RESEARCH PAPER

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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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Immunotherapies aimed at enhancing natural or endogenous antitumor T-cell immunity in patients affected by advanced malignancies are currently being implemented in the clinic with promising results. In order to optimize 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^{high} CD8+ T cells that co-expressed high levels of PD-1. Healthy donors exhibited a comparatively much lower frequency of such PD-1+CD11a^{high}CD8+ T cells. Phenotypic analyses demonstrated that CD11a^{high}CD8+ T cells are proliferating (Ki67+) and activated (CD62L-CD69+). Increased CD11a^{high}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^{high}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.

Introduction

Endogenous or naturally occurring antitumor immunity is difficult to detect and monitor,[1] 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.[2] 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.[3] 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.[4–6] In order to optimize the therapeutic protocols and monitor the effectiveness of such therapies within neoplastic lesions as well as in the peripheral blood and lymphoid tissues, reliable T-cell markers are needed. Ideally, such a marker would be stably expressed on CD8+ T cells, discriminate tumor-reactive CD8+ T cells from populations of tumor-associated or tumor-infiltrating T cells and be antigen-independent. CD11a [also known as lymphocyte functional-associated antigen 1, α chain (LFA-1α)] is an integrin expressed

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by cytotoxic T cells that binds to CD54 [intercellular adhesion molecule 1 (ICAM-1)] and hence functions as an adhesion molecule.[7] CD11a is indispensable in mediating the conjugation between cytotoxic T lymphocytes (CTLs) and target cells.[8] Blocking CD11a dramatically reduces the killing of tumor cells and rejection of transplants by CTLs,[9,10] and CD11a-deficient mice exhibit defects in rejecting immunogenic tumors.[11] Rai 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.[12] Importantly, CD11a identifies effector and memory $CD8^+$ T cells independent of antigen specificity.[12–14] Murine $CD11a^{high}CD8^+$ T cells include memory $CD8^+$ T cells capable of mounting vigorous recall responses upon a secondary antigenic challenge.[12] 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.[13,14] The utility of CD11a for studying antigen-primed $CD8^+$ T cells is demonstrated by the fact that only a portion of $CD11a^{high}CD8^+$ T cells can be detected by MHC Class I tetramer staining, while all corresponding antigen-specific $CD8^+$ T cells reside within the $CD11a^{high}CD8^+$ T-cell population.[14] The applicability of the $CD11a^{high}$ phenotype for detecting, measuring and tracking TAA-reactive $CD8^+$ T cells has not yet been established.

In this study, we identified $CD11a^{high}CD8^+$ 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 $CD11a^{high}$. $CD11a^{high}CD8^+$ T cells were proliferating ($Ki67^+$) and activated ($CD62L^-CD69^+$) 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 $CD11a^{high}CD8^+$ 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

$CD11a^{high}CD8^+$ T cells comprise an antitumor T-cell population. We monitored the presence of $CD11a^{high}CD8^+$ 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 (NDLNs). $CD11a^{high}CD8^+$ T cells were primarily detected within tumor lesions (66.7 ± 4.1% of $CD8^+$ T cells) rather than in the spleen of tumor-bearing mice (13.4 ± 1.1% of $CD8^+$ T cells, **Fig. 1A**), suggesting a tumor-associated accumulation of $CD11a^{high}CD8^+$ T cells. The spleens of naïve mice contained fewer $CD11a^{high}CD8^+$ T cells than those of tumor-bearing mice (3.9 ± 0.4% of $CD8^+$ T cells, **Fig. 1A**). As shown in **Figure S1**, the subset of $CD11a^{high}CD8^+$ T cells in both the spleen and tumor lesions also expressed high levels of CD18. In this setting, $CD11a^{high}CD8^+$ T cells did not express higher levels of CD11b and expressed slightly increased levels of CD11c than their $CD11a^{low}$ counterparts (**Fig. S1**). These results suggest that $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells using the $K^bOVA_{257–264}$ tetramer (K^bOVA-tet) and an anti-CD69 antibody. Most tumor-infiltrating $CD11a^{high}CD8^+$ T cells were $CD69^+$ (71.8%), 22.0% of which were also K^bOVA-tet^+ (**Fig. 1B**). In comparison, fewer $CD11a^{high}CD8^+$ T cells were $CD69^+$ (22.6%) in the spleen, of which < 1% also were K^bOVA-tet^+ (**Fig. 1B**). The TAA-specific CTL effector functions of $CD11a^{high}CD8^+$ 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. $CD11a^{high}CD8^+$ T cells, but not $CD11a^{low}CD8^+$ T cells, isolated from tumor lesions degranulated and secreted IFNγ in response to the OVA-derived peptide (**Fig. 1C**). In contrast, both $CD11a^{high}$ and $CD11a^{low}CD8^+$ 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, $CD11a^{high}CD8^+$ T cells mainly reside within neoplastic lesions and exert CTL effector functions in response to TAAs.

We next monitored the kinetics and distribution of $CD11a^{high}CD8^+$ T cells in tumor-bearing hosts and determined that the functional ($CD107a^+IFN\gamma^+$) $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells increased in number thereafter as the tumor grew, until the mice were euthanized on day 28. In contrast, functional $CD11a^{high}CD8^+$ T cells were largely undetectable in the spleen, DLNs and NDLNs from day 7 through day 28 after tumor-cell injection. We conclude that $CD11a^{high}CD8^+$ T cells represent TAA-specific and tumor-reactive functional CTLs that infiltrate neoplastic lesions.

