Molecular Programming of Tumor-Infiltrating CD8+ T Cells and IL15 Resistance
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Abstract

Despite clinical potential and recent advances, durable immunotherapeutic ablation of solid tumors is not routinely achieved. IL15 expands natural killer cell (NK), natural killer T cell (NKT) and CD8+ T-cell numbers and engages the cytotoxic program, and thus is under evaluation for potentiation of cancer immunotherapy. We found that short-term therapy with IL15 bound to soluble IL15 receptor α-Fc (IL15cx; a form of IL15 with increased half-life and activity) was ineffective in the treatment of autochthonous PyMT murine mammary tumors, despite abundant CD8+ T-cell infiltration. Probing of this poor responsiveness revealed that IL15cx only weakly activated intratumoral CD8+ T cells, even though cells in the lung and spleen were activated and dramatically expanded. Tumor-infiltrating CD8+ T cells exhibited cell-extrinsic and cell-intrinsic resistance to IL15. Our data showed that in the case of persistent viral or tumor antigen, single-agent systemic IL15cx treatment primarily expanded antigen-irrelevant or extratumoral CD8+ T cells. We identified exhaustion, tissue-resident memory, and tumor-specific molecules expressed in tumor-infiltrating CD8+ T cells, which may allow therapeutic targeting or programming of specific subsets to evade loss of function and cytokine resistance, and, in turn, increase the efficacy of IL2/15 adjuvant cytokine therapy. Cancer Immunol Res, 4(9), 799–811. ©2016 AACR.

Introduction

Antigen-specific immunity to intracellular pathogens requires CD8+ T cells. Inducing CD8+ T cells to mount a response against cancer cells with the same specificity and efficacy as they do against pathogen-infected cells is a long-sought goal of immunotherapy. One method to increase immune responses to cancer is to administer T-cell-trophic/activating cytokines, such as IL2 (1). IL15 closely related to IL2, and expands/activates CD8+ T cells, NK, and NKT cells (2, 3). Relative to IL2, IL15 results in less expansion of regulatory T cells, as it signals independent of IL2Rα, and IL15 has been identified as a strong candidate for clinical translation (4, 5). IL15 half-life/activity is greatly enhanced when complexed with a soluble IL15 receptor α chain coupled to an Fc fragment (IL15cx; refs. 6, 7). IL15cx has shown promise in some cancer models (6–10) and is in clinical trials for treatment of melanoma and multiple myeloma.

Upon exposure to persistent viral antigen, CD8+ T cells become “exhausted,” undergoing a progressive and hierarchical loss of function, and upregulate numerous receptors that inhibit T-cell activation, including PD-1, a process which may serve to limit immunopathology (11, 12). Exhaustion plays a role in the molecular programming of tumor-specific CD8+ T cells (13–15), and PD-1 blockade increases CD8+ T-cell responses to chronic viral or tumor antigen (16, 17). In addition to T-cell receptor (TCR)-mediated signals, T-cell activation by common γ-chain cytokines (such as IL2 and IL15) also induces proliferation and activation of the cytotoxic program (2). If exhaustion evolved as a mechanism of peripheral tolerance to limit host immunopathology, it may do so at multiple levels, including TCR- and cytokine-mediated activation. From this perspective, inhibitory receptors like PD-1 may dampen TCR-mediated cytotoxicity, whereas a parallel program of exhaustion-induced cytokine resistance may limit the abundance of antigen-specific T cells and their acquisition of cytotoxic phenotypes via TCR-independent cytokine signaling. The mechanisms underlying exhaustion-induced cytokine resistance are poorly understood.

Despite screening and current treatments, mammary cancer results in approximately 40,000 deaths annually in the United States. The transgenic, autochthonous PyMT model of mammary carcinogenesis reproduces important aspects of human breast cancer, including stage-wise histologic progression (18). Upon observing marked CD8+ T-cell infiltration of tumors in the PyMT model, we hypothesized that activation and expansion of the abundant CD8+ T cells with IL15cx would promote regression of PyMT tumors. We found, however, that PyMT mammary tumor growth was not affected in the short-term by daily treatment with IL15cx, due to tumor-mediated extrinsic and T-cell-intrinsic resistance to IL15, even when combined with PD-L1 blockade. We use the PyMT model and integration of multiple datasets to determine the molecular programming of IL15-resistant, tumor-infiltrating CD8+ T cells.

Materials and Methods

PyMT model and lymphocytic choriomeningitis virus infections
Mice were bred/housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee guidelines of the University of California, San Diego (UCSD).
Female C57BL/6J, or MMTV-PyMT+/− mice (18, 19) backcrossed to C57BL/6J (hereafter referred to as “PyMT”; ref. 20), were used for all studies. After tumors were measurable on PyMT mice at approximately 16 to 24 weeks of age, mice were randomly assigned to control or experimental groups. Tumor volumes were similar between groups before treatment. Volumes of measurable tumors (between 10 and 1,000 mm3) were calculated with the formula \[ V = \frac{1}{2} (A \times A \times B), \] where \( A \) is larger of two 90-degree caliper measurements. For PyMT MEC studies, 105 cells of a PyMT mammary epithelial cell line (Py230, provided by Dr. Lesley Ellies, UCSD) mixed 1:1 with Matrigel (BD Biosciences) were injected into the #4 mammary gland, and allowed to establish in female mice for 4 to 8 weeks. Py230 were pathogen-negative by Impact III PCR (Idexx Bioresearch), and allowed to establish in female mice for 4 to 8 weeks. Py230 were obtained in 2014, and demonstrated uniform “cobblestone” epithelial cell morphology (observed by A.L. Doedens). Acute and persistent lymphocytic choriomeningitis virus (LCMV) infections performed as in refs. 16 and 21. Tumor cell sorting, microarray, and microscopy

