

# CD103<sup>+</sup> Tumor-Resident CD8<sup>+</sup> T Cells Are Associated with Improved Survival in Immunotherapy-Naïve Melanoma Patients and Expand Significantly During Anti-PD-1 Treatment



Jarem Edwards<sup>1</sup>, James S. Wilmott<sup>2,3</sup>, Jason Madore<sup>2</sup>, Tuba Nur Gide<sup>2</sup>, Camelia Quek<sup>2</sup>, Annie Tasker<sup>1</sup>, Angela Ferguson<sup>1,3</sup>, Jinbiao Chen<sup>1</sup>, Rehana Hewavisenti<sup>1</sup>, Peter Hersey<sup>1</sup>, Thomas Gebhardt<sup>4</sup>, Wolfgang Weninger<sup>1</sup>, Warwick J. Britton<sup>1,3</sup>, Robyn P.M. Saw<sup>2,3,5</sup>, John F. Thompson<sup>2,3,5</sup>, Alexander M. Menzies<sup>2,3,6</sup>, Georgina V. Long<sup>2,3,6</sup>, Richard A. Scolyer<sup>2,3,5</sup>, and Umaimainthan Palendira<sup>1,3</sup>

## Abstract

**Purpose:** Therapeutic blockade of immune checkpoints has revolutionized cancer treatment. Durable responses, however, occur in less than half of those treated, and efforts to improve treatment efficacy are confounded by a lack of understanding of the characteristics of the cells that initiate antitumor immune response.

**Patients and Methods:** We performed multiparameter flow cytometry and quantitative multiplex immunofluorescence staining on tumor specimens from immunotherapy-naïve melanoma patients and longitudinal biopsy specimen obtained from patients undergoing anti-PD-1 therapy.

**Results:** Increased numbers of CD69<sup>+</sup>CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T cells were associated with improved melanoma-specific

survival in immunotherapy-naïve melanoma patients. Local IL15 expression levels strongly correlated with these tumor-resident T-cell numbers. The expression of several immune checkpoints including PD-1 and LAG3 was highly enriched in this subset, and these cells significantly expanded early during anti-PD-1 immunotherapy.

**Conclusions:** Tumor-resident CD8<sup>+</sup> T-cell numbers are more prognostic than total CD8<sup>+</sup> T cells in metastatic melanoma. In addition, they are likely to initiate response to anti-PD-1 and anti-LAG-3 treatments. We propose that the immune profile of these cells prior to treatment could inform strategies for immune checkpoint blockade. *Clin Cancer Res*; 24(13); 3036–45. ©2018 AACR.

## Introduction

Immune checkpoints have become a focus of intense research due to their ability to suppress T-cell-mediated immune

responses (1, 2). In the context of cancer, these receptors facilitate escape from antitumor immune responses by tumor cells. In recent years, therapies that block these immune checkpoints, thereby enhancing antitumoral immune responses, have revolutionized the treatment of many solid malignancies. For example, treatment with anti-programmed cell death-1 receptor (PD-1) antibodies in patients with advanced-stage metastatic melanoma leads to objective and durable responses in approximately 40% of the patients (3–6). Nevertheless, it remains largely unknown why a large proportion of patients fail to respond. Although the mechanism of durable response is believed to be through reinvigoration of quiescent T cells (7), only a small subset of tumor-infiltrating T cells (TILs) express these inhibitory receptors (8), and only a proportion of tumor cells express the ligands (9). The characteristics of the tumor-infiltrating CD8<sup>+</sup> T cells that express immune checkpoints, how important they are for tumor control, and whether they respond to immunotherapy are unclear. Efforts to improve the efficacy of these treatments will inevitably require a greater understanding of this population of immune cells and the factors that regulate their trafficking and function.

Although the critical role of CD8<sup>+</sup> T cells in tumor control, particularly in melanoma, has long been recognized (8, 10), mechanistically, effective responses were viewed in the context of continuous recruitment of effector lymphocyte populations from the circulation. Recent evidence from animal models, however, clearly shows that a subset of antigen experienced CD8<sup>+</sup> T

<sup>1</sup>Centenary Institute, the University of Sydney, Sydney, New South Wales, Australia.

<sup>2</sup>Melanoma Institute Australia, the University of Sydney, North Sydney, New South Wales, Australia. <sup>3</sup>Sydney Medical School, the University of Sydney, Sydney, New South Wales, Australia. <sup>4</sup>Department of Immunology and Microbiology, the Peter Doherty Institute for Infection and Immunity, the University of Melbourne, Melbourne, Victoria, Australia. <sup>5</sup>Royal Prince Alfred Hospital, Sydney, New South Wales, Australia. <sup>6</sup>Royal North Shore Hospital, Sydney, New South Wales, Australia.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

G.V. Long, R.A. Scolyer, and U. Palendira contributed equally to this article.

Current address for J. Edwards: Melanoma Institute Australia, North Sydney, NSW, Australia.

**Corresponding Authors:** Umaimainthan Palendira, Centenary Institute of Cancer Medicine and Cell Biology, Building 93, Royal Prince Alfred Hospital Grounds, Camperdown, NSW 2050, Australia. Phone: 61286276115; E-mail: m.palendira@centenary.org.au; or Richard A. Scolyer or Georgina V. Long, Melanoma Institute of Australia, 40 Rocklands Road, Wollstonecraft, NSW 2065, Australia. Phone 612-99117200; Fax: 612-9954 9290; E-mail: richard.scolyer@sswahs.nsw.gov.au.

doi: 10.1158/1078-0432.CCR-17-2257

©2018 American Association for Cancer Research.

### Translational Relevance

It remains unknown whether all tumor-infiltrating CD8<sup>+</sup> T cells are critical for initiating response to immunotherapy. Using rare human tumor biopsy specimens, we demonstrate that tumor-resident cytotoxic T cells are not only associated with protective immunity against metastatic melanoma in treatment-naïve patients, but also likely to be critical for response to anti-PD-1 therapy. We show that the expression of several checkpoint receptors is enriched in these tumor-resident CD8<sup>+</sup> T cells, and local IL15 could be a factor that regulates their numbers. Understanding the characteristics of antitumor CD8<sup>+</sup> T-cell subsets, the factors that regulate their retention within human tumors, and mechanisms to reactivate them against tumors will enable the development and utilization of better systemic treatments to improve outcomes for patients with cancer.

cells become resident within tissue environments, facilitated by local cytokines (11, 12). These resident T cells are not only distinct from their circulating counterparts, but have also been implicated in protective immune responses against many pathogens in both animal models (13–16) and humans (17–20). We therefore hypothesized that these resident T cells could be of great importance in cancers. Indeed, there is emerging evidence from different cancers, both in animal models and humans, that there are resident CD8<sup>+</sup> T cells within the tumor microenvironment (21–23). There is also some evidence that these resident cells could have a prognostic value in some human cancers (21, 22). However, the exact role of these resident T cells in tumor control and whether they respond to immunotherapy are unknown.

Tissue resident T cells are characterized by their constitutive surface expression of CD69 and CD103. CD69 has long been viewed as an early T-cell activation marker; however, it is now clear that tissue resident T cells can constitutively express this receptor in the absence of T-cell receptor stimulation. Whether all CD69<sup>+</sup> T cells in tissues are long-term residents remains controversial. We recently showed in humans that CD103<sup>+</sup>CD69<sup>+</sup> T cells were more reminiscent of tissue-resident T cells described in mouse models than CD103<sup>-</sup>CD69<sup>+</sup> T cells as the former had the highest level of downregulation of the tissue-exit signals (20). It is however possible that CD103<sup>-</sup>CD69<sup>+</sup> T cells are also retained in tissues for a limited period of time as an intermediate population. The development and persistence of tissue-resident T cells depend on local cytokines such as IL15. We have shown that IL15 can not only induce CD69 expression, but also downmodulate tissue-exit signals on circulating human CD8<sup>+</sup> T cells (20).

