Endogenous tumor-reactive CD8+ T cells are differentiated effector cells expressing high levels of CD11a and PD-1 but are unable to control tumor growth

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Introduction

Endogenous or naturally occurring antitumor immunity is difficult to detect and monitor, largely due to the absence of established biomarkers and the unknown identity of the tumor-associated antigens (TAA) that are targeted. When TAAs are known, antigen-specific T-cell receptor (TCR) tetramers can be employed. However, TCR tetramers have limitations in measuring antitumor immunity. Indeed, the downregulation of the TCR as induced by persistent antigen stimulation and the generally weak affinity of CD8+ T cells for TAAs both contribute to the propensity of this technique to underestimate antitumor responses. In addition, TCR tetramers are not amenable for detecting CD8+ T-cell responses to subdominant epitopes. TCR tetramers therefore are not capable of detecting the complete spectrum of CD8+ tumor-specific T cells, even if the TAAs are known.

Immunotherapies aimed at improving natural or endogenous antitumor T-cell immunity are currently being implemented in the clinic with promising results. In order to optimize the therapeutic protocols and monitor the effectiveness of such therapies, reliable biomarkers are needed. We used CD11a, an integrin that is upregulated on the surface of effector and memory CD8+ T cells, and PD-1, an immunoregulatory receptor expressed by activated T cells, as biomarkers to identify, quantify and monitor endogenous tumor-reactive cytotoxic T lymphocytes (CTLs) in two mouse tumor models and in the peripheral blood of 12 patients affected by Stage IV melanoma. High expression levels of CD11a and PD-1 were detected among CD8+ T cells residing within primary and metastatic murine tumor sites, as well as in spontaneous murine breast cancer tissues. In the peripheral blood of melanoma patients, tumor antigen-specific CD8+ T cells were associated with a population of CD11a+PD1+ CD8+ T cells that co-expressed high levels of PD-1. Healthy donors exhibited a comparatively much lower frequency of such PD-1+CD11ahighCD8+ T cells. Phenotypic analyses demonstrated that CD11a+PD1+ CD8+ T cells are proliferating (Ki67+) and activated (CD62L−CD69+). Increased CD11a+PD1+ CD8+ T cells and delayed tumor growth were observed in PD-1 deficient mice, suggesting that the antitumor effector functions of CD8+ T cells is compromised by an elevated expression of PD-1. The CD11a+PD1+ CD8+ T-cell population expresses high levels of PD-1 and presumably constitutes the cellular target of PD-1 blockade therapy. The expression level of CD11a and PD-1 by CD8+ T cells may therefore represent a novel biomarker to identify and monitor endogenous tumor-reactive CTLs. This may not only provide an immunological readout for evaluating the efficacy of immunotherapy but also contribute to the selection of cancer patients who are likely to benefit from anti-PD-1 therapy.
by cytotoxic T cells that binds to CD54 [intercellular adhesion molecule 1 (ICAM-1)] and hence functions as an adhesion molecule. CD11a is indispensable in mediating the conjugation between cytotoxic T lymphocytes (CTLs) and target cells. Blocking CD11a dramatically reduces the killing of tumor cells and rejection of transplants by CTLs, and CD11a-deficient mice exhibit defects in rejecting immunogenic tumors. Dai et al. first reported that an increased expression of CD11a can distinguish naïve T cells from antigen-experienced effector and memory CD8+ T cells in the peripheral blood and tissues upon infection or antigen stimulation. Importantly, CD11a identifies effector and memory CD8+ T cells independent of antigen specificity. Murine CD11a<sup>hi</sup>CD8<sup>+</sup> T cells include memory CD8+ T cells capable of mounting vigorous recall responses upon a secondary antigenic challenge. Building upon these studies, the elevated expression of CD11a on CD8+ T cells has been used to track antigen-primed effector and memory CD8+ T cells as induced by viral vaccination. The utility of CD11a for studying antigen-primed CD8+ T cells is demonstrated by the fact that only a portion of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells can be detected by MHC class I tetramer staining, while all corresponding antigen-specific CD8+ T cells reside within the CD11a<sup>hi</sup>CD8<sup>+</sup> T-cell population. The applicability of the CD11a<sup>hi</sup> phenotype for detecting, measuring and tracking TAA-reactive CD8+ T cells has not yet been established.

In this study, we identified CD11a<sup>hi</sup>CD8<sup>+</sup> T cells in both primary and metastatic lesions developing upon the inoculation of tumor cells as well as in spontaneous murine breast tumor lesions. In the peripheral blood of melanoma patients, a majority of TAA-specific (MART-1-tet+) CD8+ T cells were CD11a<sup>hi</sup>. CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were proliferating (Ki67<sup>+</sup>) and activated (CD62L<sup>+</sup>CD69<sup>+</sup>) and had differentiated to effector cells, as determined by the expression of the transcription factor T-bet and by the presence of CTL effector functions. Despite the activated effector phenotype of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells, these cells were unable to control tumor growth, presumably because they expressed elevated levels of PD-1, a negative immunoregulatory receptor. We propose that the expression levels of CD11a and PD-1 may constitute useful markers for measuring endogenous or naturally occurring tumor-reactive T-cell responses.