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Cell culture and cytokines

Cells were cultured in RPMI 1640, 10% FBS, 10 U/mL penicillin/streptomycin, 40 mmol/L HEPES, and 35 μmol/L β-mercaptoethanol (Life Technologies), with hIL2 or hIL15 where indicated (NCI Preclinical Repository). Anti-CD3/anti-CD28 activation was performed as in ref. 21. One dose of IL15cx was generated by incubating 0.5 μg hIL15 with 2.3 μg sIL15Rα-Fc (R&D systems) in PBS at 37°C for 15 minutes, and injected i.p. in 200 μL PBS.

Cell sorting, microarray, and microscopy

For microarray, single-cell suspensions were double-sorted (FACS Aria; BD Biosciences), biological triplicates of CD8+ CD44high cells from the spleen and tumor, excluding MHC II, CD4, B220, or propidium iodide expression was calculated by Robust Multiarray-Average (RMA). Microarray/GSEA/flow cytometry of single-cell suspensions of the tumors revealed a high CD8:CD4 ratio, CD8+:CD44high T-cell infiltrate (Fig. 1A, B). PyMT mice were randomly assigned to two groups, administered vehicle or IL15cx for 5 days (Fig. 1C). We observed activated CD8+ T cells and upregulated expression of cytotoxic molecules in the spleens of the PyMT mice (Supplementary Fig. S1A and S1B), yet we detected no increased tumor regression or halting of tumor growth due to IL15cx treatment over the 5-day experiment (Fig. 1D). Extending treatment over 2 weeks did not significantly change tumor volumes (data not shown).

Results

Short-term IL15cx treatment fails to affect tumor volume

All ten mammary glands in female PyMT mice develop tumors of mixed size and histologic progression by 3 to 4 months of age on the C57BL/6J background (18). Immunohistochemistry and flow cytometry of single-cell suspensions of the tumors revealed a high CD8:CD4 ratio, CD8+:CD44high T-cell infiltrate (Fig. 1A, B). PyMT mice were randomly assigned to two groups, administered vehicle or IL15cx for 5 days (Fig. 1C). We observed activated CD8+ T cells and upregulated expression of cytotoxic molecules in the spleens of the PyMT mice (Supplementary Fig. S1A and S1B), yet we detected no increased tumor regression or halting of tumor growth due to IL15cx treatment over the 5-day experiment (Fig. 1D). Extending treatment over 2 weeks did not significantly change tumor volumes (data not shown).

Intrigued by the extensive in vivo induction of a cytotoxic profile in the periphery (Fig. 2A), CD8+ T cells from spleen, lung, and tumors of PyMT mice (vehicle and IL15-treated, as in Fig. 1C) were used to identify targets rather than as a stringent enrichment test. CD8+ (53-67; eBioScience) was detected with alkaline phosphatase on 105 cells of a PyMT mammary epithelial cell line (Py230, provided by Dr. Lesley Ellies, UCSD) mixed 1:1 with Matrigel (BD Biosciences) were injected into the #4 mammary gland, and allowed to establish in female mice for 4 to 8 weeks. Py230 were pathogen-negative by Impact III PCR (Idexx Bioresearch), and allowed to establish in female mice for 4 to 8 weeks. Py230 were obtained in 2014, and demonstrated uniform “cobblestone” epithelial cell morphology (observed by A.L. Doedens). Acute and persistent lymphocytic choriomeningitis virus (LCMV) infections performed as in refs. 16 and 21. Tumor single-cell suspensions were generated by chopping, 0.05% collagenase D (Roche Diagnostics) incubation, and 20 μm straining, as in ref. 22. Spleens were disrupted with frosted glass slides and then treated identically as tumor samples.

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Flow cytometry

Immunostaining was performed with antibodies against CD8α (53-67), CD25 (PC615), CD43 (eBioR2/60), CD44 (IM7), CD69 (H1.2F3), CD71 (R17217), CD122 (TM-B1), CD127 (A7R34), CD103 (2E7), CD200R (OX110), CD244 (244F4), CXXCR3 (CXXCR3-173), GITR (DTA-1), EOMES (Dan11mag), KLRG1 (2F1), LAG-3 (C9B7W), Ly6C (HK1.4), IKZF2/HELIOS (22F6), PD-1 (J43), PD-L1 (MH5), TBE1 (4B10), TOX (1XR10), all from eBioScience; GZMB (MHGB05) from Life Technologies, and TCF1 (G63D9) from Cell Signaling Technologies. LCMV-green fluorescent protein (GFP)-33-41 virus-specific CD8+ T cells were identified with MHC I tetramers (Beckman Coulter).

