T-Cell Distribution and Adhesion Receptor Expression in Metastatic Melanoma

Carsten Weishaupt, Karla N. Munoz, Elizabeth Buzney, Thomas S. Kupper, and Robert C. Fuhlbrigge

Abstract

Purpose: Metastatic malignant melanoma is a devastating disease with a poor prognosis. Recent therapeutic trials have focused on immunotherapy to induce development of endogenous antitumor immune responses. To date, such protocols have shown success in activation of tumor-specific CTL but no overall improvement in survival. To kill tumor, antigen-specific CTL must efficiently target and enter tumor tissue. The purpose of this study was to examine the pathway of leukocyte migration to metastatic melanoma.

Experimental design: Peripheral blood and metastatic melanoma tissues (n = 65) were evaluated for expression of adhesion molecules using immunohistochemistry of tumor sections and flow cytometry of tumor-associated and peripheral blood CTL and compared with healthy controls. CTL expressing T-cell receptors for the melanoma antigen MART-1 were identified in a subset of samples by reactivity with HLA-A2 tetramers loaded with MART-1 peptide.

Results: Results show that the majority of metastatic melanoma samples examined do not express the vascular adhesion receptors E-selectin (CD62E), P-selectin (CD62P), and intercellular adhesion molecule-1 (CD54) on vessels within the tumor boundaries. Strong adhesion receptor expression was noted on vessels within adjacent tissue. Tumor-associated T lymphocytes accumulate preferentially in these adjacent areas and are not enriched for skin- or lymph node–homing receptor phenotype.

Conclusion: Expression of leukocyte homing receptors is dysregulated on the vasculature of metastatic melanoma. This results in a block to recruitment of activated tumor-specific CTL to melanoma metastases and is a likely factor limiting the effectiveness of current immunotherapy protocols.

Despite significant effort and investment in clinical care and research, effective therapeutic options for treatment of metastatic malignant melanoma remain elusive. Because trials of chemotherapeutic drugs have not been satisfactory, significant interest has been directed toward immunotherapy protocols designed to induce or augment an endogenous immune response to the tumor. A variety of such clinical trials have been published, including infusions of *ex vivo* expanded melanoma-specific CTL (1–4); vaccination with melanoma antigen–loaded antigen presenting cells (5–8); transfection of tumor cells with granulocyte macrophage colony-stimulating factor (9); treatment with granulocyte macrophage colony-stimulating factor plus FLt3 ligand (10), interleukin 12 (11, 12), or IFN-γ (13); and, most recently, transfection of CTL with a MART-1–specific T-cell receptor (14). Although activation of tumor-specific CTL and occasional complete clinical responses have been documented in many of these trials, they have not, in general, altered the mean survival times for the majority of patients.

CTL recruitment to and infiltration of metastatic tumors is an essential component of an effective immune response to melanoma. Prior studies have shown that lymphocyte infiltration is associated with spontaneous regression of primary tumor and correlates with an improved prognosis for melanoma (15–20). Interestingly, regression was rarely observed in metastases and only occurred in the presence of lymphocytic infiltration (21, 22). Trafficking of lymphocytes to specific tissues, in turn, depends on expression and interaction between specialized lymphocyte and endothelial adhesion receptors and activation pathways (23). Based on these observations, we hypothesized that the lack of benefit in tumor immunotherapy trials might reflect a defect in the process of leukocyte recruitment to melanoma metastases, preventing tumor-specific effector CTL from reaching their target and controlling tumor growth.

T-cell trafficking from blood to tissues is a multistep process (24, 25). Vascular endothelium and T cells express homing molecules on their surfaces, which allow for organ-specific

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Received 10/9/06; revised 1/3/07; accepted 2/16/07.

Grant support: NIH grants AR42689 and AI-41707 (C. Weishaupt and R.C. Fuhlbrigge), the Melanoma Research Foundation (R.C. Fuhlbrigge), the American Skin Association (K.N. Munoz), and the Medical Fellows Program of the Howard Hughes Medical Institute (K.N. Munoz and E. Buzney).

