CIITA and control tumors at early times (Figure 3a), but much higher frequencies of total and MuLV-specific CD8⁺ T cells in TS/A-hCIITA tumors on days 17 and 21. The higher frequencies of T cells were reflected in the numbers of total CD8⁺ T cells (Figure 3b) and the numbers of tumor-specific CD8⁺ T cells (Figure 3c).

We next assayed the ability of CD8⁺ T cells to produce IFN γ and granzyme B following restimulation *ex vivo*. We observed dramatically increased production of both IFN γ and granzyme B by CD8⁺ TILs from TS/A-hCIITA tumors relative to those in control tumors at all timepoints (Figure 3d). However, as we observed with CD4⁺ T cells, the CD8⁺ T cells in both groups progressively lost their ability to produce IFN γ and granzyme B (Figure 3d–e). Consistent with the idea that these cells are becoming exhausted, we observed that the expression of the inhibitory receptors, PD-1 and TIM3, increased over time on CD8⁺ T cells (Figure 3f). To demonstrate the functional significance of CD8⁺ TIL exhaustion, we injected TS/A and TS/A-hCIITA cells into BALB/c mice and allowed tumors to grow for an extended period (Figure 3g). We found that although TS/A-hCIITA tumors initially grew more slowly than control tumors, they grew at similar rates following day 21 (Figure 3g), a time that coincides with the loss in T cell effector functions.

Given the expression of PD-1 on CD8⁺ T cells, particularly those in TS/A-hCIITA tumors, we hypothesized that PD-1 blockade would preserve CD8⁺ T cell function and further impair tumor growth. However, we found minimal difference in tumor growth following treatment with anti-PD-1 (Figure 4a). To investigate why PD-1 blockade had no impact on tumor growth, we assayed the expression of the PD-1 ligands, PD-L1 and PD-L2, on TS/A and TS/A-hCIITA tumor cells. We found that PD-L1 and PD-L2 were similarly expressed by control and CIITA-expressing tumors (Figure 4b). As a control for PD-L1 staining, we showed that PD-L1 was highly expressed by a portion of CD45⁺ cells in both groups (Figure 4c). This result led us to test anti-CTLA4 and anti-TIM3 - alone and in combination with anti-PD-1. We found that anti-CTLA4 reduced the growth of control tumors and eradicated MHCII-expressing tumors (Figure 4d), either alone or in combination with anti-PD1. In contrast, anti-TIM3, either alone or in combination with anti-PD1, failed to alter tumor growth (Figure 4e). These data demonstrate the expression of MHCII on tumor cells potentiates the effects of at least some checkpoint inhibitors.

CD4⁺ T cell depletion diminishes the benefit of MHCII expression

To directly test whether CD4⁺ T cells supported CD8⁺ T cell responses, we depleted CD4⁺ cells prior to tumor cell implantation and evaluated tumor growth as well as the number and function of CD8⁺ T cells. As expected, we found that CD4 depletion restored the growth of MHCII-positive tumors to nearly that of MHCII-negative tumors in non-depleted mice (Figure 5a). However, we also found that CD4 depletion modestly increased the growth of MHCII-negative tumors, suggesting that CD4⁺ T cells have some role in promoting anti-tumor immunity via interactions with other, non-tumor, MHCII-expressing cells. Importantly, CD4 depletion notably reduced the frequency of CD8⁺ T cells that produced IFN γ and granzyme B (Figure 5b), albeit not to the levels found in CD8⁺ T cells from

MHCII-negative tumors. Surprisingly, the numbers of CD8⁺ T cells were increased in both MHCII-positive and MHCII-negative tumors following CD4 depletion (Figure 5c). Consequently, the numbers of CD8⁺ T cells producing IFN γ and granzyme B were also greatest in CD4-depleted TS/A-hCIITA tumors (Figure 5d). We confirmed elimination of CD4⁺ T cells following CD4 depletion (Figure 5e). These data suggest that despite the ability of CD4⁺ T cells to provide help to CD8⁺ T cells and facilitate the functional control of tumor growth, their absence does not entirely negate CD8⁺ T cell function and may even allow additional CD8⁺ T cell expansion via homeostatic mechanisms [26, 27].

Expression of MHCII expands the responding T cell clones in tumors

To test whether local MHCII expression promoted T cell clonal expansion or accumulation, we harvested whole tumor and matched blood samples and sequenced the CDR3 region of the TCR β chain to identify unique T cell clones. We found fewer unique sequences in tumors relative to blood but there were no differences between groups (Figure 6a). Although the proportions of individual clones were uniformly distributed in blood, we found many expanded clones in tumors (Figure 6b). Moreover, we observed more clones expanded to a greater magnitude in TS/A-hCIITA tumors than in control tumors, whereas no differences were observed between blood samples from these same mice (Figure 6b).