In this study, we have examined the prognostic value of tumor-resident CD8<sup>+</sup> T cells in metastatic melanoma patients prior to immunotherapy and in patients undergoing anti-PD-1 immunotherapy. Using multiparameter flow cytometry and multiplex immunofluorescence staining on patient samples selected from the Cancer Genome Atlas (TCGA) study, we have accurately quantified the numbers of tumor-resident CD8<sup>+</sup> T cells and also determined their phenotype. In addition, we have also examined the local factors that regulate these tumor-resident CD8<sup>+</sup> T-cell numbers. Here, we show that tumor-resident CD8<sup>+</sup> T cells are stronger predictors of melanoma-specific patient survival than

total CD8<sup>+</sup> T cells in patients who are immunotherapy naïve. Our data also show that their numbers may be dependent on local IL15 expression levels. Importantly, tumor-resident CD8<sup>+</sup> T cells were enriched in many immune checkpoints, and these cells significantly expanded early during anti-PD-1 treatment. We therefore propose that immune profiling of these cells prior to immunotherapy could predict outcomes and help determine targets for treatments.

### Patients and Methods

#### Melanoma samples

Archival formalin-fixed and paraffin-embedded tumor samples from patients treated at Melanoma Institute Australia who were also analyzed in TCGA project were used for immunofluorescence analysis. These samples were obtained as described previously (24). Briefly, patients diagnosed with either primary or metastatic cutaneous melanoma or metastatic melanoma of unknown primary treated at Melanoma Institute Australia were recruited to the TCGA study. These patients did not receive any prior systemic treatments, and the site from which the biopsy specimens were obtained had not been previously treated at any time with radiotherapy. Biopsy specimens from resected primary and/or metastatic melanomas were obtained from patients with appropriate informed consent and Institutional Review Board or ethics board approval. Biopsy specimens used for flow cytometry analysis were obtained from patients with stage III regional lymph node metastatic melanoma. Tumor dissociates were prepared by digesting with liberase (Sigma) at 37° for 1 hour. Thirteen biopsy specimens, including those from five good responders and eight poor responders, were obtained from patients who were treated with anti-PD-1 inhibitor alone (pembrolizumab). Patient selection was dependent on the availability of multiple tumor biopsies, before treatment (PRE biopsy) and within 14 days of commencing treatment (early during treatment, EDT biopsy) for 10 patients. Biopsies from three patients were obtained during treatment (Supplementary Table S1). Patients were classified as "responders" if they had durable stable disease (SD, greater than 6 months), partial response (PR), or complete response (CR) to anti-PD-1 antibodies as their best response by RECIST or immune-related response criteria (irRC) assessed at 6 to 12 weekly intervals. The study was conducted in accordance with the National Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research. The study was undertaken with institutional Human Ethics Review Committee approval and patient's informed consent. Samples were acquired from the Melanoma Biospecimen Tissue Bank, which included patients from Royal Prince Alfred Hospital, Westmead Hospital, and Melanoma Institute Australia (Protocol No. X15-0454 and HREC/11/RPAH/444).

#### Quantitative multiplex immunofluorescence assays

All immunofluorescence staining was carried out on 4- $\mu$ m-thick sections using an Autostainer Plus (Dako, Agilent Technologies) with appropriate positive and negative controls. Opal Multiplex IHC Assay kit (PerkinElmer) was used as per the manufacturer's protocol. Briefly, paraffin-embedded tissue sections were first deparaffinized, rehydrated, and treated with antigen retrieval buffer. Antigen retrieval was performed by boiling in either basic (10 mmol/L Tris base, 1 mmol/L EDTA, 0.05% Tween 20, pH 9.0) or acidic (10 mmol/L sodium citrate, 0.05% Tween

20, pH 6.0) antigen retrieval buffers. Sections were then incubated with 3% hydrogen peroxide for 10 minutes at room temperature in order to block endogenous peroxidase activity. Following this, sections were washed in 1XTBST and then incubated with a single primary antibody (made up in Da Vinci Green Diluent solution; Biocare Medical) for 35 minutes. Sections were then washed and incubated with a probe antibody (Rabbit or Mouse MACH3 Probe; Biocare Medical) specific to the species of the primary antibody for 10 minutes, which were then washed and incubated for a further 10 minutes with a horseradish peroxidase (HRP)-conjugated antibody (Rabbit or Mouse MACH3 HRP; Biocare Medical) specific to the probe. Following this, sections were washed and then incubated with opal fluorophores at a 1:50 dilution made up in tyramide signal amplification reagent (provided in the opal kit). For every additional marker, the process was repeated by treating the slides with an antigen retrieval step followed directly by primary antibody staining and then downstream steps. Following this, sections were stained with DAPI for 3 minutes and then mounted using Vectashield. The following primary antibodies were used to identify CD8 expression (Ab4055 clone; Abcam), CD103 expression (Ab129202 clone; Abcam), SOX10 expression (BC34 clone from Biocare Medical), and PD-1 expression (NAT205 clone from Cell Marque). For imaging, a standard fluorescent microscope fitted with an automated quantitative pathology imaging system (Vectra) was used in conjunction with Vectra 3.3 software. Images were unmixed and annotated in Phenochart 1.0.4 and inForm 2.2.0.

#### Flow cytometry analysis

Lymphocytes from freshly isolated or cryopreserved tumor samples were stained with fluorochrome-conjugated mAbs for 20 minutes at 4°C. T-cell subsets were identified using the following mAbs: anti-CD3, CD8, CD69, and CD103 (all from BioLegend) to identify CD8<sup>+</sup> T cells and resident subsets, whereas anti-CCR7 (R&D Systems) and anti-CD45RA (BD Biosciences) were used for characterizing naïve and memory T cells. Activation and differentiation of T cells were determined using mAbs against CD25, CD137, HLA-DR (all from BD Biosciences), and KLRG1 (BioLegend), whereas expression of inhibitory receptors was determined using mAbs against PD-1, LAG3, GITR, CD244, TIM-3 (all from BioLegend), and CTLA-4 (BD Biosciences). CD8<sup>+</sup> T cells specific for the melanoma antigen Melan-A were identified using an HLA-A\*0201-restricted MHC class 1 tetramer (ELAGI-GILTV; from IMMUDEx). Cells were labeled with Zombie Aqua or Zombie UV to exclude dead cells from analysis. Stained cells were then washed and fixed with 2% paraformaldehyde before analysis on an LSRFortessa flow cytometer (BD biosciences). The data were then processed using FlowJo software (Treestar).

#### Detection of granzyme B, perforin, and eomes by intracellular staining

Following extracellular staining, cells were fixed and permeabilized using a Transcription Factor Buffer Set (BD Pharmingen) and then intracellularly stained with mAbs to granzyme B and Eomes (eBioscience), and Perforin (BioLegend) for 20 minutes at 4°C before analysis on a flow cytometer.

#### Statistical analysis

Graphical and statistical analyses were performed using either Prism version 6.0f (GraphPad Software), TIBCO Spotfire v6.5.2, or CutoffFinder version 2.0. The mean of fluorescent intensity was

calculated using the geometric mean within FlowJo v9.7.6. *P* values were performed either by a nonparametric Kruskal–Wallis matched pairs test, nonparametric log-rank test, or Spearman rho test, where appropriate. *P* values less than 0.05 were considered significant. All variability in the data is shown as SEM. Correlations between Trm/CD8 IHC score with clinical features were performed using the Spearman  $\rho$  method. Melanoma-specific survival (MSS) was calculated from the date of surgical resection of stage III melanoma specimen to date of last follow-up or death from melanoma as performed in previous studies (24). Kaplan–Meier curves were generated using cutoff values that were determined using ROC curve analysis that maximized the sensitivity and specificity of the analysis (Cutoff Finder, <http://molpath.charite.de/cutoff/>; ref. 25). The significance for the survival curves was determined using the log-rank test.