Tumor-infiltrating CD8+ T-cell IL15cx resistance

We first determined whether PyMT mice had systemic, global suppression of T-cell responsiveness to IL15cx, or a local, tumor-specific resistance. We analyzed CD8+ T cells from spleen, lung, and tumors of PyMT mice (vehicle and IL15-treated, as in Fig. 1C). The treatment regimen expanded CD8+ T-cell absolute numbers in the spleen (Fig. 1E), but not in tumors (Fig. 1F; P = 0.64 by Student unpaired t test); CD8+ T-cell relative abundance (%) increased 4.5-fold in the spleen and 7-fold in the lung, whereas tumors failed to show an increase (Fig. 1G). The experiments in Fig. 1E–G and Supplementary Fig. S1A and S1B were all performed in PyMT mice, precluding global suppression of IL15cx signaling in tumor-bearing mice as a mechanism for poor intratumoral IL15cx activity.

We measured expression of GZMB in the tumor and a nonlymphoid tissue, to determine if IL15cx was driving acquisition of a cytotoxic profile in the periphery (Fig. 2A). CD8+ T cells from the lungs of vehicle-treated animals were largely negative for GZMB, consistent with a resting/noneffecter phenotype. In the IL15cx treatment group, nearly all lung CD8+ T cells had upregulated GZMB (Fig. 2A). However, only half of CD8+ T cells isolated from PyMT tumors upregulated GZMB after IL15cx treatment (Fig. 2A). Thus, resistance to IL15cx is tumor-specific, and blocked increases in CD8+ T-cell number, percentage, and cytotoxic phenotype.

Extrinsic and intrinsic resistance to IL15 by tumor CD8+ T cells

T cells can be suppressed by tumors via multiple mechanisms (for example, refs. 22 and 26); we tested whether IL15 resistance was dependent on the tumor environment, and thus cell-extrinsic, or if the resistance was a property of the T cells, and thus cell-intrinsic. Tumor single-cell suspensions were cultured in vitro for 3 days with or without IL15. The transferrin receptor
(CD71) is an established lymphocyte activation marker (27) that correlates with GZMB protein upregulation after IL15 treatment (data not shown). High concentrations of IL15 (1 μg/mL) for 3 days did not activate the CD8⁺ T cells within the PyMT tumor single-cell suspension, whereas separately cultured splenic CD8⁺ T cells uniformly upregulated CD71 in response to IL15 (Supplementary Fig. S2A). Furthermore, PyMT single-cell suspensions suppressed the cytokine responsiveness of wild-type (WT) splenocytes in a dose-dependent fashion (Supplementary Fig. S2B and S2C). Thus, T-cell–extrinsic, tumor-mediated mechanisms of suppression were in part limiting T-cell activation.

To determine if intratumoral CD8⁺ T cells also exhibited cell-intrinsic resistance to IL15, we sort-purified CD8⁺ T cells from tumor suspensions, as well as splenic CD8⁺ T cells as a positive control, and tested their responsiveness to IL15 in vitro. Only approximately 50% of the tumor-derived, sort-purified CD8⁺ T cells responded to IL15, even after incubation in tumor cell-free media for 3 days with an excess of IL15 (1 μg/mL; Fig. 2B and C). We also observed a defect in
accumulation after anti-CD3/anti-CD28 stimulation, further indicating hypo-responsiveness of PyMT tumor-infiltrating CD8⁺ T cells (Fig. 2C). We then focused on what molecular programs underlie the cell-intrinsic component of CD8⁺ T-cell resistance to IL15.

**Exhausted tumor CD8⁺ T-cell signature despite ample effector transcripts**

We isolated PyMT tumor CD8⁺ cells and control splenic CD8⁺ T cells, and performed microarray analysis alongside samples of the Immunological Genome Project, Immgen (28).
Figure 3.
Tumor-infiltrating CD8+ T cells express both abundant effector transcripts and an exhaustion-associated gene-expression signature. A–E and G, CD8+CD44high T cells sorted from tumors and spleens of PyMT mice; gene expression determined by microarray. A, fold change of selected cytotoxic regulators/effector transcripts. B, PyMT spleen/tumor CD8+ T-cell microarray data coregionalized with Immgen OT-I/LM-OVA (GSE15907); Gzmb transcript abundance in PyMT tumor and splenic CD8+CD44high T cells and virus-specific OT-I CD8+ T cells after LM-OVA infection; expression values directly comparable; error bars, SEM. C, gene set-enrichment analysis (GSEA) of immunologic signatures database using PyMT tumor CD8+ T cells relative to OT-I T cells responding to VSV-OVA, day 8 after infection (GSE15907). D, fold change of PyMT tumor CD8+CD44high relative to splenic CD8+CD44high T cells, plotted versus mean class P value. Highlighted transcripts regulated ≥2-fold in persistent versus acute infection or tumor versus spleen cells. E, select exhaustion-associated transcripts (persistent vs. acute infection, day 15; GSE41870) and PyMT tumor versus splenic CD8+CD44high; datasets normalized independently. F, indicated molecule abundance on CD8+ T cells by flow cytometry. "CD44high" indicates this gate was used instead of tetramer gate. G, fold change versus fold change plot of PyMT tumor CD8+CD44high T cells and OT-I T cells responding to VSV-OVA (day 6), both versus splenic CD8+CD44high T cells from PyMT mice. Transcripts regulated ≥2-fold in persistent versus acute infection, or in B16 melanoma relative to splenic CD8+CD44high T cells, are highlighted.
First, we tested the hypothesis that poor CD8+ T-cell antitumor activity in the PyMT model is a result of failure to initiate or sustain the cytotoxic effector program. Contrary to our hypothesis, we found abundant transcripts associated with CTL effector differentiation and function in PyMT tumor T cells, including granzyme family members and factors essential for effector differentiation including Tbx21 (TBET), Idd2, and Prdm1 [(BLIMP1) Fig. 3A]. The relative expression of GZMB mRNA in PyMT tumor CD8+ T cells was nearly as high as that expressed in CD8+ effector T cells near the peak of the cellular response to Listeria monocytogenes-OVA infection (Fig. 3B; ref. 29). Thus, the lack of CD8+ T-cell antitumor activity is not explained by a lack of transcripts coding for effector programming or cytotoxic mediators. The abundant GZMB mRNA (Fig. 3A and B) was not reflected in protein expression in PyMT tumor CD8+ T cells (Fig. 2A).