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

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doi:10.1158/1078-0432.CCR-06-2450

lymphocyte trafficking (26, 27). In brief, T cells circulating in the blood tether to vascular walls in post-capillary venules via selectins and roll in response to the shear force imparted by flowing blood. As the cells roll, they sample the surface for chemokines bound to the endothelial cells. Rolling cells bearing appropriate receptors for the chemokines expressed at that site become activated and up-regulate integrin avidity, allowing firm adhesion to the vascular wall and transmigration. Cells that successfully complete each of these steps can enter into the underlying tissue. Absence of the appropriate receptor/ligand interaction at any step results in disruption of this process and the cells remain in circulation. Expression of tissue-specific homing receptors on T cells is up-regulated, or imprinted, during naive to memory transition following antigen-specific activation in tissue draining lymph nodes (28). These cells then leave the lymph node and traffic via the blood to their target tissue. Because melanoma arises from skin, it might be expected that T cells responding to metastatic melanoma would traffic to tumor using skin-homing pathways. Effector memory T cells found in normal and inflamed skin express cutaneous T-cell receptor–associated antigen (CLA), which binds to endothelial E-selectin (CD62E) expressed on post-capillary venules in skin (26, 29). Receptors for chemokines expressed in skin [e.g., CCR4 (CD194)] bind to the chemokine ligands TARC (CCL17) and MDC (CCL22) on T cells. T-cell integrins LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_1\beta_4$) bind to endothelial ligands intercellular adhesion molecule 1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106), respectively. Naive and central memory T cells, which home to lymph nodes, characteristically express L-selectin (CD62L), chemokine receptor CCR7 (CD197), and LFA-1 (CD11a).

To examine the hypothesis that lymphocyte homing to melanoma is not effective, we characterized the expression of homing receptors and ligands on T cells and in tumor tissue samples from patients with metastatic melanoma. We show here that human melanoma metastases, in general, do not express the vascular adhesion receptors E-selectin (CD62E), P-selectin (CD62P), and ICAM-1(CD54) and that tumor-associated T lymphocytes in melanoma metastases accumulate primarily in the tissues immediately adjacent to, rather than within, the tumor. Peripheral blood and tumor-associated CTL, including tumor-specific CTL, from patients with metastatic melanoma showed no selection bias in homing receptor expression. Based on these observations, we postulate that lack of vascular homing receptor expression in melanoma metastases leads to an insufficient recruitment of activated tumor-specific CTL and is a factor that limits the development of antitumor immune responses and the effectiveness of current immunotherapy protocols.

Materials and Methods

Metastatic melanoma tissue and blood samples. This study was approved and all materials were collected in accordance with the Partners Research Management Institutional Review Board. Sections of metastatic melanoma tissue from various organs and/or peripheral venous blood from patients with metastatic malignant melanoma were provided by the Tissue Bank/Pathology Core of the Dana-Farber/Harvard Cancer Center Specialized Program in Research Excellence in Skin Cancer over a 2-year period. Materials were collected from an unselected population presenting to the associated clinics for diagnosis and treatment of melanoma. Nine patients from whom tissue was received and five from whom blood was received had been treated in various protocols within 1 year before sample collection. Treatments received included interleukin 2, IFN, granulocyte macrophage colony-stimulating factor, Taxol, temozolomide, and, for one patient, melanoma lysate vaccine (Melacine). In general, large blood samples were not available from patients presenting for surgical procedures. As such, the blood and tissues available represent an overlapping but nonidentical population of patients. Blood samples from consenting healthy volunteers were used as normal controls. Fresh tissue samples were divided for immunohistochemistry and for recovery of tumor-infiltrating T cells either by EDTA chelation with mechanical disruption or by culture on collagen-coated carbon matrix grids (Cellfoam, Cell Sciences Pte Ltd.) in the presence of 100 units/mL human recombinant interleukin 2 (Peprotech, Inc.) as described (30). Nontumor tissues (lymph node, tonsil, skin, and lung) were used as normal controls. Peripheral blood mononuclear cells were isolated by density gradient separation (Ficoll-Histopaque 1.077, Sigma-Aldrich) of blood, and T cells were further separated by depletion of non–T cells (Pan T-cell isolation kit II or CD8 T-cell isolation kit, Miltenyi Biotec).