An elevated expression of PD-1 by $CD11a^{high}CD8^+$ T cells compromises their ability to control tumor growth. Although functional TAA-specific $CD11a^{high}CD8^+$ T cells can be identified within tumors, they are unable to control tumor growth. Such a compromised antitumor activity of $CD11a^{high}CD8^+$ T cells may be due to exhaustion, as triggered by chronic antigen exposure

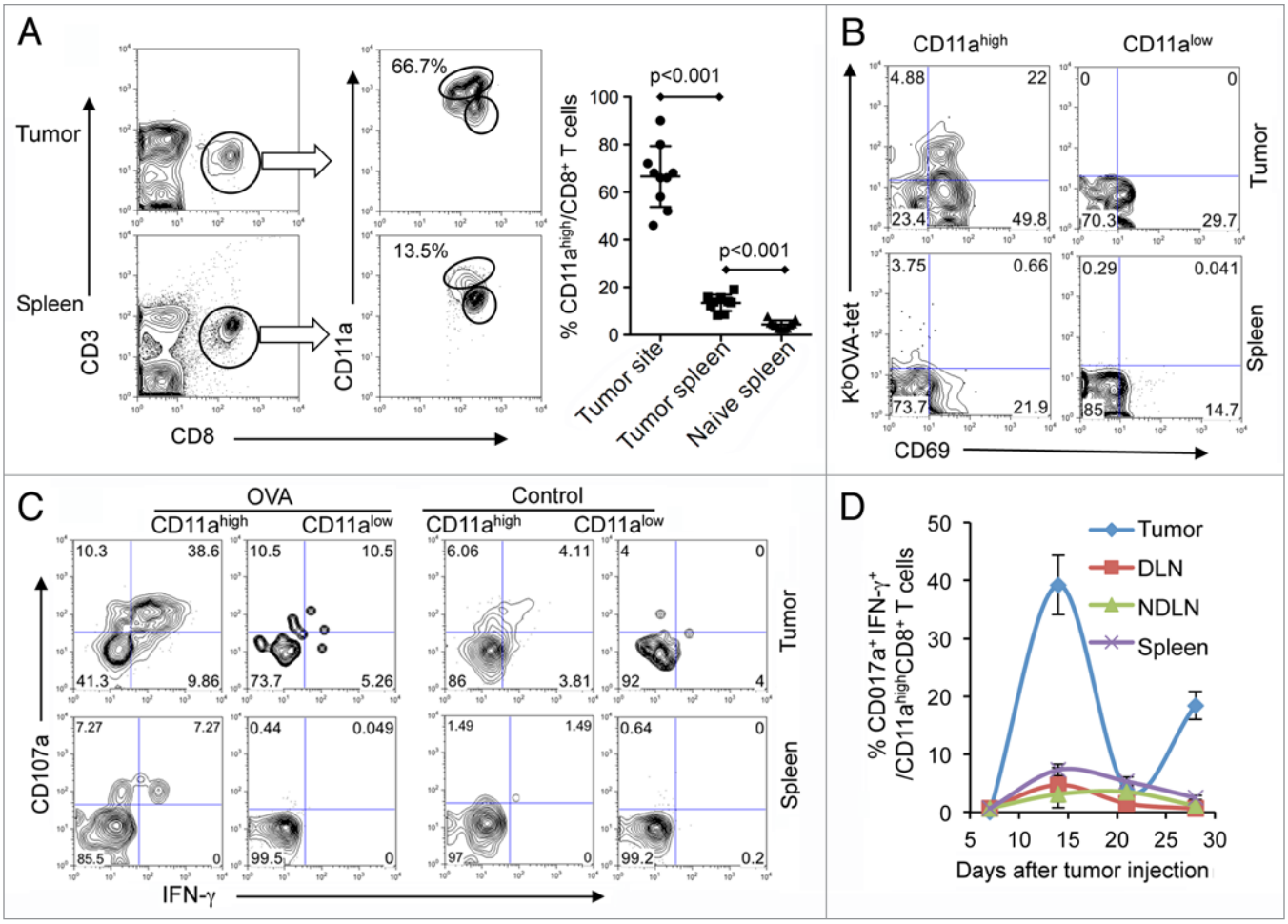

Figure 1. Endogenous tumor-specific CD8⁺ T cells in neoplastic lesions. (**A–D**) B16-OVA tumor cells were subcutaneously injected into naïve C57BL/6 mice. (**A**) On day 14 after tumor-cell injection, lymphocytes were isolated from neoplastic lesions and spleens and were stained with antibodies specific for CD3, CD8, CD11a, CD69 as well as with a K^bOVA tetramer (K^bOVA-tet). The frequency of $CD11a^{high}$ cells among CD8⁺ T cells in the tumor tissue and spleens of tumor-bearing or naïve mice is reported (n = 10). (**B**) Antigen specificity of $CD11a^{high}$ and $CD11a^{low}CD8^+$ T cells. (**C**) Cytotoxic T lymphocyte (CTL) activity of $CD11a^{high}$ and $CD11a^{low}CD8^+$ T cells following re-stimulation with OVA or control peptide for 5 h ex vivo. One of three independent experiments is shown. (**D**) Kinetics and distribution of functional $CD11a^{high}CD8^+$ T cells in tumor tissues, draining lymph nodes (DLNs), non-draining lymph nodes (NDLNs) and spleen. The average percentage ± SD of $CD107a^+IFN\gamma^+$ cells among $CD11a^{high}$ $CD8^+$ T cells is shown (n = 3).

within the tumor site.[15] To examine this possibility, we assayed $CD11a^{high}CD8^+$ T cells for the expression of PD-1, an immunoregulatory receptor expressed by exhausted and tumor-associated T cells.[16–20] PD-1 expression was elevated on $CD11a^{high}CD8^+$ T cells isolated from neoplastic lesions 14 d after tumor-cell injection (70.6% of $CD11a^{high}$ $CD8^+$ T cells) as compared with $CD11a^{low}CD8^+$ T cells (5.6% of $CD11a^{low}$ $CD8^+$ T cells, **Fig. 2A**). Intratumoral $CD11a^{high}CD8^+$ T cells maintained PD-1 expression until day 28, when mice were euthanized (**Fig. 2B**). In contrast, a low percentage of CD8⁺ T cells in the spleen, DLNs and NDLNs were $PD\text{-}1^+CD11a^{high}$ upon tumor-cell injection. To test whether the early and persistent expression of PD-1 by intratumoral $CD11a^{high}CD8^+$ 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,[18,19] our results indicate that PD-1 expression by $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells, we determined the frequency of $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells to control tumor growth is compromised due to the persistent expression of PD-1.