To exclude that the absence of cytokine receptors caused the poor PyMT CD8+ T-cell response to IL15cx, we characterized gene expression and protein abundance of IL2 and IL15 receptors on PyMT tumor CD8+ T cells: We find PyMT tumor CD8+ T cells expressed abundant receptor molecules for...
We then tested PyMT tumor CD8<sup>+</sup> T cells versus those responding to acute infection with vesicular stomatitis virus-OVA (VSV-OVA, day 8) for gene-set enrichment using GSEA (30). The top gene set (S) returned was persistent versus acute infection (GSE30962), with a normalized enrichment score of 3.25 (Fig. 3C; Supplementary Fig. S4). This result links the PyMT tumor CD8<sup>+</sup> T cell gene signature with that of chronic versus acute viral infection. As a resource, we present PyMT tumor CD8<sup>+</sup> T-cell gene expression relative to Immgen datasets for CD8<sup>+</sup> T cells from naive and postinfection populations (Supplementary Fig. S5 and Supplementary Tables S1–S4).

To further explore the contribution of T-cell exhaustion to tumor T-cell programming, we plotted fold change versus P value of the gene-expression data from PyMT tumor versus splenic CD8<sup>+</sup> CD4<sup>+</sup> T cells, and highlighted genes differentially expressed in persistent versus acute LCMV infection (GSE41870; ref. 31). The transcripts differentially regulated by PyMT tumor compared with splenic CD8<sup>+</sup> T cells shared approximately 80% identity with genes up or down in persistent versus acute infection (Fig. 3D). Exhaustion-associated cell-surface markers (32) were also highly expressed (Fig. 3E).

To validate the gene-expression data linking exhaustion to the PyMT tumor CD8<sup>+</sup> T-cell phenotype, and further characterize their activation state, we directly compared the cell-surface protein abundance of exhaustion-associated markers on PyMT tumor CD8<sup>+</sup> T cells with virus-specific CD8<sup>+</sup> T cells in acute or persistent infection. We found marked upregulation of PD-1, PD-L1, LAG3, and CD244 expression on both tumor-infiltrating CD8<sup>+</sup> T cells and those from chronic viral infection, but not on those from acute viral infection or uninfected mice (Fig. 3F). In many cases, these markers were more abundant on the PyMT tumor CD8<sup>+</sup> T cells than those from persistent viral infection. To test how results in the PyMT murine model of breast cancer compared with human disease, we performed flow cytometry on CD8<sup>+</sup> T cells infiltrating human breast cancers versus control peripheral samples (Supplementary Fig. S6). We found human breast cancer–associated CD8<sup>+</sup> T cells to highly express CD69, PD-1, and CD244.

To identify how CD8<sup>+</sup> T-cell transcription varied from competent CD8<sup>+</sup> T cells versus those in PyMT tumors, we plotted gene expression in CD8<sup>+</sup> T cells in response to VSV-OVA infection and PyMT tumor (Fig. 3G; tabular data in Supplementary Table S5). We then highlighted genes regulated ≥2-fold in persistent infection (GSE41870) and in CD8<sup>+</sup> T cells infiltrating B16 melanomas (GSE15907). This plot demonstrates T-cell activation in tumors or during infection similarly regulates many genes, which appear along the 45-degree axis line. In addition, the highlighting illustrates multiple transcripts regulated in PyMT tumor CD8<sup>+</sup> T cells are similarly regulated in B16 melanoma tumor CD8<sup>+</sup> T cells, and to a lesser degree, in response to infection. These include upregulation of effector molecule GzmB and the phosphatase PtpR1, and the downregulation of transcription factor Myb. Despite similarities, tumors and acute infection up-regulate and downregulate specific subsets of genes, such as the tumor- and persistent virus-specific upregulation of Cd200r1, or the acute and persistent infection–specific upregulation of Itgam (Fig. 3G). Therefore, although the T-cell exhaustion program is a major contributor to tumor CD8<sup>+</sup> T-cell gene expression, there remain a small number of tumor- and virus-specific genes. We wondered what factors or differentiation pathways might be responsible for genes regulated in tumor, but not in the acute or persistent infection datasets.