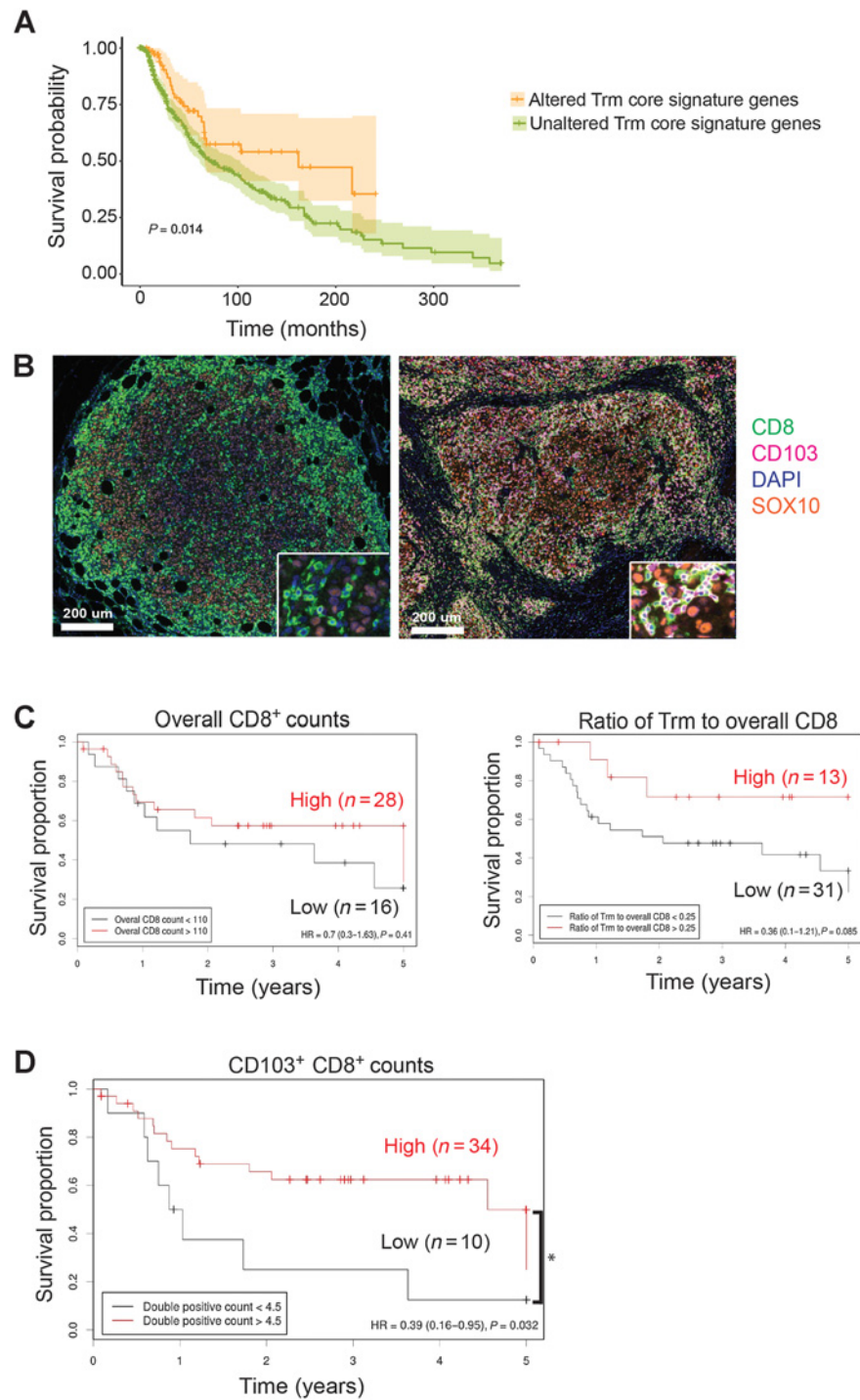
## Results

### CD103<sup>+</sup> tumor-resident cytotoxic T-cell numbers are strongly associated with increased MSS

We began by analyzing TCGA melanoma database (26) to determine whether altered expression of the core signature tissue-resident T-cell genes provided a prognostic advantage in immunotherapy-naïve patients with melanoma. This showed that increased expression of one or more of the following genes *CD69*, *CD103*, *TNFRSF18*, *CD8a*, or *2B4* (Fig. 1A; Supplementary Fig. S1) indeed was associated with improved survival for patients. In order to separate the role of tumor-resident CD8<sup>+</sup> T cells from nonresident CD8<sup>+</sup> T cells, we then performed multiplex quantitative immunofluorescence staining on a subset ( $n = 44$ ) of stage III metastatic melanoma samples used for the same TCGA analysis. This showed a high degree of heterogeneity for the number of CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T-cell numbers between patients, with some having no resident cells and others having significant numbers of them (Fig. 1B). Although it is known that CD8<sup>+</sup> TILs are important for survival in melanoma, we reasoned that it is the CD103<sup>+</sup> CD8<sup>+</sup> T cells that are of primary importance and therefore determined the effect of these cell numbers on patient survival. Although the number of infiltrating CD8<sup>+</sup> T cells was not significantly correlated with survival in this cohort of patients [HR = 0.7 (0.3–1.63);  $P = 0.41$ ], there was a trend toward better survival in patients who had a ratio of tumor-resident T cells to overall CD8<sup>+</sup> T cells of >0.25 [HR = 0.36 (0.1–1.21);  $P = 0.085$ ]. The enumeration of CD103<sup>+</sup> tumor-resident T cells yielded the strongest association with survival [HR = 0.39 (0.16–0.95);  $P = 0.032$ ; Fig. 1C and D], with a 5-year MSS of 50% in those with higher counts compared with MSS of 20% in those with lower counts.

### Expression of immune checkpoints is highly enriched in CD103<sup>+</sup> tumor-resident cytotoxic T cells

Having established the importance of tumor-resident CD8<sup>+</sup> T cells, we next determined the phenotypic characteristics of these cells in order to gain insights into their potential role in tumor control. We isolated TILs from freshly resected metastatic melanoma specimens ( $n = 10$ ) and determined the phenotype of CD8<sup>+</sup> T cells by multiparameter flow cytometry. As previously reported (27), this showed that most of the CD8<sup>+</sup> TILs were effector memory T cells (TEM, CCR7<sup>−</sup>CD45RA<sup>−</sup> or TEMRA CCR7<sup>−</sup>CD45RA<sup>+</sup>; Supplementary Fig. S2a). Interestingly, on average 30% of the CD8<sup>+</sup> T cells were CD69<sup>+</sup>CD103<sup>+</sup> tumor-resident



**Figure 1.**

Description of the protective effect of tumor-resident CD8<sup>+</sup> T cells. **A**, Kaplan-Meier survival curves of melanoma patients from the TCGA database. Patients with improved MSS show altered expression of one or more of the following genes, which are known to be expressed on tumor-resident T cells: CD69, CD103, TNFRSF18, CD8A, or 2B4. The confidence intervals (shaded area) and the *P* value for the log-rank test are shown on the plot. The cutoff used was mRNA expression z-scores (RNA Seq V2 RSEM) ± 1.5. **B**, Using multiplex tissue immunofluorescent staining on formalin-fixed paraffin-embedded sections of tumors from a subset of the matched metastatic melanoma specimens utilized in the TCGA analysis, CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers were enumerated. Figure shows the examples of a patient with a few CD103<sup>+</sup>CD8<sup>+</sup> T cells (left plot) and a patient with high CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers (right). Tissues were stained with antibodies for CD8<sup>+</sup> T cells (green), CD103 (magenta), the melanoma marker SOX10 (orange), and DAPI (blue). Colocalization of CD8 and CD103 appears as a light pink/white staining. **C**, Kaplan-Meier MSS curves for patients with high (red) counts of CD8<sup>+</sup> T cells against those with low (black) counts (cutoff 110; left plot) and patients with high (red) ratios of CD103<sup>+</sup>CD8<sup>+</sup> T cells to CD8<sup>+</sup> T cells against those with low (black) ratios (cutoff 0.25; right plot). Statistical differences were calculated using a nonparametric log-rank test and displayed as \* for *P* ≤ 0.05. **D**, Kaplan-Meier MSS curves for patients with high (red) counts of CD103<sup>+</sup>CD8<sup>+</sup> T cells and those with low (black) counts (cutoff 4.5). A tissue microarray slide (TMA) that contained 44 individual regional lymph node melanoma metastases from high TIL areas of the tumor were analyzed. The number of CD103<sup>+</sup>CD8<sup>+</sup> and CD8<sup>+</sup> T cells in each tumor core was counted and analyzed in order to determine the prognostic effect of each.