Tumor-induced $CD11a^{high}CD8^+$ T cells are present at primary and metastatic tumor sites. We next wanted to extend our study and investigate the presence of tumor-reactive

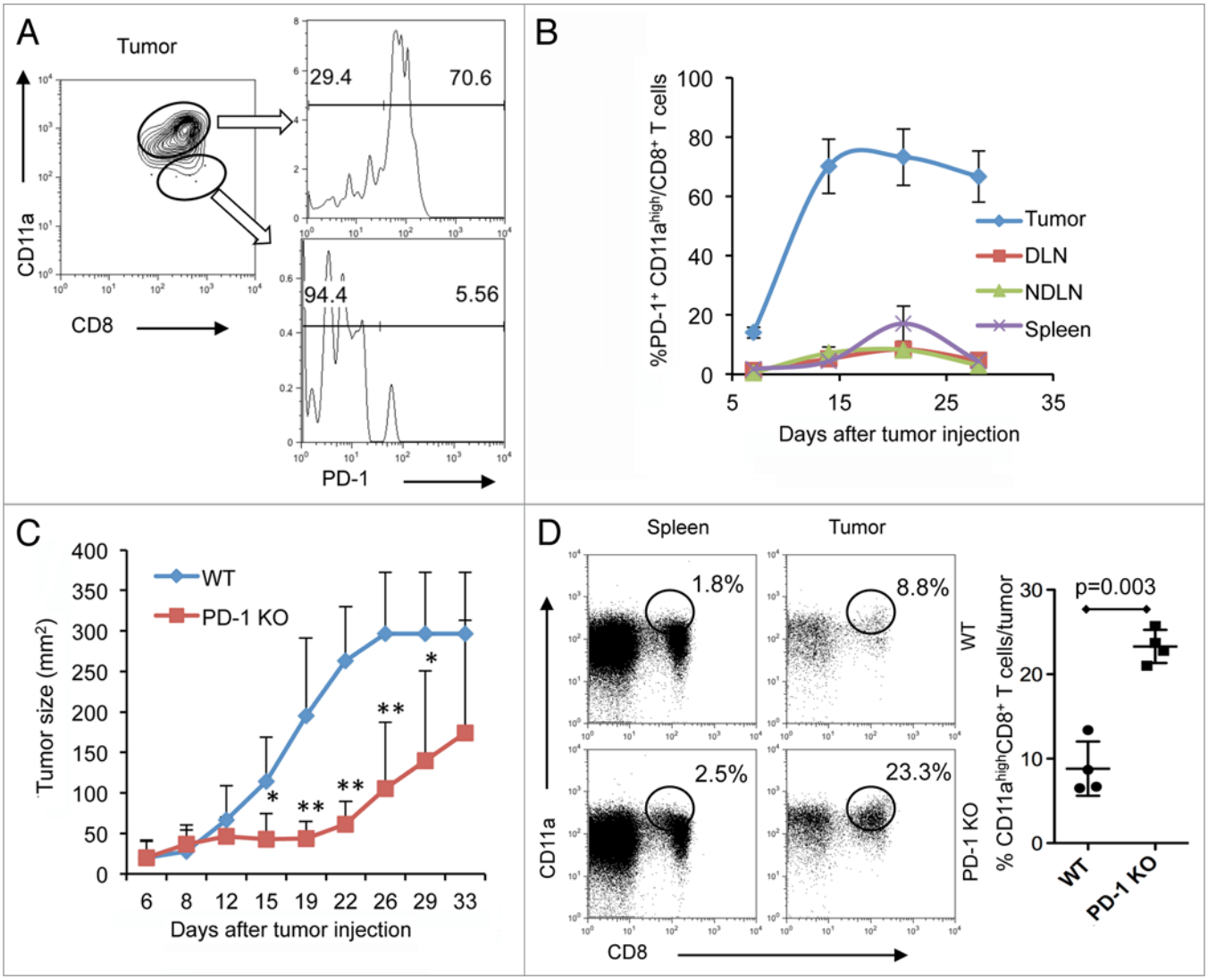

Figure 2. PD-1 limits endogenous antitumor immunity. (**A and B**) B16-OVA tumor cells were subcutaneously injected into naïve C57BL/6 mice. (**A**) PD-1 expression by CD11a^{high}CD8^{+} T cells isolated from B16-OVA tumors 7 d after tumor-cell injection. (**B**) Kinetics and distribution of PD-1^{+}CD11a^{high}CD8^{+} T cells in tumor tissues, draining lymph nodes (DLNs), non-draining lymph nodes (NDLNs) and spleen. The frequency of PD-1^{+}CD11a^{high} cells among CD8^{+} T cells in the tumor tissue, spleen, DLNs and NDLNs of tumor-bearing mice is shown (n = 3). (**C and D**) B16-OVA tumor cells were subcutaneously injected into naïve wild-type (WT) and PD-1-deficient (KO) mice. (**C**) Mean size of tumors plus SD (n = 5). *p < 0.05, **p < 0.01, as compared with tumors developing in WT mice. (**D**) Frequency of CD11a^{high}CD8^{+} T cells in the tumor tissue and spleen 12 d after tumor-cell injection. The average percentage ± SD of CD11a^{high}CD8^{+} T cells in tumor tissues is reported (n = 4).

CD11a^{high}CD8^{+} T cells at metastatic tumor sites, using a tumor model with undefined TAAs. 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^{high}CD8^{+} 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^{high}CD8^{+} 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^{high}CD8^{+} T cells continued to increase in frequency from day 21 to day 27, when mice were eventually euthanized (**Fig. 3A**). CD11a^{high}CD8^{+} 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^{+} T cells isolated from tumor lesions would manifest alterations in their adherence to ICAM-1 by performing an in vitro adhesion assay.[21] To this aim, CD8^{+} T cells were isolated from tumors 12–15 d after the subcutaneous inoculation of 4T1 tumor cells. Additionally, we isolated CD8^{+} 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 $CD11a^{high}CD8^+$ T cells to control tumor growth.

To investigate the presence of $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells in the livers of tumor-bearing and naïve mice, suggesting that not all liver-resident $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells represent antigen-primed T cells.