**Results**

CD11a<sup>hi</sup>CD8<sup>+</sup> T cells comprise an antitumor T-cell population. We monitored the presence of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells in mice harboring a subcutaneous tumor and analyzed TAA-specific CTL effector functions. B16-OVA tumor cells (engineered to express ovalbumin as a surrogate TAA) were injected subcutaneously into naïve C57BL/6 (B6) mice. Fourteen days after tumor-cell injection, lymphocytes were isolated from the spleen, tumor, tumor-draining lymph nodes (DLNs) and non-draining lymph nodes (NDLN). CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were primarily detected within tumor lesions (66.7 ± 4.1% of CD8<sup>+</sup> T cells) rather than in the spleen of tumor-bearing mice (13.4 ± 1.1% of CD8<sup>+</sup> T cells, Fig. 1A), suggesting a tumor-associated accumulation of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells. The spleens of naïve mice contained fewer CD11a<sup>hi</sup>CD8<sup>+</sup> T cells than those of tumor-bearing mice (3.9 ± 0.4% of CD8<sup>+</sup> T cells, Fig. 1A). As shown in Figure S1, the subset of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells in both the spleen and tumor lesions also expressed high levels of CD18. In this setting, CD11a<sup>hi</sup>CD8<sup>+</sup> T cells did not express higher levels of CD11b and expressed slightly increased levels of CD11c than their CD11a<sup>lo</sup> counterparts (Fig. S1). These results suggest that CD11a<sup>hi</sup>CD8<sup>+</sup> T cells are a subset of CD8+ T cells exhibiting a selective increase in the expression of LFA-1 (CD11a/CD18) rather than other β2 integrins including macrophage 1 antigen (Mac1, CD11b/CD18) or p150/95 (CD11c/CD18). We investigated the expression levels of ICAM-1 (CD54), the LFA-1 ligand, in B16-OVA tumor cells and endothelial cells within neoplastic lesions. B16-OVA cells expressed modest levels of ICAM-1 before inoculation into mice, which did not change in vivo. Conversely, endothelial cells within the tumor lesions, identified by CD31 expression, expressed high ICAM-1 levels (Fig. S2).

We next assessed the antigen specificity and activation status of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells using the K<sup>+</sup>OVA<sub>257–264</sub> tetramer (K<sup>+</sup>OVA-tet) and an anti-CD69 antibody. Most tumor-infiltrating CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were CD69<sup>+</sup> (71.8%), 22.0% of which were also K<sup>+</sup>OVA-tet<sup>+</sup> (Fig. 1B). In comparison, fewer CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were CD69<sup>+</sup> (22.6%) in the spleen, of which <1% also were K<sup>+</sup>OVA-tet<sup>+</sup> (Fig. 1B). The TAA-specific CTL effector functions of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were analyzed by measuring degranulation (CD107a expression) and interferon (IFN)γ production following a brief re-stimulation ex vivo with an OVA-derived or control peptide. CD11a<sup>hi</sup>CD8<sup>+</sup> T cells, but not CD11a<sup>lo</sup>CD8<sup>+</sup> T cells, isolated from tumor lesions degranulated and secreted IFNγ in response to the OVA-derived peptide (Fig. 1C). In contrast, both CD11a<sup>hi</sup> and CD11a<sup>lo</sup>CD8<sup>+</sup> T cells isolated from the spleens of tumor-bearing mice exhibited poor CTL effector functions, as detected by IFNγ production and CD107a expression (Fig. 1C). Our results suggest that, in tumor-bearing mice, CD11a<sup>hi</sup>CD8<sup>+</sup> T cells mainly reside within neoplastic lesions and exert CTL effector functions in response to TAAs.

We next monitored the kinetics and distribution of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells in tumor-bearing hosts and determined that the functional (CD107a IFNγ+) CD11a<sup>lo</sup>CD8<sup>+</sup> T-cell population peaked in neoplastic lesions 14 d after tumor-cell injection, then progressively diminished over the next 7 d (Fig. 1D). Interestingly, functional intratumoral CD11a<sup>hi</sup>CD8<sup>+</sup> T cells increased in number thereafter as the tumor grew, until the mice were euthanized on day 28. In contrast, functional CD11a<sup>hi</sup>CD8<sup>+</sup> T cells were largely undetectable in the spleen, DLNs and NDLNs from day 7 through day 28 after tumor-cell injection. We conclude that CD11a<sup>hi</sup>CD8<sup>+</sup> T cells represent TAA-specific and tumor-reactive functional CTLs that infiltrate neoplastic lesions.