T cells (Supplementary Fig. S2b). In line with what has been reported in mice for tissue-resident memory T cells (12), these tumor-resident CD8<sup>+</sup> T cells were KLRG1<sup>low</sup> TEM-like phenotype (Fig. 2A; Supplementary Fig. S2c), suggesting that they were not recently activated effector populations. Also, consistent with this, we found a moderate increase in the expression of some activation markers (CD137 and HLA-DR; Fig. 2A), but not others (CD25; Supplementary Fig. S2d), and a moderate increase in the expression of the lytic granules granzyme B in the absence of perforin or granzyme K (Supplementary Fig. S2d), all of which are highly expressed on recently activated effector CD8<sup>+</sup> T-cell populations.

Studies in mice and our recent work in humans have also shown that tissue-resident memory T cells are enriched for the expression of several inhibitory receptors, including PD-1, CTLA-4, and 2B4 (12, 20). We therefore hypothesized that tumor-resident CD8<sup>+</sup> T cells could be a critical population that expresses immune checkpoints. Indeed, the expression of PD-1 was highest on CD69<sup>+</sup>CD103<sup>+</sup> CD8<sup>+</sup> T cells, followed by CD69<sup>+</sup>CD103<sup>-</sup>CD8<sup>+</sup> T cells (Fig. 2B). The proportion of PD-1<sup>+</sup>CD103<sup>+</sup>CD69<sup>+</sup> cells was significantly higher than PD-1<sup>+</sup>CD103<sup>-</sup>CD69<sup>+</sup> or PD-1<sup>+</sup>CD103<sup>-</sup>CD69<sup>-</sup> CD8<sup>+</sup> T cells. Similarly, the expression levels of other inhibitory checkpoints LAG-3, 2B4, and TIM-3 were highest in CD69<sup>+</sup>CD103<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 2B; Supplementary Fig. S2e), with LAG-3<sup>+</sup> population significantly higher in CD103<sup>+</sup>CD69<sup>+</sup> subset when compared with the rest. There was, however, no difference in the expression of GITR between the three populations (Fig. 2B), and the surface expression of CTLA-4 was minimal (Supplementary Fig. S2e). When we examined for the expression of two T-box transcription factors that regulate T-cell responses, we found a trend toward a higher expression of Eomesodermin (Eomes) and Tbet in the CD103<sup>+</sup>CD69<sup>+</sup> subset (Supplementary Fig. S2f) when compared with the other two subsets. In order to verify that these phenotypes were indeed represented in tumor-specific CD8<sup>+</sup> T cells, we stained TILs with soluble peptide-MHC complex to identify Melan A-specific CD8<sup>+</sup> T cells. This showed that tumor-specific resident CD8<sup>+</sup> T cells were also enriched for PD-1 and 2B4 (Fig. 2C).

#### CD103<sup>+</sup> tumor-resident T cells significantly expand early during anti-PD-1 treatment

Previous studies examining the T-cell populations responding to anti-PD-1 treatment found an expansion of CD8<sup>+</sup> TEM cells (28) or PD-1<sup>+</sup>CD8<sup>+</sup> T cells (7) early during treatment. Based on our data, we predicted that the target population for such immunotherapy could be the tumor-resident CD8<sup>+</sup> T-cell population. To test this hypothesis, we analyzed tumor biopsy specimens from advanced stage metastatic melanoma patients being treated with anti-PD-1 monotherapy (nivolumab, pembrolizumab), procured before and early during treatment. We grouped patients who had CR, PR, and SD for greater than 6 months as determined by RECIST response criteria as "responders" and patients who had progressive disease and SD for less than 6 months as "non-responders" (Supplementary Table S1). As described before, total CD8<sup>+</sup> T-cell numbers expanded significantly during anti-PD-1 treatment (Fig. 3A and B), and there was a significant difference in their numbers at EDT ( $P = 0.039$ ) between those who responded to the treatment against those who did not respond. Quantification of the tumor-resident CD8<sup>+</sup> T-cell numbers showed that, when compared with the baseline, there was significant expansion

of CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T cells early during treatment in the majority of the patients (Fig. 3A and C). We then examined whether there were differences in the number of tumor-resident CD8<sup>+</sup> T cells between responders to anti-PD-1 therapy compared with nonresponders. Despite the small number of responders ( $n = 5$ ) analyzed with early-during treatment biopsies, there was a trend toward a greater magnitude of expansion of these cells in responding patients ( $P = 0.07$ ) compared with nonresponders ( $n = 8$ ). This suggests that a threshold of tumor-resident CD8<sup>+</sup> T-cell numbers might be necessary for the effectiveness of anti-PD-1 treatment.

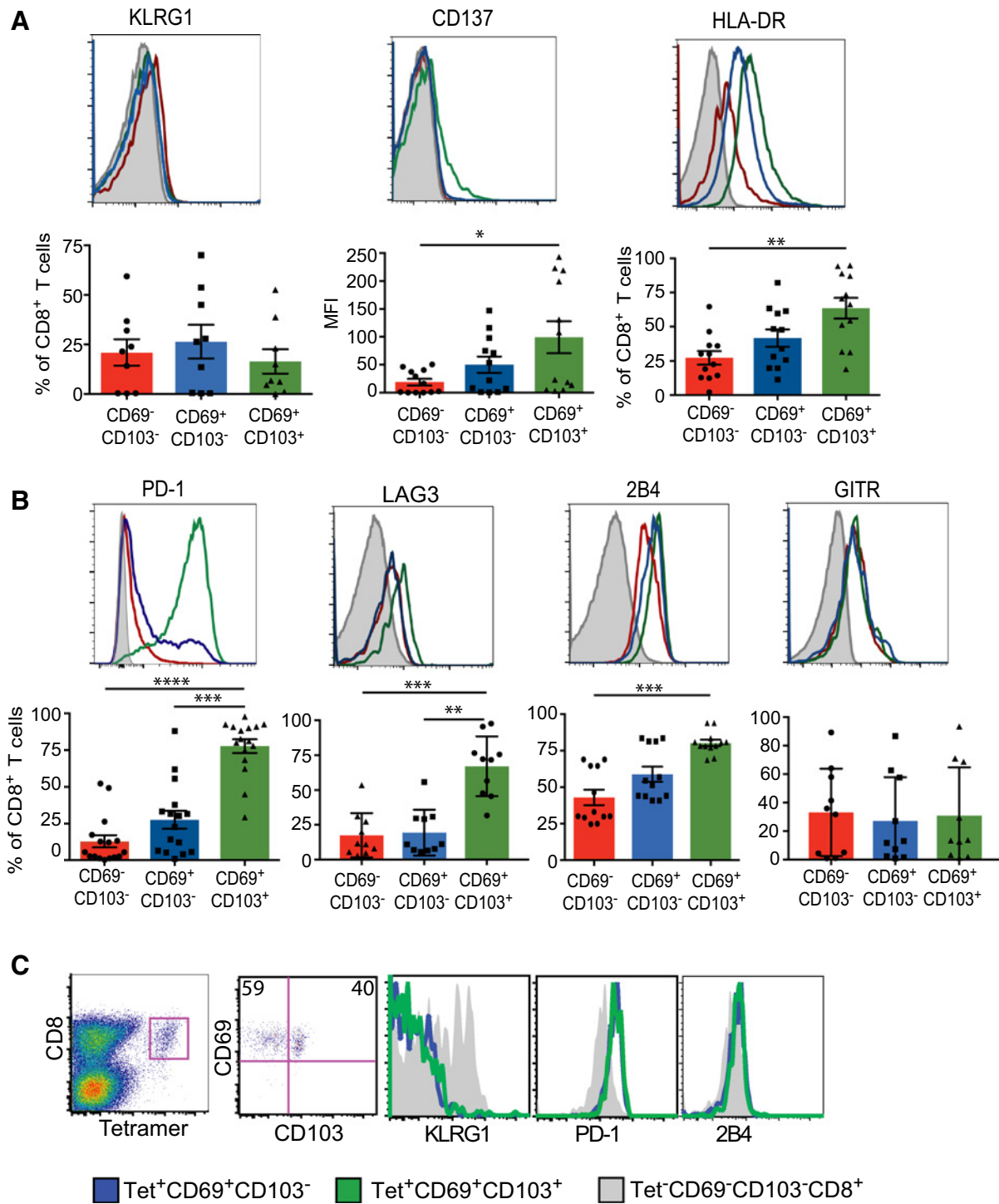
#### Local IL15 expression levels are associated with CD103<sup>+</sup> tumor-resident T-cell numbers