Immunohistochemistry. Tumor specimens were fixed in 10% formalin for 20 to 24 h, embedded in paraffin, and cut into sections of 5-μm thickness. After deparaffinization, rehydration, and antigen retrieval by incubation in a pressure cooker (3 min at 120°C), samples were stained using the Envision+ Systems HRP kit (DakoCytomation). Slides were photographed with a Nikon digital photomicroscope. To determine the frequency of infiltrating cells in various areas of the tissues, pictures were taken at a magnification of ×200 and then loaded at 35% into Adobe Photoshop 6.0. A fixed-width border (2 cm on the computer screen) was defined at the junction between tumor tissue and normal appearing tissue. Samples in which a clear border could not be defined were excluded from this analysis. Adjacent to this border, five randomly picked 2 × 2 cm fields were counted for T cells that showed clear reactivity above background. The following antibodies were used to stain sections: E- and P-selectin (16G4 and C34; Novocastra Laboratories Ltd.), CD31 (JC70A; DakoCytomation), ICAM-1 (P2A4; Chemicon International), S-100 (DakoCytomation), MART-1 (M2-7C10; Signet Laboratories), CD3 (A0452; DAKOCytomation), and CD8 (C8/144B; Chemicon International). A human E-selectin–specific antibody for staining of paraffin-embedded tissue was not available. All commercially available monoclonal antibodies (mAb) to E-selectin tested, including clones 16g4 (Novocastra), BBIG-E4 (5D11; R&D Systems), and P2H3 (Chemicon International), were found to equally stain paraffin-embedded Chinese hamster ovarian cells transfected with either human E- or P-selectin. Each of these antibodies was specific for E-selectin when used to stain frozen samples of the same transfected Chinese hamster ovarian cells. Results describing reactivity of paraffin-embedded tissues are therefore expressed as E- or P-selectin. Similar findings (absence of both E-selectin and P-selectin) were observed on cryosections prepared from a subset of tumors (data not shown).

Flow cytometry analysis of T cells isolated from tissue and peripheral blood. Phycoerythrin-conjugated MHC class I HLA-A*0201 tetramer bound to MART-1 peptide (ELAGIGILTV; iTag MHC tetramer, Beckman Coulter) was used to identify melanoma-specific $CD8^+$ T cells from tissue and blood. Tetramer function was verified with a T-cell line expressing MART-1–specific T-cell receptor (courtesy of David Panka, Dana-Farber Cancer Institute, Boston, MA). Phycoerythrin-conjugated MHC class I tetramer without peptide (Negative tetramer, Beckman Coulter) was used as a negative control. Patient samples were tested for HLA-A2 expression before using the anti–HLA-A2 tetramer (BB7.2; BD PharMingen) and approximately half of our patient population was positive. Tetramer staining was done for 20 min at room temperature and samples were simultaneously counterstained with antibodies to T-cell surface molecules: CD3 mAb (UCHT1), CD4 mAb (RPA-T4), CD8 mAb (HIT8A), CLA mAb (HECA-452), and CCR4 mAb (1G1) from BD PharMingen; CD62L mAb (DREG56) and CD49d mAb (HP2.1) from