**An elevated expression of PD-1 by CD11a<sup>hi</sup>CD8<sup>+</sup> T cells compromises their ability to control tumor growth.** Although functional TAA-specific CD11a<sup>hi</sup>CD8<sup>+</sup> T cells can be identified within tumors, they are unable to control tumor growth. Such a compromised antitumor activity of CD11a<sup>hi</sup>CD8<sup>+</sup> T cells may be due to exhaustion, as triggered by chronic antigen exposure
PD-1 expression by CD11a\textsuperscript{high}\textsuperscript{CD8+} T cells negatively regulates endogenous antitumor immunity. To investigate whether the increased endogenous antitumor immunity observed in PD-1-deficient tumor-bearing mice is related to the presence of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells, we determined the frequency of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells in neoplastic lesions established in WT and PD-1-deficient mice 12 d after the inoculation of malignant cells (when tumor size is equivalent in WT and PD-1-deficient hosts). We found that the frequency of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells was 2–3-fold higher within tumors growing in PD-1-deficient mice than within lesions developing in WT mice (p = 0.003, Fig. 2D). Our results suggest that the capacity of tumor-reactive CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells to control tumor growth is compromised due to the persistent expression of PD-1.

Tumor-induced CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells are present at primary and metastatic tumor sites. We next wanted to extend our study and investigate the presence of tumor-reactive CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells in primary and metastatic tumor sites. To examine this possibility, we assayed CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells for the expression of PD-1, an immunoregulatory receptor expressed by exhausted and tumor-associated T cells. PD-1 expression was elevated on CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells isolated from neoplastic lesions 14 d after tumor-cell injection (70.6% of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells) as compared with CD11a\textsuperscript{low} CD8\textsuperscript{+} T cells (5.6% of CD11a\textsuperscript{low} CD8\textsuperscript{+} T cells, Fig. 2A). Intratumoral CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells maintained PD-1 expression until day 28, when mice were euthanized (Fig. 2B). In contrast, a low percentage of CD8\textsuperscript{+} T cells in the spleen, DLNs and NDLNs were PD-1\textsuperscript{+} CD11a\textsuperscript{high} upon tumor-cell injection. To test whether the early and persistent expression of PD-1 by intratumoral CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells would impair their antitumor activity, B16-OVA tumor cells were injected subcutaneously into naïve PD-1-deficient mice and tumor growth was monitored. As shown in Figure 2C, tumor growth was significantly delayed in PD-1-deficient mice as compared with wild type (WT) mice. In agreement with previous reports, our results indicate that PD-1 expression by CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells negatively regulates endogenous antitumor immunity.

To investigate whether the increased endogenous antitumor immunity observed in PD-1-deficient tumor-bearing mice is related to the presence of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells, we determined the frequency of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells in neoplastic lesions established in WT and PD-1-deficient mice 12 d after the inoculation of malignant cells (when tumor size is equivalent in WT and PD-1-deficient hosts). We found that the frequency of CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells was 2–3-fold higher within tumors growing in PD-1-deficient mice than within lesions developing in WT mice (p = 0.003, Fig. 2D). Our results suggest that the capacity of tumor-reactive CD11a\textsuperscript{high} CD8\textsuperscript{+} T cells to control tumor growth is compromised due to the persistent expression of PD-1.

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CD11a<sup>high</sup>CD8<sup>+</sup> T cells at metastatic tumor sites, using a tumor model with undefined TAAAs. We utilized the 4T1 mouse breast carcinoma cell line, which forms a solid primary tumor after subcutaneous inoculation or metastatic foci (preferentially in the lung) upon intravenous injection. We first investigated the presence of CD11a<sup>high</sup>CD8<sup>+</sup> T cells after subcutaneous inoculation of 4T1 tumor cells into naïve BALB/c mice. Similar to what we determined for B16-OVA tumors growing in C57BL/6 mice, CD11a<sup>high</sup>CD8<sup>+</sup> T cells were detectable within neoplastic lesions 7 d after the inoculation of 4T1 cells, reaching a maximum frequency on day 14 and declining thereafter (Fig. 3A). As with the B16-OVA system, CD11a<sup>high</sup>CD8<sup>+</sup> T cells continued to increase in frequency from day 21 to day 27, when mice were eventually euthanized (Fig. 3A). CD11a<sup>high</sup>CD8<sup>+</sup> T cells were detected only transiently in DLNs on day 21 after tumor-cell injection and were not appreciably detectable in the spleen, lung, NDLNs or peripheral blood (Fig. 3A). 4T1 tumor cells expressed modest levels of ICAM-1 before inoculation into mice, which modestly increased in vivo. Endothelial cells within neoplastic lesions, as identified by CD31 expression, exhibited high levels of ICAM-1 (Fig. S3). We went on to investigate whether CD8<sup>+</sup> T cells isolated from tumor lesions would manifest alterations in their adherence to ICAM-1 before inoculation into mice, which modestly increased in vivo. Endothelial cells within neoplastic lesions, as identified by CD31 expression, exhibited high levels of ICAM-1 (Fig. S3).