Finally, we examined the local factors that could influence the number of resident T cells within metastatic tumors. In mouse models, cytokines such as IL15, TGF $\beta$ , IL7, and IL33 have been implicated in the development of tissue-resident memory T cells. We recently showed in humans that IL15 could induce the expression of CD69 and downmodulate the exit signals to potentiate the retention of T cells within tissues (20). Consistent with this, we found a strong correlation between the expression of IL15 and CD8 mRNA in the TCGA database ( $P = 8.3 \times 10^{-7}$ ; Fig. 4A) and more importantly that enhanced IL15 expression was also associated with better patient survival ( $P = 0.0012$ ; Fig. 4B). We therefore determined whether local IL15 levels influenced the CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T-cell numbers within the tumors. Quantitative analysis of samples that either had high or low expression of IL15 RNA in the TCGA study suggests that local IL15 levels may influence CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T-cell numbers within metastatic melanoma (Fig. 4C and D). This suggests that the heterogeneity in these cell numbers between patients could be largely due to local factors that are important for T-cell retention.

## Discussion

Although therapeutic blockade of immune checkpoints remains a promising treatment strategy for many types of cancers, there is an urgent need to improve its efficacy, reduce the associated toxicity, and develop accurate biomarkers to predict response. Critical to this is a greater understanding of the immune cells that initiate the response and the factors that regulate their numbers. Our collective data demonstrate that CD103<sup>+</sup> tumor-resident CD8<sup>+</sup> T cells are not only associated with tumor control in untreated metastatic melanoma patients, but may also be important for determining responses to anti-PD-1 immunotherapy. Here, we show that the expression of PD-1 and LAG-3 is highly enriched to this subset of CD8<sup>+</sup> T cells in vast majority of patients, suggesting that these cells are likely to be the initial target of anti-PD-1 treatment. In addition, we also found these cells significantly expanded early during anti-PD-1 treatment in melanoma specimens taken from patients with metastatic melanoma, further supporting the fact that these tumor-resident CD8<sup>+</sup> T cells could be initiating the response. In an attempt to identify the factors that regulate these cells, we found local IL15 expression levels to strongly correlate with tumor-resident CD8<sup>+</sup> T-cell numbers.

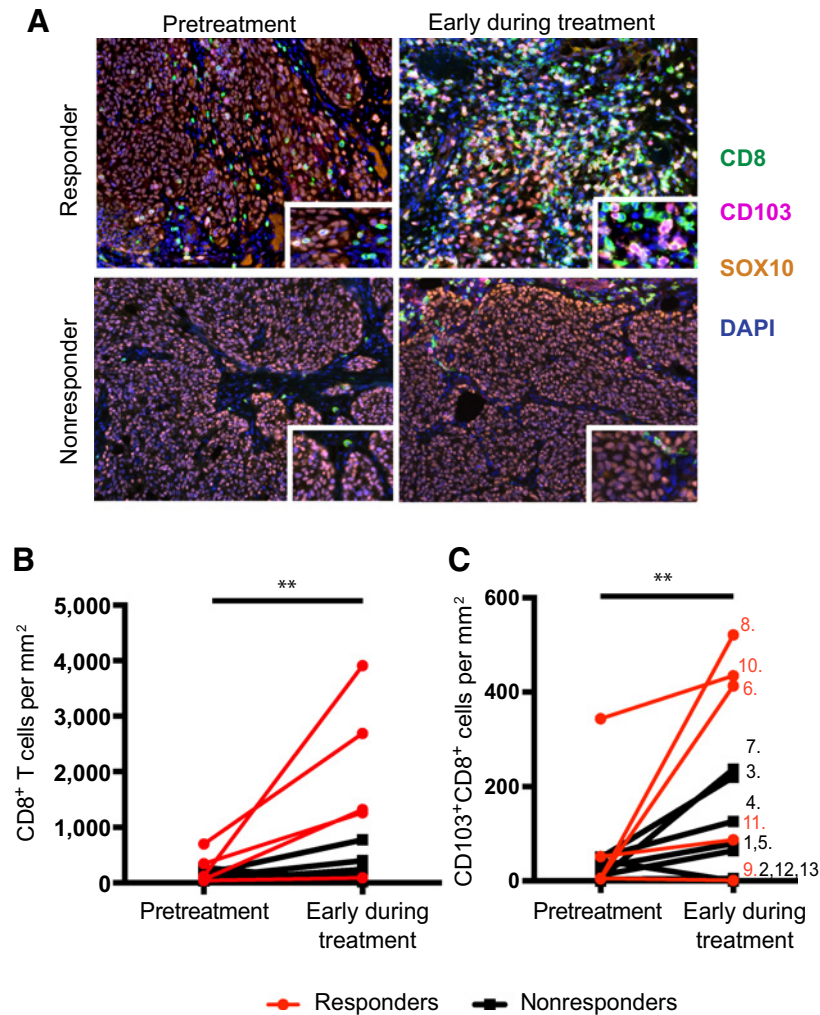
We have found that tumor-resident CD8<sup>+</sup> T-cell numbers were more prognostic than the total CD8<sup>+</sup> T-cell counts in untreated