Using the Inverse Simpson Index as a normalized measure of diversity, we found that blood samples had high index scores that were not significantly different between TS/A-hCIITA and control tumors, whereas tumor samples had low diversity measures and MHCII-expressing tumors were significantly lower than control tumors (Figure 6c). This result can be attributed to the local accumulation of the most abundant clonotypes, especially of the top 10 clones (data not shown). These data suggested that expanded T cell clones accumulated in tumors, but did not recirculate.

To determine which T cell compartment contained expanded clones, we sorted CD4⁺ and CD8⁺ T cells from tumors on day 17 and performed repertoire analysis. Although we found some clonal expansion of CD4 T cells in TS/A and TS/A-CIITA tumors (Supplemental Figure 4a), it was more evident in CD8 T cells from TS/A-CIITA tumors. These differences were not due to the total CDR3 reads (Supplemental Figure 4b), but were more evident in the number of unique reads (Supplemental Figure 4c). However, we found that, after normalization with the Inverse Simpson Index, reduced diversity was evident in only the CD8⁺ T cell compartment (Supplemental Figure 4d).

We next tested whether clonotypes of identical amino acid sequence were expanded in multiple mice. By performing pairwise comparisons of normalized numbers of sequences, we found increased sharing of CDR3 sequences between TILs within MHCII-expressing tumors (Figure 6d). In addition, overlap indexes showed a significantly greater number of common sequences in TS/A-hCIITA tumors relative to controls (Figure 6e). Moreover, weighting sequence overlap by shared clones' abundance using Horn's pairwise overlap index also yielded a significant difference (Figure 6f). Finally, comparing sequences common to multiple mice (rather than two in pairwise comparison) revealed a significant increase in the number of shared sequences amongst TILs from MHCII-expressing tumors (Figure 6g). Collectively, these data suggested that MHCII expression on TS/A-hCIITA

tumors increased intratumoral T cell clonal expansion, including clonotypes shared between mice, suggesting that they are responding to particular tumor antigens.

DISCUSSION

Consistent with previous studies [20], our data show that expression of MHCII on breast cancer cells expands local CD4⁺ and CD8⁺ T cells and impairs tumor growth. Our data extend those studies to show that T cells infiltrating MHCII-expressing tumors produce more effector molecules for longer periods, suggesting that locally-stimulated CD4⁺ T cells promote CD8⁺ T cell accumulation and function. Surprisingly, clonal expansion is primarily observed in CD8⁺ T cells, rather than CD4⁺ T cells. Despite the local activation of both CD4⁺ and CD8⁺ T cells in MHCII-expressing tumors, both cell types eventually become exhausted – a process that can be circumvented using antibodies against CTLA4, but not PD-1 or TIM3. These findings suggest that MHCII expression on tumor cells is an important factor in the success of anti-tumor immune responses.

MHCII molecules are typically expressed by professional antigen-presenting cells, like dendritic cells, which activate naïve CD4⁺ T cells in lymphoid organs. As a result, many tumors contain relatively few MHCII-expressing cells other than immunosuppressive cells of the myeloid lineage [28, 29]. However, if tumor cells themselves express MHCII, then they should be able to directly stimulate CD4⁺ T cells. In fact, previous studies show that MHCII expression enables tumor cells to directly present intracellular antigens to CD4⁺ T cells [20, 30] and promotes dendritic cell accumulation, which may also enhance T cell stimulation. Together, MHCII-expressing tumor cells and dendritic cells should allow CD4⁺ T cells to recognize cryptic antigens and provide help to CD8 T cells [31, 32]. Interestingly, CD4⁺ T cells in MHCII-expressing tumors strongly expressed GZB, suggesting that they may also be directly cytotoxic to tumor cells. However, previous studies from Motara et al [20] show that both CD4⁺ and CD8⁺ T cells are required to eliminate CIITA-expressing tumors, suggesting that CD4⁺ and CD8⁺ T cells have non-redundant activities. Therefore, the most likely explanation is that stimulation of CD4⁺ T cells by tumor-specific MHCII expression provides local help to CD8⁺ T cells, which expand and more effectively kill tumor cells.