We went on to investigate whether CD8<sup>+</sup> T cells isolated from tumor lesions would manifest alterations in their adherence to ICAM-1 by performing an in vitro adhesion assay. To this aim, CD8<sup>+</sup> T cells were isolated from tumors 12–15 d after subcutaneous inoculation of 4T1 tumor cells. Additionally, we isolated CD8<sup>+</sup> T cells from the spleens of the same tumor-bearing mice. We performed the adhesion assay in the presence or absence of
100 ng/mL phorbol myristate acetate (PMA) to consider the impact of T-cell activation on adhesion. As shown in Figure S4, CD8+ T cells isolated from neoplastic lesions exhibited a decreased adhesion to ICAM-1 than CD8+ T cells isolated from spleens, both in the presence and in the absence of PMA. As expected, the addition of an anti-CD11a antibody to the system universally abrogated adhesion (Fig. S4). Our data demonstrates that even though a majority of CD8+ T cells isolated from neoplastic lesions express high levels of CD11a, their ability to adhere to ICAM-1 is impaired, presumably contributing to the failure of CD11a+CD8+ T cells to control tumor growth.

To investigate the presence of CD11a+CD8+ T cells at metastatic tumor sites, we analyzed lymphocytes isolated from multiple organs after the intravenous injection of 4T1 tumor cells. Seven days after 4T1 tumor-cell injection, the frequency of CD11a+CD8+ T cells was significantly increased in the lung (22.7% of CD8+ T cells, p < 0.01) and in the peripheral blood (3.9% of CD8+ T cells, p < 0.05), as compared with naïve mice, but remained unchanged in the spleen, liver and bone marrow (Fig. 3B). We observed the highest frequency of CD11a+CD8+ T cells in the livers of tumor-bearing and naïve mice, suggesting that not all liver-resident CD11a+CD8+ T cells are tumor-induced. As the liver is the main site for the deposition and depletion of endogenously activated CD8+ T cells,22,23 this finding corroborate the notion that CD11a+CD8+ T cells represent antigen-primed T cells.

We next wanted to check whether intratumoral CD11a+CD8+ T cells develop in situ, or rather are recruited to neoplastic lesions from lymphoid organs. To this aim, we simultaneously injected 4T1 tumor cells and FTY720, a molecule that inhibits the emigration of lymphocytes from lymphoid organs,24 then compared the frequency of CD11a+CD8+ T cells in the lungs of naïve and tumor-bearing mice. In naïve mice, FTY720 blocked the accumulation of CD11a+CD8+ T cells in the lung (7.1% of lung CD8+ T cells were CD11a+ vs. 17.6% in PBS-treated controls, *p < 0.05), this trend was also reflected in the absolute number of CD11a+CD8+ T cells that was detected in the lungs of FTY720-treated mice (2.8 ± 1.6 × 10^3 cells vs. 6.5 ± 1.2 × 10^3 cells in PBS-treated controls, p = 0.014, Fig. 4B). This suggests that CD11a+CD8+ T cells migrate to the lungs from lymphoid organs, while CD11a+CD8+ T cells represent a lung-resident T-cell population. In tumor-bearing mice, both the frequency (Fig. 4A) and the number (Fig. 4B) of CD11a+CD8+ T cells in the lung increased upon the injection of FTY720 (49.3 ± 13.9 × 10^3 cells vs. 12.6 ± 2.2 × 10^3 cells in PBS-treated controls, p = 0.013), while there was a significant decrease in CD11alowCD8+ T cells (12.6 ± 4.2 × 10^3 cells vs. 27.8 ± 10.1 × 10^3 cells in PBS-treated controls, p = 0.023). Since the increase of the CD11a+CD8+ T-cell population in tumor-bearing mice receiving FTY720 could not be due to the migration of these cells from lymphoid organs, these findings suggest that within the lung tissue CD11a+CD8+ T cells are induced by tumor cells to become antigen-experienced CD11a+CD8+ T cells.

We next asked if the priming of CD11a+CD8+ T cells in situ required only the presence of TAAs or if the active infiltration of tumor cells was necessary. To investigate this issue, we lethally irradiated 4T1 tumor cells and then injected them into naïve mice. Seven days after the inoculation of irradiated 4T1 tumor cells, we did not detect an increase in the frequency (Fig. 5A) or absolute cell number (Fig. 5B) of CD11a+CD8+ T cells in whole lung, spleen, liver, bone marrow and peripheral blood of irradiated mice. As expected, the addition of an anti-CD11a antibody to the system universally abrogated adhesion (Fig. S4). Our data demonstrates that even though a majority of CD8+ T cells isolated from neoplastic lesions express high levels of CD11a, their ability to adhere to ICAM-1 is impaired, presumably contributing to the failure of CD11a+CD8+ T cells to control tumor growth.
the subcutaneous injection of tumor cells, CD11ahigh CD8+ T cells isolated from the spleen and neoplastic lesions produced IFN-γ, yet failed to degranulate or produce granzyme B (data not shown). This indicates that—despite their differentiated effector cell and proliferative phenotype—CD11ahigh CD8+ T cells lack robust CTL effector functions.