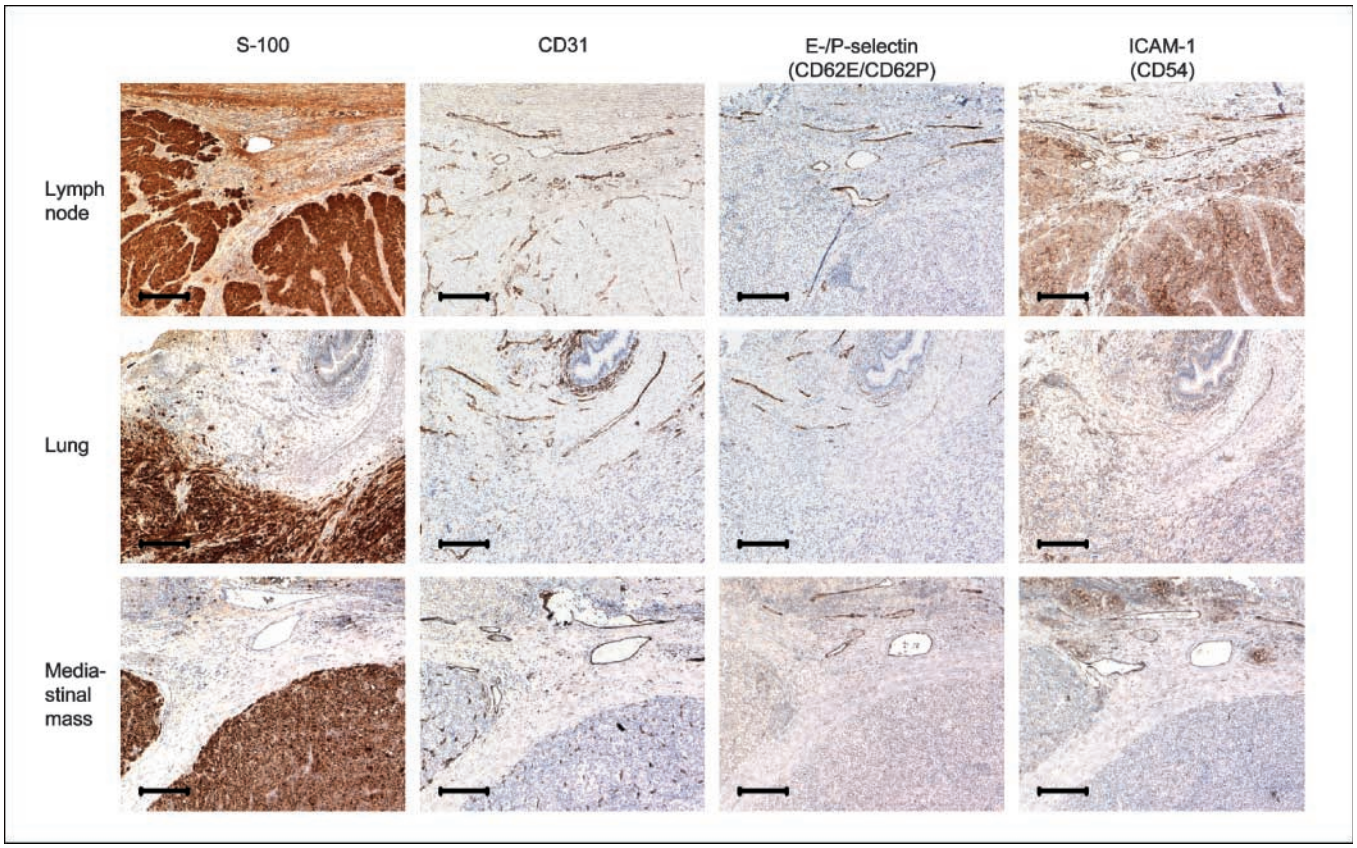

Fig. 1. Expression of adhesion molecules is decreased on the vessels in melanoma metastasis relative to surrounding tissues. Melanoma borders were defined by S-100 staining in paraffin sections and the distribution of vessels was determined by staining with CD31. Intratumoral vessels showed little or no E- or P-selectin (CD62E, CD62P) or ICAM-1 (CD54) in comparison with expression on peritumor vessels. Staining is indicated by red-brown color. Bar, 200 μm.