**Figure 2.** Phenotype of tumor-resident CD8<sup>+</sup> T cells. Freshly isolated tumor-infiltrating lymphocytes from melanoma patients were stained with antibodies and analyzed by flow cytometry. Antibodies to CD3, CD8, CD103, and CD69 were used to identify tumor-resident CD8<sup>+</sup> T cells. **A**, The expression levels of KLRG1, CD137, and HLA-DR were compared between CD103<sup>+</sup>CD69<sup>+</sup> (green), CD103<sup>-</sup>CD69<sup>+</sup> (blue), and CD103<sup>-</sup>CD69<sup>-</sup> (red) CD8<sup>+</sup> T-cell populations. Top plot shows the representative histogram overlay plots with FMO controls (gray), and the bottom plot shows the plots representing a minimum of 9 different patient samples with  $\pm$  SEM. **B**, Similarly, the expression levels of PD-1, LAG3, 2B4, and GITR were compared between CD103<sup>+</sup>CD69<sup>+</sup> (green), CD103<sup>-</sup>CD69<sup>+</sup> (blue), and CD103<sup>-</sup>CD69<sup>-</sup> (red) CD8<sup>+</sup> T-cell populations. Top plot shows the representative histogram overlay plots with FMO controls (gray), and the bottom plot shows the plots representing a minimum of 10 different patient metastatic melanoma specimens with  $\pm$  SEM. Statistical differences were calculated using a nonparametric Kruskal-Wallis test and displayed as \* for  $P \leq 0.05$ , \*\* for  $P \leq 0.01$ , \*\*\* for  $P \leq 0.001$ , and \*\*\*\* for  $P \leq 0.0001$ . **C**, Soluble peptide-MHC complexes (tetramers) were used to identify CD8<sup>+</sup> T cells specific for Melanin-A antigen. Plots show the tetramer stain, the expression of CD69 and CD103 on tetramer-positive cells, and representative histogram overlay plots of tetramer<sup>+</sup>CD103<sup>+</sup>CD69<sup>+</sup> (green) and tetramer<sup>+</sup>CD103<sup>+</sup>CD69<sup>-</sup> (blue) CD8<sup>+</sup> T cells for indicated receptors. Tumor-specific T-cell plots are representative of two different patient samples.

Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/24/13/3036/204549/3036.pdf> by guest on 27 August 2022



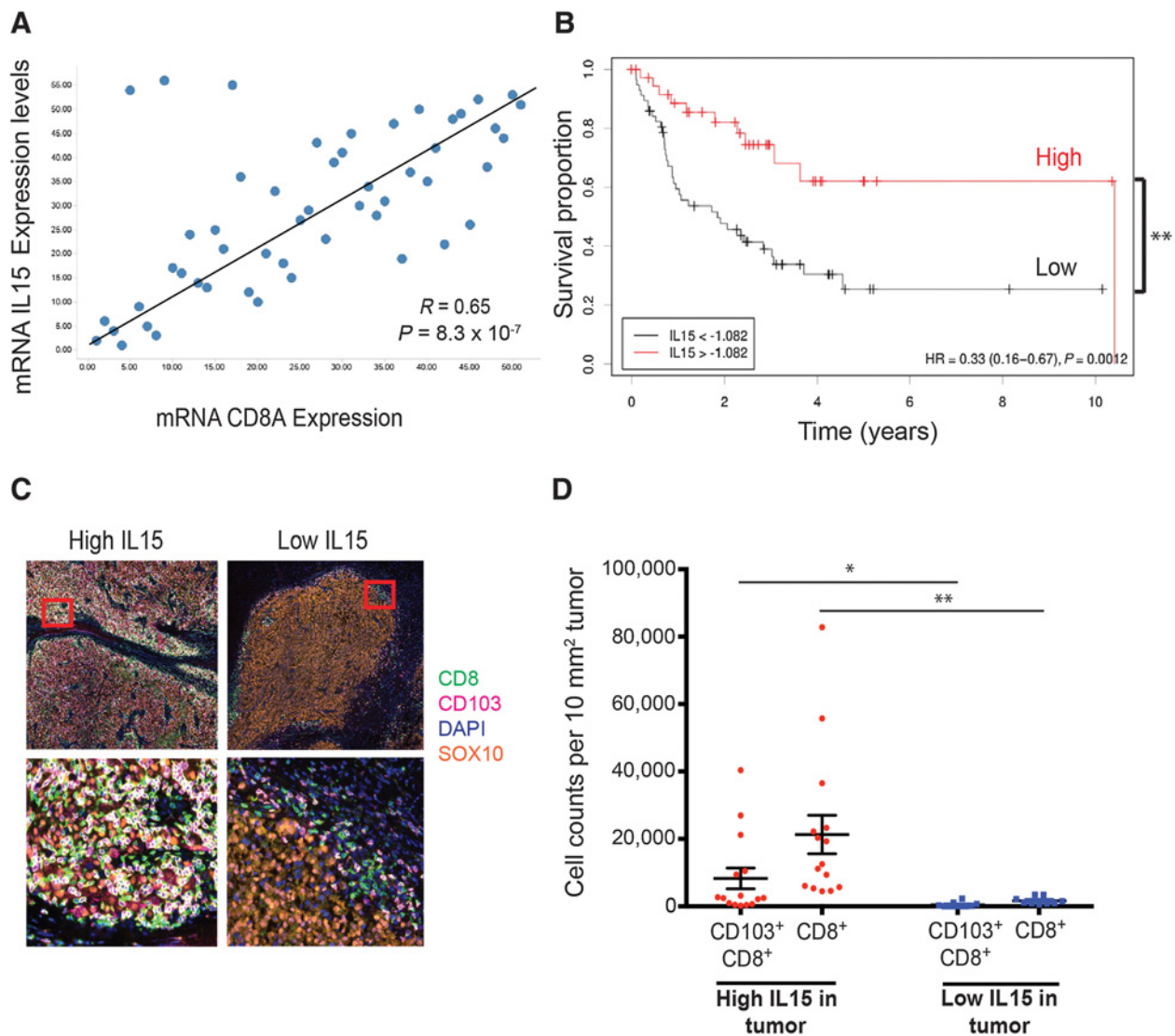


**Figure 3.** Tumor-resident CD8<sup>+</sup> T cells respond to immunotherapy. Tumor biopsies were obtained prior to (pretreatment) and early during treatment from metastatic melanoma patients undergoing anti-PD-1 therapy. Paraffin-embedded sections were then stained for CD8<sup>+</sup> T cells (green), CD103 (magenta), the melanoma marker SOX10 (orange), and DAPI (blue) and CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers. **A**, Example sections from a patient who responded to anti-PD-1 treatment (top plots) and a patient who failed to respond (bottom plots) are shown. Quantification of total CD8<sup>+</sup> T cells (**B**) and CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers (**C**) in responders ( $n = 5$ ) and nonresponders ( $n = 8$ ) shows a significant expansion of these cells early during treatment. Statistical differences were calculated using nonparametric Wilcoxon matched-pairs signed rank test, and \*\* indicates a  $P$  value of  $< 0.01$ . Graph points are indexed and matched to patient clinical data in Supplementary Table S1.

melanoma patients. Further studies are required to evaluate the predictive value of these cells. Although total CD8<sup>+</sup> T-cell counts have previously been associated with patient survival, in the absence of information on subsets, it is difficult to determine whether the protective response was associated with any particular subset. Recruitment of tumor-specific effector CD8<sup>+</sup> T cells to the site of tumors is essential for tumor rejection. It has recently become evident that some of these CD8<sup>+</sup> T cells that infiltrate tissues become resident (29, 30). Here, we show that human tumors also contain populations of tumor-resident CD8<sup>+</sup> T cells, and their numbers are highly variable between patients. Importantly, our data clearly demonstrate that the increased presence of tumor-resident CD8<sup>+</sup> T cells is strongly associated with better MSS in untreated patients. This suggests that tumor-resident CD8<sup>+</sup> T cells could be critical for immune control of metastatic tumors. Tissue-resident CD8<sup>+</sup> T cells are characterized by the constitutive expression of CD69 and CD103 (31). In humans, early reports described two populations of resident T cells, CD69<sup>+</sup>CD103<sup>+</sup>CD8<sup>+</sup> and CD69<sup>+</sup>CD103<sup>-</sup>CD8<sup>+</sup> T cells (32, 33). CD69 expression, however, can also be induced on T cells upon T-cell receptor activation, therefore relying on CD69 expression alone to identify resident memory T cells and differentiate them from recently activated effector cells may not be accurate. We

recently showed that CD69<sup>+</sup>CD103<sup>+</sup>CD8<sup>+</sup> T cells in human tissues were more characteristic of the tissue-resident memory T cells described in mouse models than CD69<sup>+</sup>CD103<sup>-</sup>CD8<sup>+</sup> T cells (20). Among the other features that characterize resident cells are a set of core signature genes that are either enhanced (*RGS1*, *TNFRSF18*, *CD244*, *ICOS*) or suppressed (*KLF2*, *S1PR1*, *KLRG1*) in resident cells when compared with their circulating counterparts (12).