CD11ahigh CD8+ T cells are induced by spontaneous tumors. Although the expression levels of CD11a in CD8+ T cells are not affected by inflammation,12 in our studies, the injection of tumor cells may have caused an acute CD8+ T-cell response due to the sudden exposure to large TAA amounts. To get further insights into this issue, we investigated the accumulation of CD11ahigh CD8+ T cells within spontaneous tumors, which provide a model for chronic and persistent antigen exposure. To this aim, we isolated lymphocytes from female BALB-neuT mice (which transgenically express the activated HER-2/neu oncogene29) aged 15–18 weeks (that is, when mammary glands display visible invasive carcinomas).30 In this setting, we detected CD11a high CD8+ T cells within neoplastic lesions but not in the spleen (Fig. 7A).

Intratumoral CD11ahigh CD8+ T cells exhibited an effector phenotype (CD69high PD-1high) (Fig. 7B). Nevertheless, CD11ahigh CD8+ T cells isolated from spontaneous neoplastic lesions lacked CTL effector functions, as indicated by the lack of CD107a expression and limited production of IFN-γ upon stimulation with PMA/ionomycin ex vivo (Fig. 7B). Furthermore, CTL effector functions could not be induced by a high affinity Neu-derived peptide (p66)30 (data not shown). The dysfunction of the CD11ahigh CD8+ T-cell population infiltrating spontaneous breast carcinomas is consistent with their failure to control tumor growth.

Tumor-reactive PD-1+ CD11ahigh CD8+ T cells are detectable in the blood of melanoma patients. To test whether...
CD11a<sup>high</sup>CD8<sup>+</sup> T cells represent tumor-specific CD8<sup>+</sup> T cells in cancer patients, we analyzed CD8<sup>+</sup> T cells from the peripheral blood of 10 patients affected by Stage IV melanoma. We used the HLA-A2/MART-1 tetramer (MART-1-tet) to define tumor-specific CD8<sup>+</sup> T cells. The CD11a<sup>high</sup> subset of CD8<sup>+</sup> T cells contained a significantly higher frequency of MART-1-tet<sup>-</sup> T cells as compared with the CD11a<sup>low</sup>CD8<sup>+</sup> subset (p = 0.001, Fig. 8A and B), suggesting that CD11a<sup>high</sup>CD8<sup>+</sup> T cells represent tumor-specific CD8<sup>+</sup> T cells. The CD11a<sup>high</sup>CD8<sup>+</sup> T-cell compartment of these patients expressed elevated levels of PD-1 (but not of the other prominent immunoregulatory receptor CTLA-4) compared with CD11a<sup>low</sup>CD8<sup>+</sup> T cells (Fig. 8C). The frequency of PD-1<sup>-</sup>CD11a<sup>high</sup>CD8<sup>+</sup> T cells was significantly increased in the peripheral blood of the cancer patients (18.7 ± 1.8%) as compared with healthy donors (1.7 ± 0.4%, p < 0.0001, Fig. 8D). When we used a CD11a antibody that specifically recognizes a unique epitope of CD11a implicated in the activation of LFA-1 complex (MEM-83), we identified a similarly increased population of PD-1<sup>-</sup>CD11a<sup>high</sup>CD8<sup>+</sup> T cells in these melanoma patients (data not shown), suggesting that this population of CD8<sup>+</sup> T cells is TAA-primed. Our results suggest that most tumor-specific CD8<sup>+</sup> T cells of advanced melanoma patients are CD11a<sup>high</sup>CD8<sup>+</sup> T cells that co-express elevated levels of PD-1, presumably accounting for the dysfunctional state of tumor-reactive CD8<sup>+</sup> T cells that normally characterizes these patients.

**Discussion**

Our results demonstrate that upregulation of CD11a by CD8<sup>+</sup> T cells can be used as a marker to track endogenous tumor-reactive CD8<sup>+</sup> T cells. CD11a<sup>high</sup>CD8<sup>+</sup> T cells exist within primary and metastatic neoplastic lesions that develop after tumor-cell inoculation, as well as within spontaneously arising tumors. We also detected TAA-specific CD11a<sup>high</sup>CD8<sup>+</sup> T cells in the peripheral blood of advanced-stage melanoma patients. Although CD11a<sup>high</sup>CD8<sup>+</sup> T cells are antigen-primed, proliferating effector cells, they are unable to control tumor growth. The elevated expression of the immunoregulatory receptor PD-1 by tumor reactive CD11a<sup>high</sup>CD8<sup>+</sup> T cells may provide an important inhibitory influence to the effector functions of these cells. The role of PD-1 in limiting endogenous antitumor immunity was demonstrated by the significant delay in tumor growth observed in PD-1-deficient mice as compared with their WT counterparts. Thus, CD11a<sup>high</sup>CD8<sup>+</sup> T cells comprise a population of endogenous tumor-reactive effector T cells that are sensitive to PD-1-mediated immunosuppression.