Beckman Coulter; CCR7 mAb (150503) from R&D Systems; and CD43 mAb (1D4) from Medical & Biological Laboratories International Corp. Appropriate isotype antibodies were used as negative controls (mouse immunoglobulin G1, mouse immunoglobulin G2b, and rat immunoglobulin M from BD PharMingen; mouse immunoglobulin G2a from R&D Systems; and mouse immunoglobulin G2a from Medical & Biological Laboratories). $CD8^-$ T cells from peripheral blood and tissue as well as $CD8^+$ T cells from healthy donors were also stained and used as normal controls. Samples were acquired and analyzed using BD FACScan flow cytometer and CellQuest software.

Statistical analysis. Statistical analysis was done by using either the Mann-Whitney *U* test or the Wilcoxon signed rank test.

Results

Melanoma metastases express little or no vascular E-selectin, P-selectin, or ICAM-1. To examine the expression of homing receptors on blood vessels in metastatic melanoma tissues, we obtained metastatic melanoma tissue samples ($n = 65$) from 41 patients and stained sections including both tumor and surrounding normal tissue with antibodies to E-selectin, P-selectin, and ICAM-1 (Fig. 1). The boundaries of the tumor were confirmed by staining of sequential sections for S-100 (Fig. 1) and MART-1 (data not shown). Vascular structures, identified by staining for CD31, were apparent both within tumor and the adjacent tissues, as shown in representative pictures of a lymph node metastasis, a lung metastasis, and a mediastinal tumor mass (Fig. 1). Immunohistochemical staining for adhesion molecules, however, revealed a striking absence of E-selectin, P-selectin, and ICAM-1 expression on vessels within tumor metastases and strong expression of selectin and ICAM-1 on vessels in adjacent tissue areas. Of 32 samples examined, 30 (93.8%) showed little or no expression of E- or P-selectin on $CD31^+$ vessels within the tumor with significant staining evident in the adjacent areas of almost all samples. Although ICAM-1 staining of tumor cells obscured the vessels in approximately half of the samples, expression of ICAM-1 on vessels within tumors was reduced relative to vessels outside of tumors in 17 of 18 (94.4%) samples suitable for analysis.

T lymphocytes associated with melanoma metastases accumulate preferentially in the tissues adjacent to the tumor and not within the tumor boundaries. To characterize T-cell homing in the context of the vascular adhesion receptor patterns described above, we stained consecutive sections of tumor samples for CD3. In a large majority of melanoma metastases tested, immunohistochemical staining revealed a striking accumulation of $CD3^+$ cells within the tissue immediately adjacent to the tumors, whereas low numbers of $CD3^+$ cells were observed inside the boundaries of the tumor (Fig. 2A). To obtain an objective measure, the number of $CD3^+$ cells per high-power field was determined on each side of a border zone between tumor and the surrounding tissue (Fig. 2B). Examination of melanoma metastases from skin ($n = 2$), as well as from lung ($n = 4$) and lymph nodes ($n = 3$), consistently showed greater numbers of T cells in the normal

tissue adjacent to tumor than within the tumor structure (Fig. 2A). Although the numbers of patients with comparable specific therapeutic histories were small, we were unable to discern any difference between the variously treated and untreated populations (data not shown). Comparing all samples, >5-fold more $CD3^+$ cells were observed in the zones of adjacent normal tissue (8.3 ± 0.6 per field) compared with within the tumor boundary (1.6 ± 0.2 per field; $P < 0.0001$; $n = 17$; Fig. 2C). This pattern of T-cell distribution matches the pattern of vascular adhesion receptor expression and suggests that T cells may be restricted from entering melanoma metastases directly.

Expression of homing receptors is not altered on peripheral blood $CD8^+$ T cells from melanoma patients. To determine if the pattern of T-cell migration observed reflected a global dysfunction of T-cell homing, we assessed expression of homing receptors on peripheral blood CTL from patients with metastatic melanoma. As noted above, expression of CLA and CCR4 is characteristic of the skin-homing T-cell population, whereas expression of CD62L and CCR7 directs homing of