A subset of tumor CD8<sup>+</sup> T cells upregulate a resident memory T-cell gene-expression signature

A CD8<sup>+</sup> T-cell memory subset residing in tissues (33), called tissue-resident memory T cells (T<sub>RM</sub>), typically expresses Igαe (CD103) and Cd69, along with a set of other transcripts including Cbx1 (E-cadherin; ref. 34). PyMT tumor CD8<sup>+</sup> T cells expressed multiple transcripts associated with T<sub>RM</sub> cells, including CD103. Using the “core” T<sub>RM</sub> signature (34), PyMT tumor CD8<sup>+</sup> T cells similarly up- or downregulate approximately 80% of the transcripts versus splenic controls (Fig. 4A). We further compared our PyMT tumor CD8<sup>+</sup> T-cell data with gene-expression data of brain CD103<sup>+</sup> T<sub>RM</sub> versus conventional splenic memory T cells (35) and persistent versus acute viral infection (ref. 31; Supplementary Fig. S7A). Again, PyMT tumor CD8<sup>+</sup> T cells express many T<sub>RM</sub> transcripts, with few shared with exhausted cells, such as Chm2 and Cd244 (Supplementary Fig. S7A). Thus, both an exhausted and T<sub>RM</sub> gene-expression signature are present in the PyMT tumor CD8<sup>+</sup> T-cell population. We then plotted the transcription factors/regulators differentially expressed ≥2-fold between PyMT tumor versus spleen CD8<sup>+</sup> T cells, where exhaustion and T<sub>RM</sub> programming appear to explain much of the gene expression in PyMT tumor CD8<sup>+</sup> T cells (Fig. 4B).

To distinguish whether PyMT tumor CD8<sup>+</sup> T cells have a mixed differentiation comprising exhausted and T<sub>RM</sub>–like cells, all within a uniform population, or whether PyMT tumor CD8<sup>+</sup> T cells consist of a mixture of distinct exhausted and T<sub>RM</sub>–like cells, we immunostained CD103, CD69, and PD-1 on PyMT

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**Table 1. Tumor-infiltrating CD8<sup>+</sup> T-cell functional annotation clusters**

<table>
<thead>
<tr>
<th>DAVID cluster</th>
<th>Enrichment score</th>
<th>Cluster description</th>
<th>Number of genes</th>
<th>Selected genes composing cluster</th>
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<td><strong>Upregulated in tumor CD8</strong>&lt;sup&gt;+&lt;/sup&gt; <strong>T cells</strong></td>
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<tr>
<td>1</td>
<td>6.88</td>
<td>Surface/plasma membrane</td>
<td>58 Pdeccl, Cdsb, Cba4, Llyf, Adora3, Innp5, Stc1C6a10, Igtav</td>
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<tr>
<td>2</td>
<td>2.65</td>
<td>Negative regulation of cell signaling/G-protein regulation</td>
<td>13 Cis8, Rgs13, Sry1, Spryl, Spryl2, Soci2, Tyfb3, Atxn1</td>
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<tr>
<td>8</td>
<td>2.52</td>
<td>SH2 domain</td>
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<tr>
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<td>Ig-like domain</td>
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<tr>
<td>19</td>
<td>1.78</td>
<td>Phosphatases</td>
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<td></td>
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<tr>
<td><strong>Downregulated in tumor CD8</strong>&lt;sup&gt;+&lt;/sup&gt; <strong>T cells</strong></td>
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<tr>
<td>1</td>
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<td>Transmembrane/receptor</td>
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<td>16 Trem2o, Slamf6, Emb, Fcgtr, Icam2, Sei, Pecam1, Sem4f</td>
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tumor CD8⁺ T cells (Fig. 4C), using a slow-growing PyMT mammary epithelial cancer cell line (PyMT MEC) injected into mammary glands of female mice. We found that CD103 and PD-1 were largely mutually exclusive whereas CD69 was expressed at a moderate level on PD-1⁺ cells. This result suggests that T engagements and exhausted cells were two different tumor-infiltrating subsets in PyMT MEC tumors, which can be distinguished with CD103 and PD-1.

**Molecular characterization of IL15-resistant population**

Transcription factors/regulators play central roles in lymphocyte programming (36). T-box transcription factors TBET and EOMES play nonredundant, essential roles in CD8⁺ effector T-cell differentiation, whereas TCF1 is required for normal thymic T-cell development and memory cells, but is markedly downregulated in effector CD8⁺ T cells (29, 36). Many T engagement highly express IKZF2/HELIOS, which is essential for full suppressive function (37); this factor is absent from splenic CD8⁺ T cells (29). TOX is an HMG-box factor essential for T-cell development (38) with an unknown role in peripheral T cells. To further characterize the exhausted and T engagements-like infiltrate, we assessed protein levels of transcription factors/regulators found to be regulated in the PyMT transcript-expression data (Fig. 4B). We distinguished the T engagements-like and exhausted subsets with PD-1 and CD103, and immunostained for transcription factors/regulators TBET, TCF1 (product of Tcf7), IKZF2/HELIOS, TOX, and EOMES (Fig. 4D). The exhausted subset, using PD-1 as a marker, was always TOX⁺, TCF1⁻, EOMES⁻, and intermediate for IKZF2 (Fig. 4D). We found that CD103⁺ cells, uninvolved with the T engagements-like subset, had mixed IKZF2 expression and intermediate-high TOX expression (4D). As in Fig. 4C, these data further suggest there is a distinct CD103⁺ T engagements-like component of the PyMT ME tumor CD8⁺ T-cell population that does not exhibit high PD-1 or TOX.