The engagement of the TCR upon antigen stimulation triggers an inside-out signaling cascade that promotes CD11a expression and increases its affinity for ICAM-1. These events lead to the formation of the so-called “peripheral supramolecular activation cluster” (p-SMAC) in the immune synapse, allowing for stable interactions between T cells and antigen-presenting cells. In addition, CD11a is essential for CD8<sup>+</sup> T cells to penetrate into tissues and kill target cells. Studies in T cells and NK cells have revealed that LFA-1 (CD11a/CD18) delivers outside-in signaling upon recognition of ICAM-1. This signaling is critical for the initiation of T-cell and NK cell cytotoxicity, leading to actin polymerization and granule polarization. In addition, LFA-1 signaling stimulates the production of tumor necrosis factor α (TNFα) by activated NK cells, as well as T-cell activation and survival. Here, we found that tumor-reactive CD8<sup>+</sup> T cells express increased levels of CD11a, suggesting an endogenous...
activation of CD8+ T cells by TAAs and an increased potential of CD8+ T cells to receive co-stimulatory signals from LFA-1. Moreover, the co-expression of CD11a and PD-1 by CD8+ T cells suggests that tumor-reactive effector T cells are balanced by both positive (LFA-1) and negative (PD-1) regulatory signals.

High levels of CD11a expression are maintained for long periods of time upon antigen exposure.12–14 As naïve CD8+ T cells express low levels of CD11a, CD11a upregulation clearly specifically characterize antigen-primed effector and memory CD8+ T cells,12 CD44 and CD69 are also used as markers for T-cell activation, but these molecules are not implicated in CTL function. An advantage of using CD11a over MHC Class I tetramers as a marker for antigen-specific CD8+ T cells is that the immunostaining of CD11a is compatible with fixation and permeabilization procedures for the cytfluorometric detection of intracellular cytokines and other signaling molecules.36 In contrast, the binding of MHC Class I tetramers to CD8+ T cells is dramatically decreased following these harsh procedures (data not shown). In this study, we demonstrated that tumor-induced CD11ahigh CD8+ T cells are differentiated effector cells, as they downregulate CD62L and exhibit an increased expression of T-bet (Fig. 6), a master transcriptional regulator for TH1 and CTL differentiation.37 Functional assays showed that CD11ahigh CD8+ T cells are potentially functional CTLs, as they can be induced to undergo degranulation and/or produce IFNγ upon brief stimulation with antigenic peptides or PMA/ionomycin ex vivo (Fig. 1; Fig. 7; Fig. S5).

The detection of an endogenous, differentiated, proliferative effector population of CD11ahigh CD8+ T cells infiltrating tumor lesions prompted us to investigate where this effector T-cell population originated. CD11ahigh CD8+ T cells could either develop within the tumor or represent T cells that migrate to the tumor upon activation in lymph nodes. Since CD11a is involved in the extravasation of CD8+ T cells into ICAM-1-expressing tissues, it seems plausible that the CD11ahigh CD8+ T cells detected within neoplastic lesions that express ICAM-1 (Fig. S2 and Fig. S3) migrated to the tumor tissue upon activation in local lymph nodes. We performed immunofluorescence staining of tumor tissues to identify the extravasation of CD11ahigh CD8+ T cells. We identified CD11a high CD8+ T cells within neoplastic lesions (data not shown) and observed some CD8+ T cells that were in close contact with claudin-5+ endothelial cells in the tumor vasculature (data not shown). As claudin-5 is a transmembrane protein associated with tight junctions in blood vessels, our data suggests that these CD8+ T cells may be in the process of extravasation via firm adhesions with endothelial cells that express ICAM-1.

We further investigated the migration of CD11ahigh CD8+ T cells using FTY720, which blocks the emigration of lymphocytes from DLNs. In naïve mice, FTY720 blocked the accumulation of CD11ahigh CD8+ T cells within the lungs (Fig. 4), indicating that in untreated mice this cell population migrates to the lung tissue. Interestingly, we found that in tumor-bearing mice the accumulation of CD11ahigh CD8+ T cells within neoplastic lesions (data not shown) and observed some CD8+ T cells that were in close contact with claudin-5+ endothelial cells in the tumor vasculature (data not shown). As claudin-5 is a transmembrane protein associated with tight junctions in blood vessels, our data suggests that these CD8+ T cells may be in the process of extravasation via firm adhesions with endothelial cells that express ICAM-1.