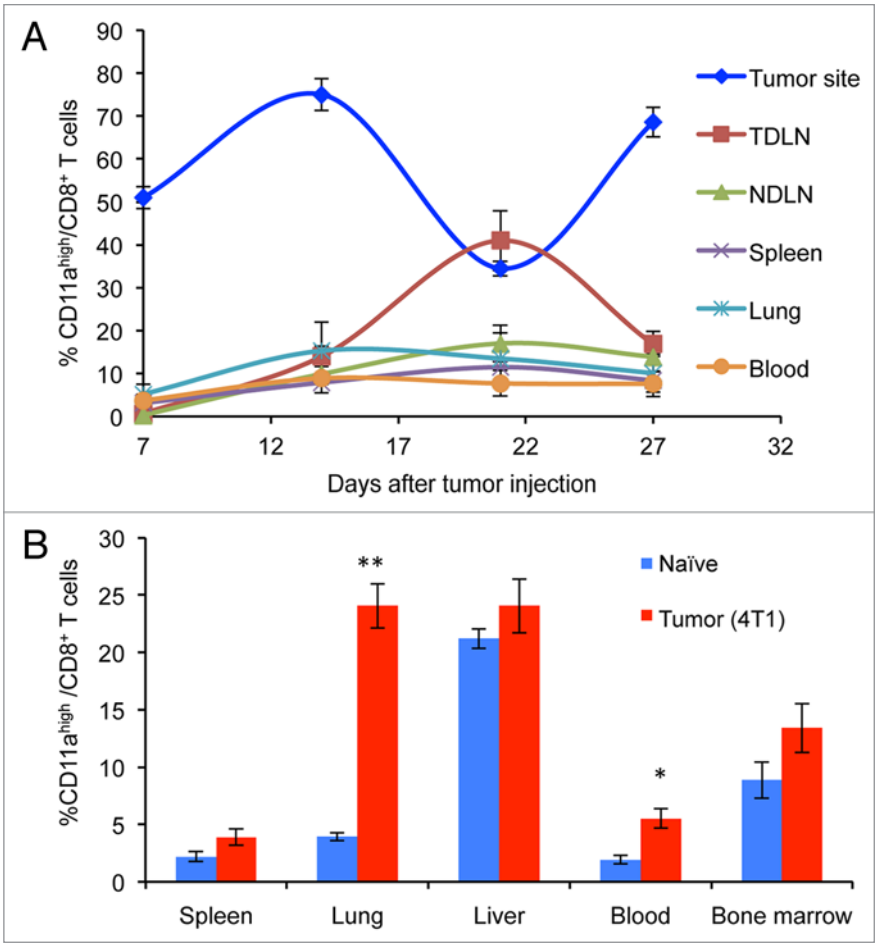

Figure 3. $CD11a^{high}CD8^+$ T cells identified at primary and metastatic tumor sites. (**A and B**) 4T1 mouse breast tumor cells (1×10^5) were injected subcutaneously (**A**) or intravenously (**B**) into BALB/c mice. (**A**) Kinetics and distribution of $CD11a^{high}CD8^+$ T cells in multiple tissues of mice with subcutaneously implanted 4T1 tumors. The average percentage ± SD of $CD11a^{high}$ cells among $CD8^+$ T cells is shown (n = 3). (**B**) Distribution of $CD11a^{high}CD8^+$ T cells in multiple tissues of naïve mice and mice intravenously administered with 4T1 tumors 7 d after tumor-cell injection. The average percentage ± SD of $CD11a^{high}$ cells among $CD8^+$ T cells is shown (n = 3). *$p < 0.05$, **$p < 0.01$, as compared with naïve mice. Results from one out of three representative experiments are shown.

We next wanted to check whether intratumoral $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells in the lungs of naïve and tumor-bearing mice. In naïve mice, FTY720 blocked the accumulation of $CD11a^{high}CD8^+$ T cells in the lung (7.1% of lung $CD8^+$ T cells were $CD11a^{high}$ vs. 17.6% in PBS-treated controls), but not that of $CD11a^{low}$ $CD8^+$ T cells (**Fig. 4A**). This trend was also reflected in the absolute number of $CD11a^{high}CD8^+$ T cells that was detected in the lungs of FTY720-treated mice ($2.8 \pm 1.6 \times 10^3$ cells vs. $6.5 \pm 1.2 \times 10^3$ cells in PBS-treated controls, $p = 0.014$, **Fig. 4B**). This suggests that $CD11a^{high}CD8^+$ T cells migrate to the lungs from lymphoid organs, while $CD11a^{low}CD8^+$ T cells represent a lung-resident T-cell population. In tumor-bearing mice, both the frequency (**Fig. 4A**) and the number (**Fig. 4B**) of $CD11a^{high}CD8^+$ T cells in the lung increased upon the injection of FTY720 ($49.3 \pm 13.9 \times 10^3$ cells vs. $12.6 \pm 2.2 \times 10^3$ cells in PBS-treated controls, $p = 0.013$), while there was a significant decrease in $CD11a^{low}CD8^+$ T cells ($12.6 \pm 4.2 \times 10^3$ cells vs. $27.8 \pm 10.1 \times 10^3$ cells in PBS-treated controls, $p = 0.023$). Since the increase of the $CD11a^{high}CD8^+$ 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 $CD11a^{low}CD8^+$ T cells are induced by tumor cells to become antigen-experienced $CD11a^{high}CD8^+$ T cells.

We next asked if the priming of $CD11a^{high}CD8^+$ 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 $CD11a^{high}CD8^+$ T cells in