We previously observed cell-intrinsic IL15 resistance in a subset of tumor CD8⁺ T cells (Fig. 2). Having established T engagements-like and exhausted subsets in the tumor, we then determined their IL15 responsiveness. First, we distinguished that IL15 receptors CD122 and CD132 were similarly expressed on PD-1 high and low tumor-infiltrating CD8⁺ T cells (Supplementary Fig. S3). We then sorted PyMT MEC tumor T cells based on CD103 expression and spleocytes as a control, and cultured with or without IL15 for 60 hours (Supplementary Fig. S6). The spleocyte CD8⁺ T cells responded to IL15 culture with near uniform upregulation of CD71 and were >97% negative for IKZF2, TOX, and PD-1 (Supplementary Fig. S7B). In contrast, a subset of sorted tumor CD8⁺ T cells did not upregulate CD71, as was previously observed in Fig. 2B. We found that the CD71⁺ cells were almost entirely PD-1⁻ TOX⁺, and exhibited low side scatter, suggesting a failure to proliferate. CD103⁺ cells were less likely to be PD-1⁻ after IL15 treatment (Supplementary Fig. S7B), as was observed previously in untreated PyMT tumor CD8⁺ T cells (Fig. 4C). We found that the CD103⁺ tumor-infiltrating cells were more likely to appear IKZF2⁻ CD71⁺ after IL15 culture; 45% of the sorted CD103⁺ appeared IKZF2⁺ CD71⁺ versus 14% from the sorted CD103⁻ population. Taken together, these data indicate the PD-1⁻, TOX⁺ tumor-infiltrating population exhibits a failure to induce blastogenesis in response to IL15. IKZF2 was not predictive of cytokine responsiveness, but was enriched in sorted IL15-treated CD103⁺ T cells from the tumor.

Given that the IL15-resistant tumor CD8⁺ T-cell population resembled exhausted CTL responding to persistent viral infection, we wondered whether IL15 could also fail to expand PD-1⁺ cells in persistent viral infection with LCMV-cl13. Although IL15 could result in a dramatic expansion of CD8⁺ T cells, virus-specific cells were vastly under-represented in the expanded population (Supplementary Fig. S8A and S8B). This result is unlikely to be explained by cytokine receptors, as IL2rb (CD122) and Il2rg (CD132) are expressed by virus-specific cells in persistent infection (Supplementary Fig. S3D). In vitro, only the PD-1⁺ subset from LCMV-cl13-infected mice exhibited hallmarks of activation after IL15 treatment (Supplementary Fig. S8C), similar to that observed in PyMT tumor CD8⁺ T cells stimulated with IL15 (Supplementary Fig. S7B). Therefore, these results extend those of previous reports (39) and demonstrate virally exhausted CD8⁺ T cells are poorly responsive to IL15 in vitro and in vivo.

Blockade of PD-1 enhances adaptive immune responses to persistent antigen such as cancer and LCMV-cl13 infection (16). However, cytokine resistance may not be directly addressed by PD-1 pathway blockade; indeed, when administered to PyMT mice, systemic in vivo anti-PD-1 increased the percentage of intratumoral CD8⁺ T cells producing IFNγ, but did not affect cytokine resistance as gauged GZMB protein expression or tumor volume in our short-term assay (Supplementary Fig. S9A–S9D). Therefore, overcoming exhaustion-associated, cell-intrinsic cytokine resistance may require therapeutic strategies beyond PD-1 blockade.

**Targeting regulators of tumor CD8⁺ T-cell responsiveness and function**

To identify novel regulators of tumor-infiltrating CD8⁺ T cells, we returned to the gene-expression data (Figs. 3 and 4). We found 504 genes with increased and 358 with decreased abundance in PyMT CD8⁺ T cells versus spleen (Fig. SA; Supplementary Tables S6 and S7). We collated results from DAVID (23), which determines enrichment among ontology, localization, and other
properties (Table 1). We then incorporated additional datasets profiling exhausted CD8+ T cells exposed to persistent antigens: Chronic viral infection (GSE41870) and PD-1high tumor–infiltrating T cells (GSE76362). Metascape (24) was then used to perform ontological meta-analysis of all three datasets, and the overlap of genes upregulated 2-fold was diagrammed in Fig. 5A. We focused on three classes of genes: transcription factors and regulators which may program the exhausted state, signaling regulators potentially underlying exhaustion-induced trophic cytokine resistance, and accessible cell-surface/membrane proteins to reverse T-cell dysfunction. For the selected candidates, we present gene expression from multiple datasets of T-cell differentiation (Fig. 5B).

We first focused on cell-surface/membrane-localized and immunoglobulin-like domain-containing transcripts (Table 1); these included known exhaustion-associated negative regulators such as Pdcd1 (PD-1) and Tigit (40), as well as Cld200r1 and myeloid-associated Cish (Supplementary Fig. S10) and Tnbc-associated adenosine receptor Aorda3. Expression data for exemplar transcript Pdcd1 as well as Lirhb4 and Cld200r1 are presented in Fig. 5B. Lirhb4 is an ITIM-containing negative regulator of immunity (41), and we verified elevated CD200R on PD-1high PyMT tumor T cells beyond that observed in persistent viral infection (Supplementary Fig. S10). Cld200r1 is induced in both PD-1high and KNK1.1high PyMT tumor subsets (Fig. 5B); CD200R downregulates TNF in macrophages (42).