The enhanced clonal expansion and effector functions of CD8⁺ T cells in MHCII-expressing tumors, despite unchanged expression of MHC class I molecules, suggests that locally activated CD4⁺ T cells produce factors that enhance CD8⁺ T cell responses [32]. The expanded T cell repertoire in MHCII-expressing tumors may be due to epitope spreading [33], or to the local presentation of neoantigens or cryptic antigens [34]. Although T cells may be responding to hCIITA, which is only about 75% identical to mouse CIITA, this possibility is unlikely to explain the dramatic increase in T cell function. Moreover, immunity against hCIITA-expressing tumors enhances the clearance of tumors that lack hCIITA, suggesting that T cells are primarily responding to endogenous tumor antigens, such as MuLV gp70 [25]. Importantly, CD4 depletion largely negated the benefits of MHCII expression by tumor cells. Paradoxically, CD4 depletion also increased the number of CD8⁺ T cells in tumors, possibly due to homeostatic expansion [35]. However, CD8⁺ T cell activation without help from CD4⁺ T cells can compromise their effector and memory

functions [18, 36] and lead to exhaustion [21] - phenotypes that are consistent with the poor control of tumor growth that we observed following CD4+ T cell depletion.

Despite increased activation of both CD4+ and CD8+ T cells in MHCII-expressing tumors, both cell types eventually lose effector functions and become exhausted. This exhausted phenotype is likely due to prolonged TCR signaling in the absence of inflammation or costimulation [37]. In addition, tumors often acquire immune inhibitory characteristics. For example, although IFN γ is critical for anti-tumor immunity [20], prolonged IFN γ signaling often leads to “adaptive resistance”, in which tumor cells upregulate T cell-inhibitory proteins like indoleamine oxidase (IDO) or PD-L1 [38]. As a result, the augmented IFN γ production by T cells responding to MHCII-expressing tumors may initially promote anti-tumor immunity, but later lead to immune suppression [39].

Although T cell exhaustion can sometimes be overcome with blocking antibodies to inhibitory receptors, like PD-1, in most cases, only a subset of patients respond to therapy. For example, patients whose melanoma tumors express MHCII are more likely to clinically benefit from PD-1/PD-L1 blockade [8]. However, despite the increased expression of PD-1 on CD8+ T cells in our experiments, PD-1 blockade failed to preserve T cell effector functions or promote tumor clearance, regardless of MHCII expression. This result is not entirely unexpected, as we failed to find substantial PD-L1 expression on tumor cells at any time point, a predictor of response to anti-PD-1 blockade [40]. Similarly, anti-TIM3 was also unable to promote tumor control, despite expression of TIM3 on CD8+ TILs. In contrast, anti-CTLA4 antibody led to significantly impaired growth of control tumors and complete rejection of MHCII-expressing tumors. Hence, the efficacy of checkpoint blockade is mediated by numerous factors, including tumor phenotype, pre-treatment immune response and timing of administration.

In summary, our data show that MHCII expression on tumor cells enhances and prolongs both CD4+ and CD8+ T cell responses and slows tumor growth. Although T cells eventually become exhausted, even in MHCII-expressing tumors, their functions can be maintained by checkpoint blockade with anti-CTLA4. Given the enhanced immune responses and better clinical outcomes in both mice and humans with MHCII-expressing tumors [8, 10, 13, 15], we believe that therapies triggering MHCII expression on tumor cells *in vivo* should be developed and used in combination with other immunity-enhancing strategies to prevent T cell exhaustion and promote tumor clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations.

gp70	glycoprotein 70
GZB	Granzyme B
hCITA	human class II transcriptional activator
MHCII	MHC class II
MuLV	Murine leukemia virus

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Figure 1. MHCII expression on tumor cells impairs tumor growth via adaptive immunity.

a. MHCII surface expression on live, CD45^{neg} cells from dissociated tumors was measured by flow cytometry on day 14. **b.** Tumor volume was monitored over time in at least 20 mice BALB/c per group per timepoint. **c.** Tumor mass was determined in 10 samples per group per timepoint. **d.** Tumor volume was monitored over time in 10 BALB/c.scid mice per group. **e.** RNA was extracted from TS/A and TS/A-CIITA tumors (harvest day listed across bottom) and analyzed by Nanostring assay. Heat map shows fold increases in red and fold decreases in green. **f.** Schematic of sequential tumor injections into contralateral mammary pads of the same mouse. **g.** Growth of primary tumors. **h.** Growth of secondary tumors. Error bars represent standard error of the mean. Statistical differences are expressed as * $p < 0.05$, ** $p < 0.005$, # $p < 0.0005$.