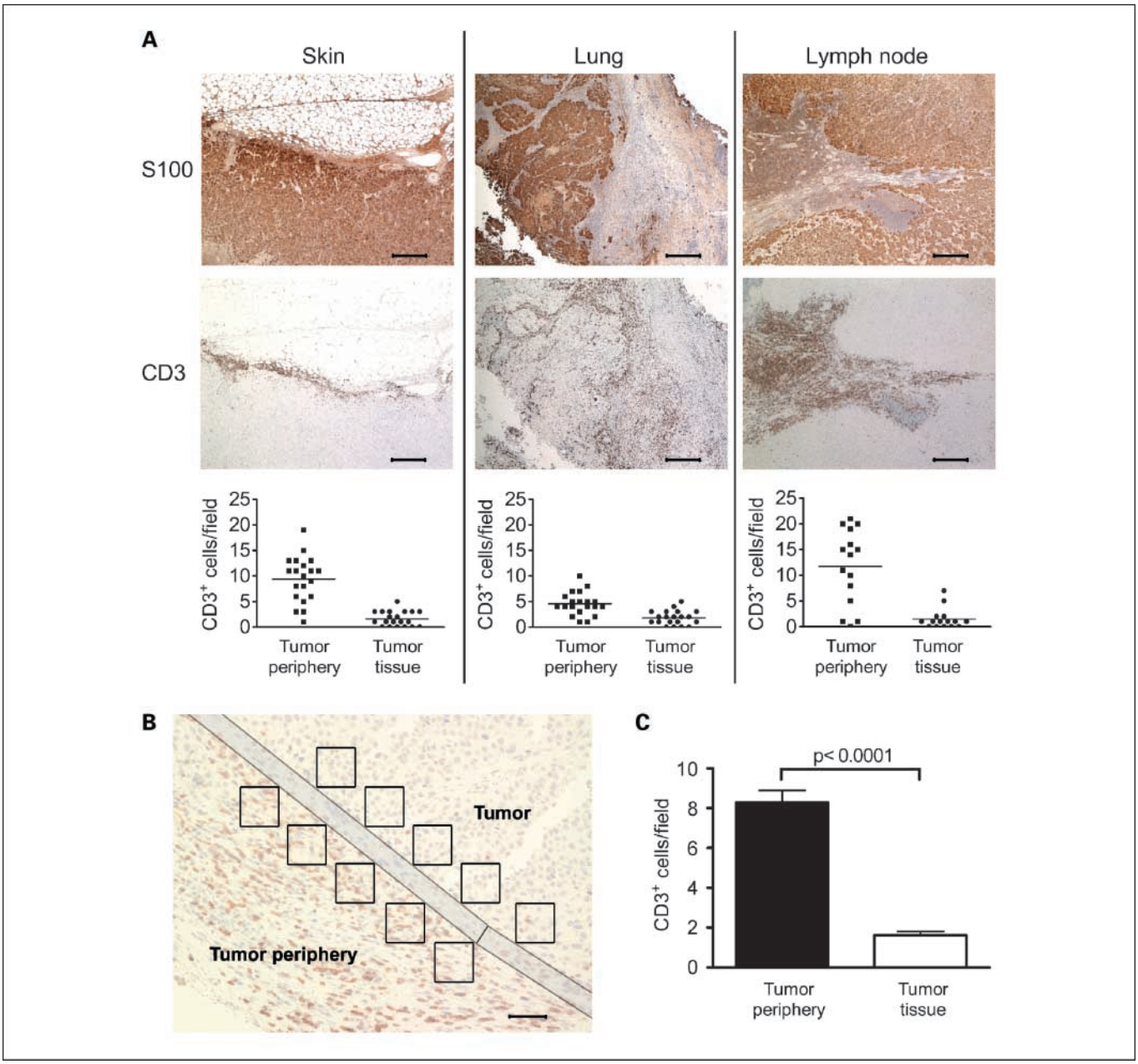

Fig. 2. T lymphocytes do not infiltrate human melanoma metastases. *A,* melanoma boundaries were identified by S100 stain in skin, lung, and lymph node metastases. Staining for CD3 reveals accumulation of Tcells in the tumor periphery and a relative absence of Tcells in tumor tissue. Staining is indicated by red-brown color. Bar, 300 μm. Statistical analysis of intratumor versus extratumor Tcells counted on immunostained sections (Mann-Whitney *U* test). Counted fields: skin mass, $n = 10$; lung, $n = 20$; lymph node, $n = 15$. Bars, mean. *B,* CD3 cells were counted by defining five fields on each side of the border between tumor and normal-appearing tissue. Counts were tested for significance with the Wilcoxon signed rank test. Bar, 50 μm. *C,* melanoma metastases of different regions showed significantly more $CD3^+$ cells in the tumor periphery compared with tumor tissue. Columns, mean (tumor samples, $n = 17$; counted fields, $n = 85$); bars, SE.

naive and central memory T cells to lymph nodes. To examine expression of homing receptors on CTL in patients with metastatic melanoma, we assessed expression of skin- and lymph node–homing receptors on $CD8^+$ lymphocytes isolated from the blood of patients with melanoma (n = 10) in comparison with healthy control subjects (n = 5; Fig. 3). Although the $CLA^+/CCR4^+$ population was slightly higher in control individuals compared with melanoma patients (10.96 ± 9.16% versus 3.55 ± 0.90%, respectively), this difference was not statistically significant (Fig. 3B, *left*). As above, we were unable to discern any differences between the variously treated and untreated populations (data not shown). Similarly, a comparison of the $CD62L^+/CCR7^+$ population among $CD8^+$ T cells showed no significant difference between healthy individuals and melanoma patients (60.4 ± 7.1% versus 54.3 ± 10.5%, respectively; Fig. 3B, *right*). Comparison of mean fluorescence intensity of T cells stained for CLA, CCR4, CD62L, and CCR7 also did not reveal significant differences between healthy individuals and melanoma patients (data not shown). Thus, the peripheral blood $CD8^+$ lymphocyte population in patients with metastatic melanoma showed no bias toward or away from expression of standard skin and lymph node homing characteristics. This would indicate that there is no inherent limitation in development of specific homing populations in these patients.