Tumor-infiltrating CD8+ T cells exhibit a unique profile of transcription factors and regulators. We selected six transcription factors that have a role in CD8+ tumor, acute infection (LCMV-Armstrong), persistent infection (LCMV-clone13), TRM, PD-1high PyMT tumor, and myeloid-associated Glccl1 (40), as well as Rgs1, Rgs3, and Rgs16 (Table 1; Supplementary Fig. S11). Carboxylase TOX and PD-1 correlated with IL15 cytokine resistance (Fig. 4D; Supplementary Fig. S7B); further, Teneurin mirrors Pdcd1 expression in persistent infection (Fig. 5B; ref. 31), appearing to mark exhausted CD8+ T cells in tumor and viral contexts. Bhlhed01 is upregulated in multiple postactivation T-cell subsets (Fig. 5B), and regulates cytokine production (43). Ikf2 expression is abundant in exhausted, TecKcd and CD8α NK1.1+ tumor-infiltrating cells (Fig. 5B; refs. 31, 44); however, it poorly correlated with cytokine resistance (Supplementary Fig. S7B). Lastly, three transcription factors/regulators in exhausted, tumor-infiltrating T cells had unexplored roles in T lymphocytes (Fig. 5B): Tisp1 is a transcriptional repressor of GATA-regulated genes (45), and Lbct has a role in myeloid cell malignancies (45), and Atax1 is a chromatin-binding protein which can repress Notch signaling (46). In summary, our analysis identifies specific transcription factors/regulators expressed in multiple exhausted and tumor-infiltrating CD8+ T-cell populations.

We then profiled several clusters with roles in negative regulation of signal transduction or proliferation (Table 1 and Fig. 5B): one such cluster contained Cish, Rgs1, Rgs3, and Rgs16 (Table 1; Fig. 5B). Cish is a SOCS and SH2 domain protein, diminishes JAK/STAT signaling (47), and affects tumor-infiltrating CD8+ TCR responsiveness (48). Regulator of G-protein signaling (RGS) molecules control immune cell migration and activation. Rgs1 is highly expressed in Tregs and regulates trafficking (49). Rgs16 is induced by cell activation, persistent infection and in Treg cells (Fig. 5B), and inhibits immune cell activation (50). Integrins serve adhesion and cell signaling regulatory roles: Itgav (Fig. 5B) binds extracellular matrix proteins, including fibronectin and laminin; integrins have been successfully targeted in preclinical studies (51). Samt3/Hascl1/ Sh2 (Fig. 5B) is a SH3-containing, immunoinhibitory adaptor which can bind ITIM's such as those in Pdcd1 and Lirhb4: ablation of Samt3 upregulates cellular tyrosine phosphorylation, whereas overexpression impedes PD4+ T-cell activation/proliferation (52–54). Glccl1 (Fig. 5B) is induced by anti-inflammatory glucocorticoids, and in humans, SNPs in Glccl1 markedly affect response to glucocorticoid therapy in asthma patients (55). Prsr1 (Fig. 5B) directly binds to mTORC2, and Prsr1 degradation by RFFL2 results in mTORC2-dependent protein kinase C activation (56, 57). Altogether, we find that specific signaling regulators with potent antiproliferative and immuno-inhibitory functions are upregulated in exhausted, tumor-infiltrating CD8+ T cells.

Phosphatases can attenuate TCR and cytokine responsiveness; we found Dusp and Ppim family members (Table 1; Supplementary Table S8), as well as phosphatase subunit Pprr2c enriched in PyMT CD8+ T cells. We further profiled Dusp4, Ppim1, and Pprr2c. We observed marked enrichment of these phosphatases in PD-1high tumor–infiltrating cells, whereas IL15-responsive Dgs8+ NK1.1+ cells (44) showed minimal enrichment (magenta and orange bars; Fig. 5B).

To facilitate further identification of cell-surface molecules, we used gene ontology and manual identification to select transcripts coding for cell-surface molecules and ordered them by fold change in PyMT tumor CD8+ T cells versus splenic controls (Fig. 5C). We then calculated fold change for multiple CD8+ T-cell datasets and depicted this via heatmap, allowing visualization of shared or differential relative expression in tumor, acute infection (LCMV-Armstrong), persistent infection (LCMV-clone13), TRM, PD-1+ NK1.1+, and NK1.1+ PD-1+ PyMT tumor-infiltrating CD8+ T cells.

Our strategy of gene-expression profiling has identified potential regulators of tumor-infiltrating CD8+ T cells that may be pursued in future studies as therapeutic targets.

Model of molecular programming of tumor-infiltrating CD8+ T cells and IL15 resistance

PyMT CD8+ T cells exhibit cell-intrinsic resistance to IL15cx therapy correlated with an exhausted phenotype. These data suggest that the predominant effect of systemic IL15cx to either tumor-bearing or persistent virus-infected mice was expansion of irrelevant CD8+ T cells (Fig. 6A; flow cytometry-verified molecules summarized in Fig. 6B). We observed that the adoptive transfer of activated tumor-specific CD8+ T cells resulted in rapid induction of exhaustion markers PD-1 and PD244, suggesting exhaustion-induced cytokine resistance will also apply to adoptively transferred cells (Supplementary Fig. S11). As molecules at the cell surface are readily targeted and thus candidates for clinical translation, we present a Venn diagram of cell-surface molecule relative expression in CD8+ T cells exposed to persistent antigen (Fig. 6C).