Further insight into the critical role of these tumor-resident CD8<sup>+</sup> T cells became evident when we examined their phenotype. Similar to what has been reported for tissue-resident memory T cells, the tumor-resident CD8<sup>+</sup> T cells were also enriched for several immune inhibitory receptors. It was recently reported that CD69<sup>+</sup>CD8<sup>+</sup> T cells in metastatic melanoma were highly enriched for immune checkpoint receptors (34). However, our data suggest that mainly the CD103<sup>+</sup>CD69<sup>+</sup> subset expressed the highest levels of PD-1 and LAG-3. Together with the fact that these CD103<sup>+</sup> T cells expanded significantly during anti-PD-1 treatment, it could be inferred that these cells initiate the response to anti-PD-1. Although it has been shown that preexisting CD8<sup>+</sup> T cells at the tumor-invasive margin was predictive of response to anti-PD-1 treatment (7), our data strongly suggest that tumor-resident subset of CD8<sup>+</sup> T cells is critical. Further supporting



**Figure 4.**

The impact of IL15 expression levels on tumor-resident CD8<sup>+</sup> T cells. The expression of IL15 in the tumors of patients with metastatic melanoma was analyzed with respect to CD8 expression levels and patient survival. **A**, Correlation of IL15 and CD8A mRNA expression levels in the tumors of melanoma patients as found in TCGA melanoma data ( $P = 8.3 \times 10^{-7}$ , Spearman rank correlation coefficient,  $r = 0.65$ ). **B**, Kaplan-Meier MSS curve for patients with high (red) IL15 expression levels and low (black) IL15 expression levels from TCGA melanoma cohort. The two groups were compared by analyzing the proportion of patients who survived as a function of time (years). Data were binarized using a threshold of 1.082 for IL15 expression. Statistical differences were calculated using a nonparametric log-rank test and displayed as \*\* for  $P \leq 0.01$ . **C**, Representative images of patients with either high (left) or low (right) mRNA levels of IL15 in tumors. Top images are low objective views, and bottom images are regions from highlighted (red) area of interest at high-power magnification. Paraffin-embedded sections from high IL15 mRNA expression ( $n = 14$ ) and low ( $n = 11$ ) mRNA-expressing metastatic melanoma tissues from the TCGA analysis were stained for CD8<sup>+</sup> T cells (green), CD103 (magenta), SOX10 (orange), and DAPI (blue) and CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers enumerated. Colocalization of CD8 and CD103 is represented by light pink/white staining. **D**, Quantitative analysis of CD8<sup>+</sup> and CD103<sup>+</sup>CD8<sup>+</sup> T-cell numbers in high (red) or low (blue) IL15 mRNA-expressing groups per 10 mm squared area of tumor. Each point represents an individual patient. Statistical significance was calculated using nonparametric Kruskal-Wallis test and displayed as \* for  $P \leq 0.05$  and \*\* for  $P \leq 0.01$ .

our work is another very recent study that showed the phenotype of expanding T-cell clones during immunotherapy. Wei and colleagues showed that T-cell clones that expanded during anti-PD-1 treatment expressed high levels of CD69, PD-1, LAG-3, and CD45RO (35), an identical phenotype to the tumor-resident CD8<sup>+</sup> T-cell population we have described. There is also growing evidence from mouse models that tumor-resident

T cells may play a critical role in checkpoint inhibitor efficacy. For example, recently it was shown that the effectiveness of checkpoint blockade and the expansion of effector populations during treatment may be independent of circulating T cells (36, 37), suggesting that the preexisting tumor-resident T cells were sufficient to mediate response. In addition, the failure of circulating T-cell numbers to predict response to checkpoint



blockade could also be attributed to the fact that the critical population is noncirculating and is resident within the tumor environment.

There was significant expansion of tumor-resident CD8<sup>+</sup> T cells in the vast majority of melanoma patients early during treatment with anti-PD-1 drug therapy compared with baseline tissue samples. The magnitude of expansion, however, was higher in responders than nonresponders. It is worth noting that there are some limitations to the interpretation of the data: (1) the tumor biopsies were obtained from different sites between pre and EDT samples, (2) time to EDT samples varied among patients, (3) the responding clones could have changed phenotype and therefore we underestimated their numbers, (4) we were unable to stain for CD69, and (5) there were limited number of biopsies available from patients undergoing immunotherapy. Nevertheless, our data strongly suggest that the numbers of tumor-resident CD8<sup>+</sup> T cells could be critical in determining the effectiveness of checkpoint blockade. In addition, it has become evident that combination immunotherapies that target multiple immune checkpoints can improve the success rate when compared with monotherapies. In line with this, we have found that tumor-resident CD8<sup>+</sup> T cells express multiple immune checkpoints and therefore are likely to be regulated by more than one inhibitory receptor. These data need to be confirmed in larger patient cohorts; however, the immune profile of tumor-resident CD8<sup>+</sup> T cells may provide a rational approach to the selection of therapies for patients who fail monotherapies and provide a basis for the design of clinical trials in this patient population.

Boosting tumor-resident CD8<sup>+</sup> T-cell numbers may be a strategy to improve antitumoral immune responses. Understanding the factors that regulate the retention of tumor-resident CD8<sup>+</sup> T cells is therefore critical. We have recently shown that IL15 not only downmodulates tissue-exit signals on human T cells, but also induces the expression of CD69 and hence may facilitate their retention. IL15 has also been implicated in the development of tissue-resident memory T cells in mouse models (12). Our current study shows that the expression levels of local IL15 was highly variable between patients, and importantly, the levels correlated strongly with tumor-resident T-cell numbers. In the absence of antibodies that can stain CD69 on formalin-fixed tissues, we were unable to determine the proportion of CD69<sup>+</sup>CD8<sup>+</sup> T cells. This also corroborates with the recent finding that majority of CD8<sup>+</sup> T cells in TILs express CD69 (34). Therefore, it could be inferred that the local IL15 could be a critical factor in retaining T cells within the tumor environment. Further studies are, however, required to determine the exact role of this cytokine. IL15 could have multiple effects on tumor-infiltrating T cells. It can also enhance the survival of memory T cells through the upregulation of pro-survival genes, and it can induce proliferation of T cells in the absence of T-cell receptor-mediated signals. Although the role of IL15 in antitumoral T-cell responses has long been recognized, its ability to retain cells within the tumor environment has not been appreciated. Interestingly, a recent study in human colorectal cancers suggests that IL15 gene deletion by tumor cells could be an immune escape mechanism (38). Another factor that could contribute to tissue-retention of T cells is TGFβ, which not only synergizes with IL15 to downmodulate tissue-exit signals, but is also necessary for the expression of CD103 (20). How these cytokines are regulated within the tumor environment is unclear;

however, tumor-infiltrating dendritic cells (DCs) and/or stromal cells can be a source of IL15.

Persistence of tumor-resident CD8<sup>+</sup> T cells could also be critical for long-term tumor control. One possibility is that they are better suited to survive in metabolically challenging tumor environments. A recent study has shown that tissue-resident T cells are uniquely dependent on the uptake of free fatty acids and their metabolisms for long-term survival (39). The restricted access to glucose within the tumor environment (40) could favor the survival of these resident T cells. Nevertheless, our findings will support the notion that the effectiveness of T-cell-mediated control of metastatic cancers relies not only on the recruitment of effector T cells, but also on retaining them within the tumor environment.