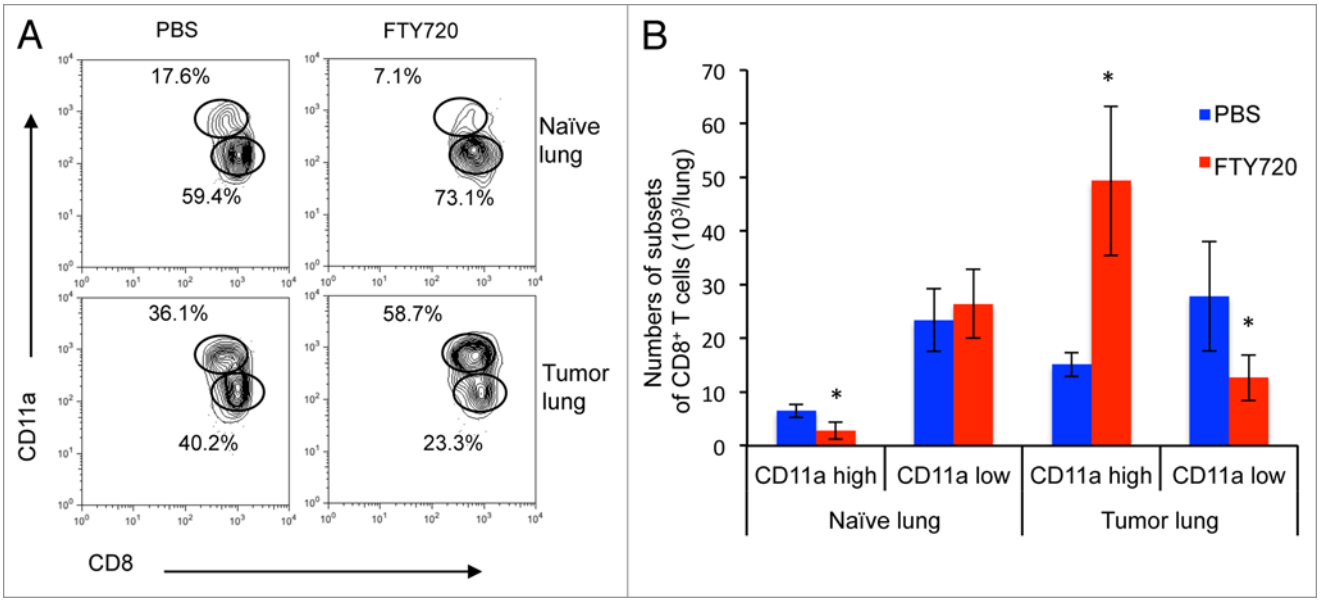

Figure 4. In situ expansion of CD11a^{high}CD8^{+} T cells within neoplastic lesions. (**A and B**) 4T1 tumor cells were intravenously injected into BALB/c mice alone or combined with the intraperitoneal injection of 1 mg/kg FTY720. Seven days after tumor-cell injection, lymphocytes were isolated from the lung of tumor-bearing mice and naïve mice that also received FTY720. (**A**) Percentages of CD11a^{high} and CD11a^{low}CD8^{+} T cells in the lungs. (**B**) Absolute numbers (mean ± SD) of CD11a^{high} and CD11a^{low}CD8^{+} T cells in the lung (n = 3). *p < 0.05, as compared with control PBS groups. Results from one out of two independent experiments are shown.

the lung as compared with naïve mice (3.4 ± 1.4 × 10^3 cells vs. 5.4 ± 0.6 × 10^3 cells in naïve mice). In contrast, mice receiving live tumor cells had a significant increase in the percentage (**Fig. 5A**) and number (**Fig. 5**) of CD11a^{high}CD8^{+} T cells in the lung seven days after tumor-cell injection (35.6 ± 5.1 × 10^3 cells vs. 5.4 ± 0.6 × 10^3 cells in naïve mice, p = 0.0005). This indicates that the priming of CD11a^{high}CD8^{+} T cells in situ requires the active infiltration of tumor cells.

Tumor-induced CD11a^{high}CD8^{+} T cells are proliferative effector cells but lack robust CTL effector functions. The accumulation of CD11a^{high}CD8^{+} T cells at both primary and metastatic 4T1 breast carcinoma lesions is striking because 4T1 tumor cells are considered to be poorly immunogenic.[25] This prompted us to investigate the phenotype and function of CD11a^{high}CD8^{+} T cells isolated from 4T1 metastatic tumor sites. As shown in **Figure 6A**, 7 d after the intravenous injection of 4T1 tumor cells, CD11a^{high}CD8^{+} T cells isolated from the lung displayed an elevated forward light scatter (FSC) and stained positive for Ki67, a nuclear protein associated with cell proliferation.[26] This population also exhibited an activated effector phenotype (CD62L^{low}CD69^{high}PD-1^{high}) and expressed high levels of the transcription factor T-bet, which determines the differentiation of CTLs[27] (**Fig. 6B**). This data suggests that CD11a^{high}CD8^{+} T cells accumulating in the lungs after the intravenous injection of 4T1 tumor cells are proliferative and have undergone effector differentiation. We next wanted to investigate the effector functions of tumor-induced CD11a^{high}CD8^{+} T cells. Since the TAAs of 4T1 cells are not defined, we used PMA and ionomycin to bypass TCR signaling.[28] We assessed CTL effector functions by measuring granzyme B secretion, degranulation (CD107a expression), and IFNγ production. As shown in **Figure S5**, 11 d after the subcutaneous injection of tumor cells, CD11a^{high} CD8^{+} T cells isolated from the spleen and neoplastic lesions produced IFNγ, yet failed to degranulate or produce granzyme B (data not shown). This indicates that—despite their differentiated effector cell and proliferative phenotype—CD11a^{high}CD8^{+} T cells lack robust CTL effector functions.

CD11a^{high}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 CD11a^{high}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* oncogene[29]) 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 CD11a^{high}CD8^{+} T cells exhibited an effector phenotype (CD69^{high}PD-1^{high}) (**Fig. 7B**). Nevertheless, CD11a^{high}CD8^{+} T cells isolated from sponatenous neoplastic lesions lacked CTL effector functions, as indicated by the lack of CD107a expression and limited production of IFNγ upon stimulation with PMA/ionomycin ex vivo (**Fig. 7B**). In addition, CTL effector functions could not be induced by a high affinity Neu-derived peptide (p66)[30] (data not shown). The dysfunction of the CD11a^{high}CD8^{+} T-cell population infiltrating spontaneous breast carcinomas is consistent with their failure to control tumor growth.

Tumor-reactive PD-1^{+}CD11a^{high}CD8^{+} T cells are detectable in the blood of melanoma patients. To test whether