Figure 6. Phenotype of tumor-induced CD11ahigh CD8+ T cells. (A and B) Lymphocytes were isolated from the lungs of mice 7 d after the intravenous injection of 4T1 tumor cells. (A) Proliferation of CD11ahigh CD8+ T cells was measured by increased forward light scatter (FSC) and intranuclear expression of Ki67. (B) Surface expression of CD62L, CD69 and PD-1 and intracellular expression of T-bet by CD11ahigh and CD11alow CD8+ T cells. Results from one of three independent experiments are shown.
fully differentiated effectors when cognate antigens are present. How a sterile tumor microenvironment can prime naive CD8+ T cells to differentiate into activated effector cells remains an open question. Recent reports describe a mechanism whereby the Toll-like receptor (TLR)-independent recognition of spontaneous tumor cell death by the innate immune system leads to IFNβ production, allowing intratumoral CD8α+ dendritic cells to prime naive CD8+ T-cell responses against TAAs. This process presumably occurs in our models as well, resulting in the accumulation of endogenous tumor-reactive CD11a$^+$CD8+ T cells.

Upon activation in tumor lesions, CD11a$^+$CD8+ T cells are proliferative, differentiated effector cells, yet they are unable to control tumor growth. We propose that the failure of antitumor immunity in our system is not due to the absence of tumor reactive CD8+ T cells, but rather to the fact that CD11a$^+$CD8+ T cells express high levels of PD-1. The detection of PD-1+ tumor-reactive CD8+ T cells is consistent with previous results, demonstrating the presence of PD-1+CD8+ T cells within neoplastic lesions and in the peripheral blood. We found that CD11a$^+$CD8+ T cells express significantly lower levels of PD-1 than their CD11a$^+$ counterparts (Fig. 6B), suggesting that TAA-driven T-cell activation might be a prerequisite for the upregulation of PD-1 on CD8+ T cells. In support of this idea, we demonstrated that CD11a$^+$CD8+ T cells that express PD-1 also express CD69 and Ki67, validating the activation status of these cells (Fig. 6). Moreover, we found that the growth of B16 tumors is significantly delayed in PD-1-deficient mice (Fig. 2A). Other groups have made similar observations using various distinct tumor models, and PD-1 also appears to limit the adhesion capacity of CD8+ T cells. Indeed, CD8+ T cells isolated from neoplastic lesions exhibited an impaired adhesion to recombiant ICAM-1 in vitro as compared with CD8+ T cells isolated from the spleens of the same mice, both in the presence and in the absence of PMA (Fig. S4). PMA induces T-cell adhesion by directly activating protein kinase C (PKC). The compromised adhesive capacity of CD8+ T cells isolated from the tumor tissue could be due to their elevated expression levels of PD-1, as PD-1 signaling is known to inhibit the activation of PKC.

Altogether, our data indicate that the early signals leading to the activation and differentiation of tumor-reactive CD8+ T cells are not impaired by PD-1-transduced signals. However, as the tumor grows, the expression of PD-1 ligand 1 (PD-L1, also known as B7-H1) by tumor cells increases, resulting in the engagement of PD-1 on CD8+ T cells and hence in the transmission of strong immunosuppressive signals. Therefore, a strategy for improving antitumor immunity may be to restore the function of tumor-reactive CD8+ T cells, rather than merely increase their abundance. In support of this idea, Phase I clinical trials involving the administration of anti-PD-1 and anti-PD-L1 antibodies to cancer patients have provided encouraging results.

Recent clinical trials involving the blockade of immune checkpoints by means of antibodies that modulate the PD-1/PD-L1 signaling pathway underscore the importance of endogenous antitumor T-cell immunity. In some patients undergoing anti-PD-1 therapy, durable responses including prolonged tumor regression upon the discontinuation of therapy have been observed, indicating the re-emergence of antitumor memory T-cell responses. Anti-PD-1/PD-L1 therapies mainly affect tumor-T-cell interactions within neoplastic lesions, but the appearance of PD-1+CD11a$^+$CD8+ T cells in the blood of melanoma patients (Fig. 8) indicates a new potential target for these approaches. We have previously reported that the PD-1/PD-L1 pathway limits the entry of effector CD8+ T cells...
be useful for identifying and tracking endogenous tumor-reactive CTLs in future studies.

Materials and Methods

Mice, cell lines and reagents. Female C57BL/6 and BALB/c mice were purchased from Taconic Farms. PD-1 knockout (Pdcd1<sup>−/−</sup>) C57BL/6 mice were provided by L. Chen (Yale University) with the permission of Dr. T. Honjo (Kyoto University). Mice were maintained under pathogen-free conditions and used at 8–12 weeks of age. Tumor lesions and spleen samples from BALB-neuT mice were provided by L. Pease (Mayo Clinic). B16-OVA murine melanoma cells were provided by R. Vile (Mayo Clinic). 4T1 tumor cells were purchased from ATCC and cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1 U/mL penicillin, 1 μg/mL streptomycin and 20 mM HEPES buffer (all from Mediatech). FTY720 was purchased from Cayman Chemical. PMA and ionomycin were purchased from Sigma-Aldrich. Studies were conducted in accordance with the National Institutes of Health guidelines for the proper use of animals in research and local Institutional Animal Care and Use Committee approval.