Discussion

Common gamma-chain cytokines induce CD8+ T-cell activation, proliferation, and cytotoxic programming (2). IL2 is FDA-approved to treat metastatic melanoma and renal cell carcinoma (1), and IL15-based therapies are under intense investigation and clinical development. However, we found the PyMT model of mammary carcinogenesis to be refractory to short-term IL15cx-mediated tumor destruction. Tumor-infiltrating CD8+ T cells exhibited extrinsic and intrinsic resistance to IL15: The majority failed to engage cytokotoxic and proliferative programs, despite...
expression of IL2/15 receptors, purification by cell sorting, and extended IL15 treatment in vitro. In humans, adjuvant cytokine therapy must be carefully managed to avoid morbidity and mortality (58). Understanding the mechanisms behind cytokine hypo-responsiveness is important to fully and safely exploit common gamma-chain cytokine therapies.

Cancer and persistent viral infection both exhibit high antigen load and the failure of antigen-specific T cells to eliminate the antigen-bearing cells (13). Our analysis confirmed the exhausted phenotype common to both viral and tumor-mediated exhaustion. Previous studies have shown exhausted CD8⁺ T cells are resistant to gamma chain cytokines (39), yet have also reported their capacity to lower viral titers and partially overcome T-cell exhaustion (recently, ref. 59). Exhaustion of virus-specific T cells depends on antigen load, and in some cases, long-term gamma-chain cytokine administration may lower viral titer indirectly. We observed poor expansion of virus-specific cells by IL15cx (Supplementary Fig. S8).

Figure 6.
Therapeutic consequences and molecular hallmarks of CD8⁺ T-cell exhaustion-associated IL15 resistance. A, intrinsic and extrinsic factors dampen tumor CD8⁺ T-cell IL15 responses, leaving systemic IL15cx to primarily activate/expand extra-tumoral CD8⁺ T cells. B, partial phenotype of exhausted, IL15-resistant tumor-infiltrating T cell; these proteins were validated by flow cytometry as shown in Figs. 3 and 4 and Supplementary Fig. S10. Exhaustion/inhibitory receptors, red; inhibitory ligands, yellow; and other, orange. C, cell-surface molecules upregulated ≥1.5-fold in PyMT tumor CD8⁺ T cells versus splenic controls (from Fig. 5C), subdivided into those upregulated ≥1.5-fold in CD8⁺PD-1⁺ and CD8⁺NK1.1⁺ T cells, versus CD8⁺PD-1⁺NK1.1⁺ T-cell controls (GSE76362), or in persistent versus acute infection, day 15 (GSE43870). Underlined transcripts are upregulated ≥1.5-fold in CD103⁺ brain T繁 relative to conventional splenic memory cells (GSE39152).
and D). CD69 is often used as a T\textsubscript{EM} marker, yet splenic exhausted cells express CD69 (Fig. 3F; ref. 60), and PD-1\textsuperscript{hi} PyMT MEC tumor CD8\textsuperscript{+} T cells expressed intermediate CD69 (Fig. 4C), suggesting CD103\textsuperscript{+} may better differentiate T\textsubscript{EM}-like and exhausted tumor CD8\textsuperscript{+} T cells. Dadi and colleagues report a CD8\textsuperscript{+} NK1.1\textsuperscript{hi} PD-1\textsuperscript{hi} tumor-infiltrating population to be cytokine-responsive and often CD103\textsuperscript{+}, calling them "ILTC1" cells (44). Here, we refer to CD8\textsuperscript{+} CD103\textsuperscript{+} PD-1\textsuperscript{+} cells as T\textsubscript{EM}-like, based on T\textsubscript{EM}-like signature (Fig. 4) and expression of CD103, whereas Dadi and colleagues identify the ILTC1 population based in part on NK signature and expression of NK1.1. Both of our reports have found CD103\textsuperscript{+} cells more likely to be PD-1 negative and to respond to IL15 (Supplementary Fig. S7B). Dadi and colleagues also found significant delay, but not elimination, of PyMT tumors with transgenic IL15. While we observed no control of tumor volume with IL15cx in our assay, our protocol administered IL15cx to mice with established tumors. Altogether, given the poor response of PyMT tumors to IL15cx despite the presence of ILTC1/T\textsubscript{EM}-like cells, we conclude targeting of novel regulators of cytokine responsiveness is essential to maximize IL15 antitumor efficacy.

The presence of CD103\textsuperscript{+}, T\textsubscript{EM}-like CD8\textsuperscript{+} T cells in tumors may present unappreciated therapeutic opportunities. In our model, CD8\textsuperscript{+} CD103\textsuperscript{+} cells appeared less likely to be PD-1\textsuperscript{hi} or to express exhaustion-associated transcription factor TOX (Fig. 4D), were more likely to be IL15-responsive (Supplementary Fig. S7B), and expressed unique activation/inhibitory receptors. Further understanding the cytotoxic capacity/activity of CD103\textsuperscript{+} T\textsubscript{EM}-like cells in the tumor will inform whether therapeutic strategies should optimally target the cytokine-resistant exhausted cells, T\textsubscript{EM}-like cells, or both.

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## Molecular Programming of Tumor-Infiltrating CD8+ T Cells and IL15 Resistance


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