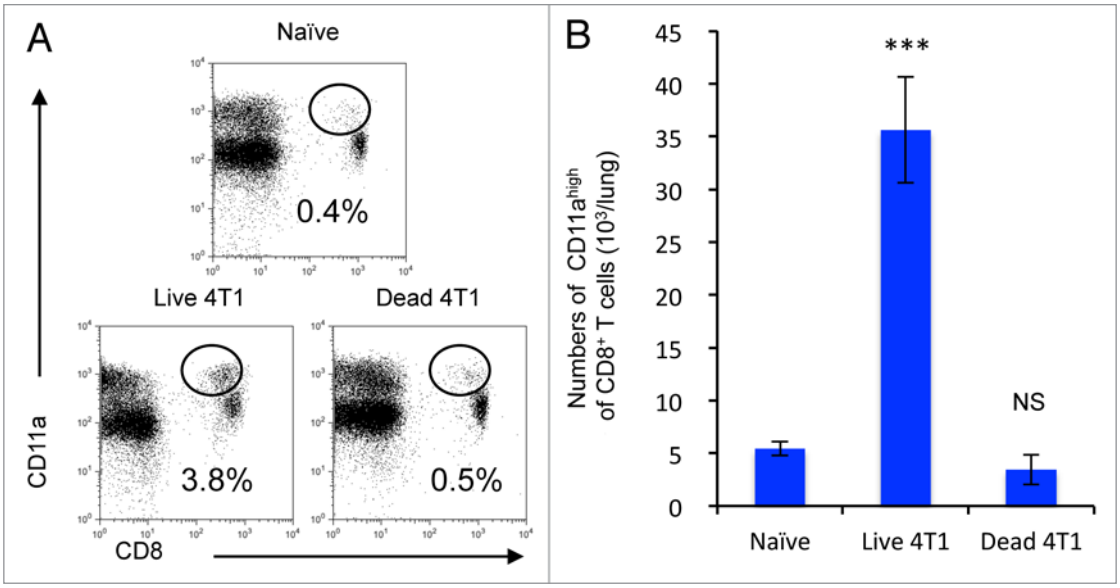

Figure 5. Live tumor cells induce CD11a^{high}CD8^{+} T-cell responses. (**A and B**) Live or lethally irradiated 4T1 tumor cells were intravenously injected into naïve BALB/c mice. Seven days after tumor-cell injection, lymphocytes were isolated from the lungs of mice injected with tumor cells and naïve control mice. (**A**) Percentages of CD11a^{high}CD8^{+} T cells in the lung (target for metastatic colonization). (**B**) Absolute numbers (mean ± SD) of CD11a^{high}CD8^{+} T cells in the lung (n = 3). ***p < 0.001, NS (non-significant), as compared with naïve mice. Results from one out of two independent experiments are shown.

CD11a^{high}CD8^{+} T cells represent tumor-specific CD8^{+} T cells in cancer patients, we analyzed CD8^{+} 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^{+} T cells. The CD11a^{high} subset of CD8^{+} T cells contained a significantly higher frequency of MART-1-tet^{+} T cells as compared with the CD11a^{low}CD8^{+} subset (p = 0.001, **Fig. 8A and B**), suggesting that CD11a^{high}CD8^{+} T cells represent tumor-specific CD8^{+} T cells. The CD11a^{high}CD8^{+} 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^{low}CD8^{+} T cells (**Fig. 8C**). The frequency of PD-1^{+}CD11a^{high}CD8^{+} 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^{+}CD11a^{high}CD8^{+} T cells in these melanoma patients (data not shown), suggesting that this population of CD8^{+} T cells is TAA-primed. Our results suggest that most tumor-specific CD8^{+} T cells of advanced melanoma patients are CD11a^{high}CD8^{+} T cells that co-express elevated levels of PD-1, presumably accounting for the dysfunctional state of tumor-reactive CD8^{+} T cells that normally characterizes these patients.

Discussion

Our results demonstrate that upregulation of CD11a by CD8^{+} T cells can be used as a marker to track endogenous tumor-reactive CD8^{+} T cells. CD11a^{high}CD8^{+} 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^{high}CD8^{+} T cells in the peripheral blood of advanced-stage melanoma patients. Although CD11a^{high}CD8^{+} 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^{high}CD8^{+} 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^{high}CD8^{+} 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.[7] 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.[7,8] In addition, CD11a is essential for CD8^{+} T cells to penetrate into tissues and kill target cells.[7] 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.[31,32] In addition, LFA-1 signaling stimulates the production of tumor necrosis factor α (TNFα) by activated NK cells,[33] as well as T-cell activation and survival.[34,35] Here, we found that tumor-reactive CD8^{+} T cells express increased levels of CD11a, suggesting an endogenous

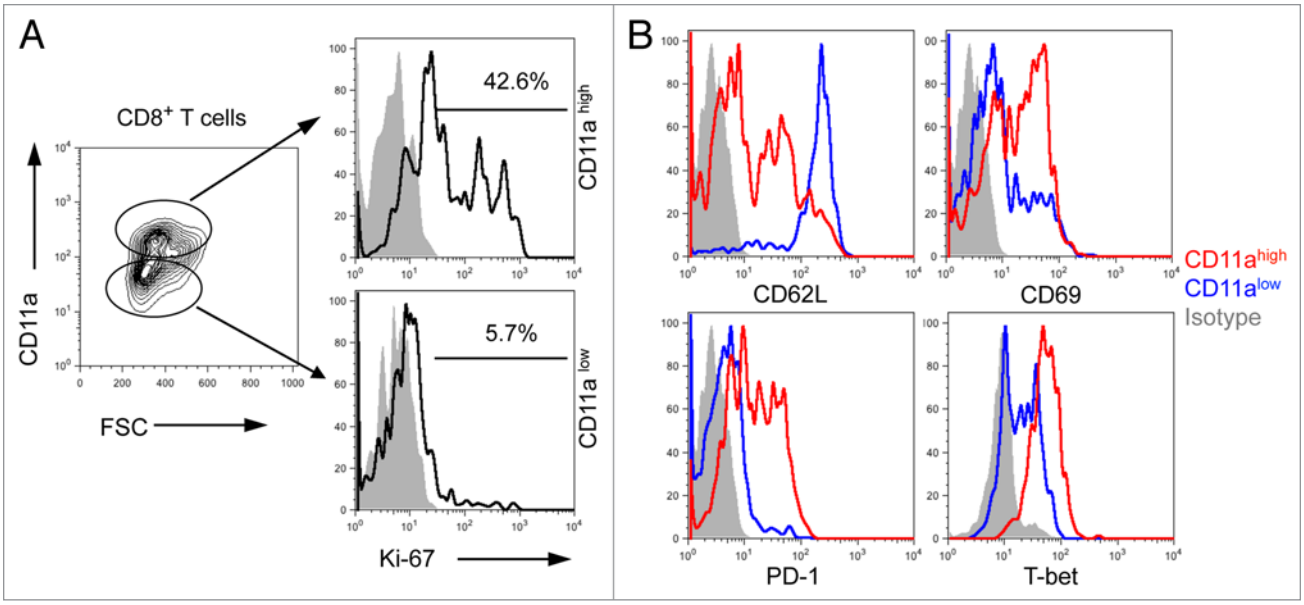

Figure 6. Phenotype of tumor-induced CD11a^{high}CD8^{+} T cells. (**A and B**) Lymphocytes were isolated from the lungs of mice 7 d after the intravenous injection of 4T1 tumor cells. (**A**) Proliferation of CD11a^{high}CD8^{+} T cells was measured by increased forward light scatter (FSC) and intranuclear expression of Ki67. (**B**) Surface expression of CD62L, CD69 and PD-1 and intracellular expression of T-bet by CD11a^{high} and CD11a^{low}CD8^{+} T cells. Results from one of three independent experiments are shown.