Flow cytometry analysis. Class I MHC (K<sup>b</sup>OVA peptide SIINFEKL) tetramer and control tetramer (mock-loaded) were
purchased from Beckman Coulter. Fluorochrome-conjugated antibodies against mouse CD3, CD8, CD11a (M17/4), CD11b, CD11c, CD18, CD31, CD54, CD69, PD-1 (RMP1-30), CD62L, T-bet, Foxp3, Ki67, CD107a and IFNγ were purchased from BD Biosciences, BioLegend or eBioscience. To detect intracellular IFNγ levels, cells were incubated with the GolgiPlug reagent (BD Biosciences) for 4 h prior to analysis. Cells were stained for surface antigens and then incubated in Fixation Buffer (BioLegend) for 20 min at room temperature, followed by permeabilization by means of the Permeabilization Wash Buffer (BioLegend). To detect the intranuclear levels of Ki67 and T-bet, T cells were first stained for surface antigens (CD8 and CD11a), fixed and then permeabilized using the Foxp3 buffer kit, according to the manufacturer's protocol (eBiosoScience). Upon staining, cells were washed three times with washing buffer before analysis. At least 100,000 viable cells were live gated on a FACScan or FACSCalibur instrumentation (BD Biosciences). Flow cytometry analysis was performed using the FlowJo software (Tree Star).

**Cytotoxic T lymphocyte (CTL) functional assays.** The degranulation of CTLs was measured by CD107a mobilization\(^9\) followed by intracellular staining for IFNγ. Briefly, lymphocytes were incubated with 1 \(\mu\)g/mL OVA\(_{257-264}\) peptide (Mayo Clinic Peptide Core) or 50 ng/mL PMA plus 500 ng/mL ionomycin for 4 h in the presence of anti-CD107a antibodies. After incubation, cells were stained for surface CD8 and CD11a, followed by intracellular staining for IFNγ.

**T-cell adhesion assays.** CD8+ T cells purified from neoplastic lesions or spleens were labeled with calcine acetoxyethyl ester and added to 96-well flat bottom plates (Costar) pre-coated with a murine recombinant ICAM-1/Fc chimera (R&D Systems) at 0.6 \(\mu\)g/well, as previously reported.\(^{21}\) Blocking anti-CD11a antibodies (clone M17/4, Biolegend) were added when appropriate at a final concentration of 10 \(\mu\)g/mL. T cells were activated with 100 ng/mL PMA for 10 min at 37°C, and non-adherent cells were removed by washing. The adherence of adherent T cells was measured on a fluorescence microplate reader (excitation wavelength = 485 nm; emission wavelength = 538 nm). The percent of adhesion was calculated as: (OD of test well/OD of input well) \(\times 100\).

**Tumor studies.** WT and Pdcd1\(^{-/-}\) C57BL/6 mice were inoculated s.c. with 5 \(\times 10^5\) B16-OVA tumor cells, BALB/c mice were inoculated s.c. or i.v. with 1 \(\times 10^4\) 4T1 tumor cells. A caliper was used to measure the size of tumors twice a week. Some mice were injected i.p. with 1 mg/Kg FTY720 every 2 d over a week. On indicated days after tumor injection, tumor tissues and lymphoid organs were removed and incubated in digestion buffer (RPMI medium supplemented with 5% FBS, 0.02% collagenase IV, 0.002% DNase I and 10 U/mL heparin) for 40 min, followed by the isolation of lymphocytes.

**Detection of melanoma specific human CD8+ T cells.** Peripheral blood mononuclear cells (PBMC) samples were collected from HLA-A2+ patients affected by Stage IV melanoma according to a protocol approved by the Institutional Review Board at Mayo Clinic. Cells were stained with antibodies specific for CD8, CD11a (TS2/4, or HI111), PD-1 (EH12.2H7, J105) and CTLA-4 (BioLegend, BD Bioscience and eBioscience, respectively). An antibody that recognizes a unique epitope of CD11a implicated in the activation of the LFA-1 complex (MEM-83, Novus), was also included in the study. To detect melanoma antigen-specific CD8+ T cells, PBMCs were stained with a HLA-A2/MART-1 tetramer (Beckman Coulter).

**Statistical analyses.** All statistical analyses were performed using the Prism software package v. 5.0 (GraphPad Software, Inc.). Two-sided, unpaired or paired Student’s t-tests were used to assess the statistical significance of differences between experimental groups. A p value < 0.05 was considered as statistically significant.

**Disclosure of Potential Conflicts of Interest**

H.D., S.N.M, C.J.K. and E.D.K. have filed a patent application for the use of PD-1/CD11a as a predictive T-cell biomarker for the assessment of cancer immunotherapies. The other authors have no financial conflicts of interest.

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**Supplemental Materials**

Supplemental materials may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/23972
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