Figure 2. MHCII-expressing tumors promote CD4 $^{+}$ TIL activation.

a. IFN γ production by CD4 $^{+}$ T cells was assayed by flow cytometry. Plots are gated on live, CD3 $^{+}$ CD4 $^{+}$ cells. **b.** The number of IFN γ -producing CD4 $^{+}$ T cells was normalized to tumor mass. **c.** The total number of CD4 $^{+}$ T cells was normalized to tumor mass. This experiment contained 5 mice per group per timepoint and was performed two times with similar results. **d.** Tumor-infiltrating Tregs were determined by flow cytometry. Plots gated on live, CD3 $^{+}$ CD4 $^{+}$ cells. **e.** Total number of Tregs normalized to mass. **f.** MFI of PD-1 on

Tregs. **g.** Intratumoral CD8 to Treg ratio. Error bars represent standard error of the mean.
Statistical differences are expressed as * $p < 0.05$, ** $p < 0.005$, # $p < 0.0005$.

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Figure 3. MHCII-expressing tumors maintain functional, tumor-specific CD8⁺ T cells.

a. The frequency of MuLV env-specific CD8⁺ T cells was determined by tetramer binding and flow cytometry. Plots are gated on live, CD3⁺CD8⁺ cells. **b.** The number of CD8⁺ T cells was normalized to tumor mass. **c.** The number of MuLV env-specific CD8⁺ T cells was normalized to tumor mass. **d.** The ability of CD8⁺ T cells to make IFNγ and granzyme B (GZB) following restimulation ex vivo was determined by intracellular staining and flow cytometry. Plots are gated on live, CD3⁺CD8⁺ cells. **e.** The number of CD8⁺IFNγ⁺GZB⁺ cells was normalized to tumor mass. **f.** The expression of PD-1 and TIM3 on CD8⁺ TILs was analyzed by flow cytometry. Histograms are gated on live, CD3⁺CD8⁺ cells. **g.** The volume of TS/A and TS/A-hCIITA tumors was measured over an extended time frame and the arrow indicates a change in tumor growth rate corresponding to T cell exhaustion. These experiments contained 5 mice per group per timepoint and were performed at least two

times. Error bars represent standard error of the mean. Statistical differences are expressed as * $p < 0.05$, ** $p < 0.005$, # $p < 0.0005$.

Figure 4: Checkpoint blockade variably affects tumor control.

a. Tumor growth in anti-PD-1 treated mice. **b-c.** The expression of PD-L1 and PD-L2 on cell suspensions from dissociated tumors was analyzed by flow cytometry. Plots are gated on live, CD45^{neg} cells (**b**) or live, CD45⁺ cells (**c**). **d.** Tumor growth in mice treated with anti-CTLA4 alone or in combination with anti-PD-1 (combo). **e.** Tumor growth in mice treated with anti-TIM3 alone or in combination with anti-PD-1 (combo). These experiments each contained 5 mice per cohort per timepoint. Error bars represent standard error of the mean. Statistical difference is expressed as * $p < 0.05$, ** $p < 0.005$, # $p < 0.0005$.

Figure 5. CD4 depletion impairs tumor growth and CD8+ T cell activity

a. Tumor volume was measured over 14 days. **b.** T cells from tumors were restimulated ex vivo and the expression of IFN γ and Granzyme B was assayed by flow cytometry on day 14. Plots shown are gated on live, CD3+, CD8+ lymphocytes. **c.** The number of CD8+ T cells was normalized to tumor mass. **d.** The number of CD8+IFN γ +GZB+ cells was normalized to tumor mass. This experiment had 5 mice per group and was performed three times with similar results. Error bars represent standard error of the mean. Statistical difference is expressed as *p<0.05, **p<0.005, #p<0.0005.

Figure 6. MHCII expression increases the number and magnitude of expanded TCR clonotypes in tumors, but not blood.

a. The numbers of unique CDR3 reads were compared and horizontal lines denote group mean. **b.** Tree-map plots show relative sizes of individual clonotypes in matched blood and tumor samples on day 14. Each colored shape corresponds to a unique clone and its relative area indicates its representation in the repertoire. **c.** Inverse Simpson Index quantifies expansion of detected clones in blood and tumor. Statistical significance assessed by the non-parametric Mann-Whitney test. **d.** Analysis of TIL CDR3 amino acid sequence similarity in all samples using pairwise overlap indexes, where legend values represent indexes. **e.** Quantification of pairwise comparison is demonstrated by plotting the overlap indexes. **f.** Overlap indexes were weighted by abundance of shared clones using Horn's index. In **e** and **f**, error bars and horizontal lines indicate standard deviation and mean, respectively. **g.** Proportion of CDR3 amino acid sequences in each repertoire that are shared by a number (mice) of other repertoires in the same group (data represents 12 mice).

Horizontal bars represent the mean and error bars are standard deviation. Statistical difference is expressed as * $p < 0.05$, ** $p < 0.005$, # $p < 0.0005$

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