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 cytofluorometric 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 CD11a^{high} 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 T_H1 and CTL differentiation.[37] Functional assays showed that CD11a^{high}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 CD11a^{high}CD8^{+} T cells infiltrating tumor lesions prompted us to investigate where this effector T-cell population originated. CD11a^{high}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 CD11a^{high} 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 CD11a^{high}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 CD11a^{high}CD8^{+} T cells using FTY720, which blocks the emigration of lymphocytes from DLNs. In naïve mice, FTY720 blocked the accumulation of CD11a^{high}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 CD11a^{high}CD8^{+} T cells within the lungs (in this case constituting a metastatic site) was not inhibited by FTY720 (**Fig. 4**). Hence, while high CD11a expression levels may facilitate the recruitment of CD8^{+} T cells into neoplastic lesions, our findings strongly suggest that the intratumoral CD11a^{high}CD8^{+} T-cell population mainly comprise locally activated cells, a conclusion that is consistent with the results of several recent studies. Thompson et al. demonstrated that naïve T cells can infiltrate tumors regardless of the presence of antigens, and can become

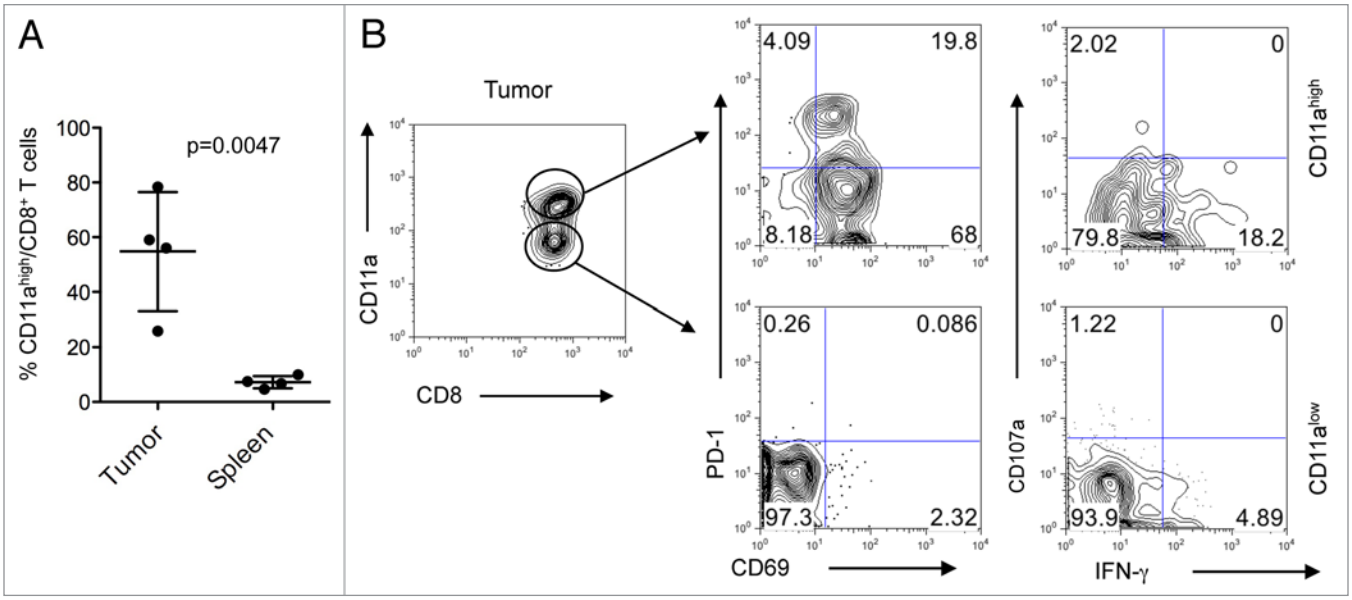

Figure 7. CD11a^{high}CD8$^+$ T cells identified within spontaneous tumors. (**A and B**) Lymphocytes were isolated from breast tissues and spleens of tumor-bearing BALB-neuT mice at 15–18 weeks of age. (**A**) Percentage of CD11a^{high}CD8$^+$ T cells within tumor lesions and the spleen. (**B**) Phenotype and function of CD11a ^{high} and CD11a^{low}CD8$^+$ T cells isolated from tumor tissues. Degranulation (CD107a expression) and IFNγ production were measured following a 4-h stimulation with PMA/ionomycin ex vivo. Results from one of three independent experiments are shown.

fully differentiated effectors when cognate antigens are present.[38] How a sterile tumor microenvironment can prime naïve CD8$^+$ T cells to differentiate into activated effector cells remains an open question. Recent reports describe a mechanism thereby 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 naïve CD8$^+$ T-cell responses against TAAs.[39,40] This process presumably occurs in our models as well, resulting in the accumulation of endogenous tumor-reactive CD11a^{high}CD8$^+$ T cells.

Upon activation in tumor lesions, CD11a^{high}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^{high}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[20,41] and in the peripheral blood.[19] We found that CD11a^{low}CD8$^+$ T cells express significantly lower levels of PD-1 than their CD11a^{high} 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^{high}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. 2**). Other groups have made similar observations using various distinct tumor models.[18,19] 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 recombinant 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).[21,42] The compromised adhesive capacity of CD8$^+$ T cells isolated from the tumor tissue could be due to their elevated expression levels of PD-1 (**Fig. 2A**), as PD-1 signaling is known to inhibit the activation of PKC.[43]

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,[44,45] resulting in the engagement of PD-1 on CD8$^+$ T cells and hence in the transmission of strong immunosuppressive signals.[46,47] 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.[5,6]

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.[4] In some patients undergoing anti-PD-1 therapy, durable responses including protracted tumor regression upon the discontinuation of therapy have been observed,[6,48] 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,[48] but the appearance of PD-1$^+$CD11a^{high}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