In conclusion, our work demonstrates that tumor-resident CD8<sup>+</sup> T cells could be critical for tumor control and are likely to be the population that initiates response to checkpoint blockade. A greater understanding of the characteristics of this population and the crucial factors that regulate their numbers will open new opportunities for novel therapies.

### Disclosure of Potential Conflicts of Interest

J.F. Thompson reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, and Provectus. A.M. Menzies is a consultant/advisory board member for Bristol-Myers Squibb, MSD, Novartis, Pierre-Fabre, and Roche. G.V. Long is a consultant/advisory board member for Amgen, Array, Bristol-Myers Squibb, Merck, Novartis, Pierre-Fabre, and Roche. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

**Conception and design:** J. Edwards, W.J. Britton, A.M. Menzies, G.V. Long, R.A. Scolyer, U. Palendira

**Development of methodology:** J. Edwards, J.S. Wilmott, T.N. Gide, A. Ferguson, J. Chen, G.V. Long, R.A. Scolyer

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J. Edwards, J.S. Wilmott, T.N. Gide, A. Tasker, R. Hewavisenti, R.P.M. Saw, J.F. Thompson, A.M. Menzies, G.V. Long, R.A. Scolyer

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J. Edwards, J.S. Wilmott, J. Madore, C. Quek, A. Tasker, A. Ferguson, T. Gebhardt, W. Weninger, W.J. Britton, G.V. Long, R.A. Scolyer, U. Palendira

**Writing, review, and/or revision of the manuscript:** J. Edwards, J.S. Wilmott, T.N. Gide, C. Quek, J. Chen, R. Hewavisenti, P. Hersey, T. Gebhardt, W. Weninger, W.J. Britton, R.P.M. Saw, J.F. Thompson, A.M. Menzies, G.V. Long, R.A. Scolyer, U. Palendira

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.S. Wilmott, A. Ferguson, J. Chen

**Study supervision:** R.A. Scolyer, U. Palendira

### Acknowledgments

This work was funded by the National Health and Medical Research Council of Australia and the University of Sydney's Sydney Research Excellence Initiative grant.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 3, 2017; revised November 9, 2017; accepted March 20, 2018; published first March 29, 2018.

## References

- Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677–704.
- Boussiotis VA. Molecular and biochemical aspects of the PD-1 checkpoint pathway. *N Engl J Med* 2016;375:1767–78.
- Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med* 2015;373:23–34.
- Robert C, Schachter J, Long G V, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med* 2015;372:2521–32.
- Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csószai T, Fülöp A, et al. Pembrolizumab versus chemotherapy for PD-L1–positive non–small-cell lung cancer. *N Engl J Med* 2016;375:1823–33.
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2449–58.
- Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515:568–71.
- Kakavand H, Vilain RE, Wilmott JS, Burke H, Yearley JH, Thompson JF, et al. Tumor PD-L1 expression, immune cell correlates and PD-1+ lymphocytes in sentinel lymph node melanoma metastases. *Mod Pathol* 2015;28:1535–44.
- Herbst RS, Soria J-C, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014;515:563–7.
- Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW, et al. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol* 2012;30:2678–83.
- Adachi T, Kobayashi T, Sugihara E, Yamada T, Ikuta K, Pittaluga S, et al. Hair follicle–derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. *Nat Med* 2015;21:1272–9.
- Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, et al. The developmental pathway for CD103(+)CD8(+) tissue-resident memory T cells of skin. *Nat Immunol* 2013;14:1294–301.
- Jiang X, Clark Ra, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* 2012;483:227–31.
- Mackay LK, Stock AT, Ma JZ, Jones CM, Kent SJ, Mueller SN, et al. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc Natl Acad Sci* 2012;109:7037–42.
- Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* 2012;491:463–7.
- Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrançois L, Farber DL. Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol* 2011;187:5510–4.
- Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8 $\alpha\alpha$ + skin-resident T cells in human herpes virus infection. *Nature* 2013;497:494–7.
- Clark Ra, Watanabe R, Teague JE, Schlapbach C, Tawa MC, Adams N, et al. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med* 2012;4:117ra7–117ra7.
- Jozwik A, Habibi MS, Paras A, Zhu J, Guvenel A, Dhariwal J, et al. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat Commun* 2015;6:10224.
- Woon HG, Braun A, Li J, Smith C, Edwards J, Sierro F, et al. Compartmentalization of total and virus-specific tissue-resident memory CD8+ T cells in human lymphoid organs. *PLoS Pathog* 2016;12:1–19.
- Djenidi F, Adam J, Goubar a, Durgeau a, Meurice G, de Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol* 2015;194:3475–86.
- Webb JR, Milne K, Watson P, DeLeeuw RJ, Nelson BH. Tumor-infiltrating lymphocytes expressing the tissue resident memory marker cd103 are associated with increased survival in high-grade serous ovarian cancer. *Clin Cancer Res* 2014;20:434–44.
- Murray T, Fuertes Marraco SA, Baumgaertner P, Bordry N, Cagnon L, Donda A, et al. Very late antigen-1 marks functional tumor-resident CD8 T cells and correlates with survival of melanoma patients. *Front Immunol* 2016;7:1–12.
- Madore J, Strbenac D, Vilain R, Menzies AM, Yang JYH, Thompson JF, et al. PD-L1 negative status is associated with lower mutation burden, differential expression of immune-related genes, and worse survival in stage III melanoma. *Clin Cancer Res* 2016;22:3915–23.
- Morello S, Capone M, Sorrentino C, Giannarelli D, Madonna G, Mallardo D, et al. Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. *J Transl Med* 2017;15:1–9.
- Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, Amin SB, et al. Genomic classification of cutaneous melanoma. *Cell* 2015;161:1681–96.
- Baitsch L, Baumgaertner P, Devèvre E, Raghav SK, Legat A, Barba L, et al. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest* 2011;121:2350–60.
- Ribas A, Shin DS, Zaretsky J, Cornish A, Avramis E, et al. PD-1 blockade expands intratumoral memory T cells. *Cancer Immunol Res* 2016;4:194–203.
- Schenkel JM, Masopust D. Tissue-resident memory T cells. *Immunity* 2014;41:886–97.
- Carbone FR, Mackay LK, Heath WR, Gebhardt T. Distinct resident and recirculating memory T cell subsets in non-lymphoid tissues. *Curr Opin Immunol* 2013;25:329–33.
- Carbone FR. Tissue-resident memory T cells and fixed immune surveillance in nonlymphoid organs. *J Immunol* 2015;195:17–22.
- Thome JJC, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, et al. Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell* 2014;159:814–28.
- Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* 2013;1–11.
- Boddupalli CS, Bar N, Kadaveru K, Krauthammer M, Pomputtpong N, Mai Z, et al. Interleukin diversity of T cell receptors in melanoma with immune checkpoints enriched in tissue-resident memory T cells. *JCI Insight* 2016;1.
- Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell* 2017;170:1120–1125.e17.
- Spranger S, Koblisch HK, Horton B, Scherle Pa, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8 (+) T cells directly within the tumor microenvironment. *J Immunother Cancer* 2014;2:3.
- Williams JB, Horton BL, Zheng Y, Duan Y, Powell JD, Gajewski TF. The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8+ T cells in the tumor microenvironment. *J Exp Med* 2017 Feb;214:381–400.
- Mlecnik B, Bindea C, Angell HK, Sasso MS, Obenaus AC, Fredriksen T, et al. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. *Sci Transl Med* 2014;6:228ra37–228ra37.
- Pan Y, Tian T, Park CO, Lofftus SY, Mei S, Liu X, et al. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature* 2017;543:252–6.
- Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* 2015;162:1229–41.