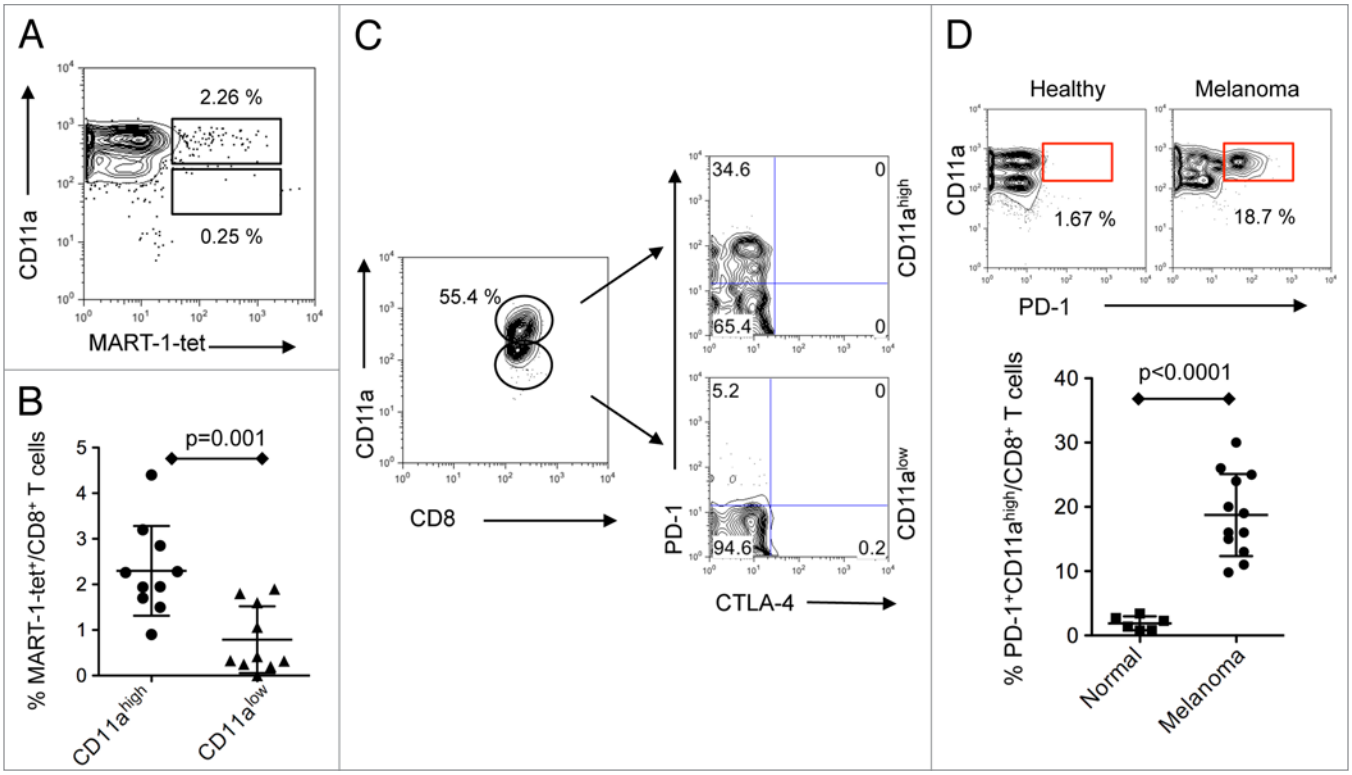

Figure 8. PD-1^{+}CD11a^{high}CD8^{+} T cells are increased in the peripheral blood of melanoma patients. (**A–D**) Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors or patients affected by Stage IV melanoma. (**A**) Tumor antigen specificity of the CD11a^{high}CD8^{+} T cells found among the PBMCs of melanoma patients. The percentage of MART-1-tet^{+} cells among CD11a^{high} and CD11a^{low}CD8^{+} T cells is reported. (**B**) Percentages of MART-1-tet^{+}CD11a^{high} and MART-1-tet^{+}CD11a^{low} cells among total CD8^{+} T cells (means ± SEM, n = 10). (**C**) Expression of PD-1 and CTLA-4 by CD11a^{high} and CD11a^{low}CD8^{+} T cells found among the PBMCs of melanoma patients. (**D**) Frequency of PD-1^{+}CD11a^{high} cells among total CD8^{+} T cells in the PBMCs of healthy donors (n = 6) and melanoma patients (n = 12).

into the memory pool by upregulating the pro-apoptotic molecule Bim.[36] Interestingly, alterations in BIM expression were only detected in CD11a^{high}CD8^{+} T cells. As the CD11a^{high}CD8^{+} T cells express high levels of PD-1 (**Fig. 2**), this cell population presumably constitutes the cellular target of PD-1/PD-L1-blocking therapies. It is possible that these immunotherapeutic approaches re-educate PD-1^{+}CD11a^{high}CD8^{+} T cells to become efficient memory T cells. Since only a small portion of cancer patients are sensitive to anti-PD-1/PD-L1 antibodies, measuring the frequency and distribution of tumor-induced PD-1^{+}CD11a^{high}CD8^{+} T cells in cancer patients may not only provide an immunological readout for evaluating the efficiency of treatment, but also contribute to selecting patients who are most likely to benefit from this therapy.

In summary, the upregulation of CD11a and PD-1 on CD8^{+} T cells can be used to identify endogenous tumor-reactive cells at both primary and metastatic tumor sites. Although CD11a^{high}CD8^{+} T cells are proliferative differentiated effector T cells and persist within neoplastic lesions, they are unable to control tumor growth. The elevated expression levels of PD-1 by CD11a^{high}CD8^{+} T cells and the delayed growth of tumors implanted in PD-1-deficient mice indicate that PD-1 is one of the major factors that undermine endogenous antitumor immunity. High expression levels of PD-1 and CD11a on CD8^{+} T cells will 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*$^{-/-}$) 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 form 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^{b}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 (eBioScience). 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 FACSCailbur 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[49] followed by intracellular staining for IFNγ. Briefly, lymphocytes were incubated with 1 μg/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 calcein acetoxymethyl 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 μg/well, as previously reported.[21] Blocking anti-CD11a antibodies (clone M17/4, Biolegend) were added when appropriate at a final concentration of 10 μg/mL. T cells were activated with 100 ng/mL PMA for 10 min at 37°C, and non-adherent cells were removed by washing. Finally, the absorbance 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) × 100.

Tumor studies. WT and *Pdcd1*$^{-/-}$ C57BL/6 mice were inoculated s.c. with 5×10^5 B16-OVA tumor cells, BALB/c mice were inoculated s.c. or i.v. with 1×10^5 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 $HLA\text{-}A2^+$ 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 immunotherapy. The other authors have no financial conflicts of interest.

Acknowledgments

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Supplemental Materials

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

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