Expression of homing receptors on MART-1 T-cell receptor–positive $CD8^+$ T cells from melanoma patients is not biased toward a skin-homing phenotype. Because the population of specific melanoma-reactive T cells in peripheral blood is expected to be low, we also examined expression of skin- and lymph node–homing receptors on CTL expressing melanoma-specific T-cell receptor by staining $CD8^+$ T cells isolated from blood or cultured from melanoma tissue of HLA $A2^+$ melanoma patients with HLA A2/MART-1 tetramers (Fig. 4). MART-1 is a dominant melanoma epitope that is expressed by the majority of malignant melanomas and is commonly used as target antigen in immunotherapy trials (7, 31, 32). Although the number of HLA $A2^+$ samples available was small (n = 11), four patients were noted to have significant MART-1 tetramer–reactive $CD8^+$ T cells (range, 0.5-5.8%). All four patients from whom MART-1 tetramer–reactive T cells were identified were confirmed to have MART-1–positive tumors (data not shown). Stains for adhesion receptors were striking for the lack of significant CLA expression on tetramer-reactive T cells. $CD8^+$ cells isolated from metastatic melanoma tissue (Fig. 4A, *bottom*) revealed a similar phenotype of low or absent CLA expression. This is in contrast to the high levels of CLA expression (>90%) routinely observed on CTL from normal skin (33). Staining for other T-cell surface markers, including CD49a (VLA-4), CD62L (L-selectin), and CD45RO, showed no apparent differences between MART-1 tetramer–reactive cells and the tetramer-negative population of tumor-associated cells (Fig. 4B). Immunochemistry of tissue sections confirmed that the $CD3^+$ T cells in the tumor adjacent tissues were predominantly CLA negative (Fig. 4C). We conclude from these studies that there was no specific recruitment of CLA-positive or MART-1 tetramer–reactive T cells to the site of these metastases. Indeed, the values observed are consistent with nonspecific recruitment of T cells from the circulating blood pool in these patients.

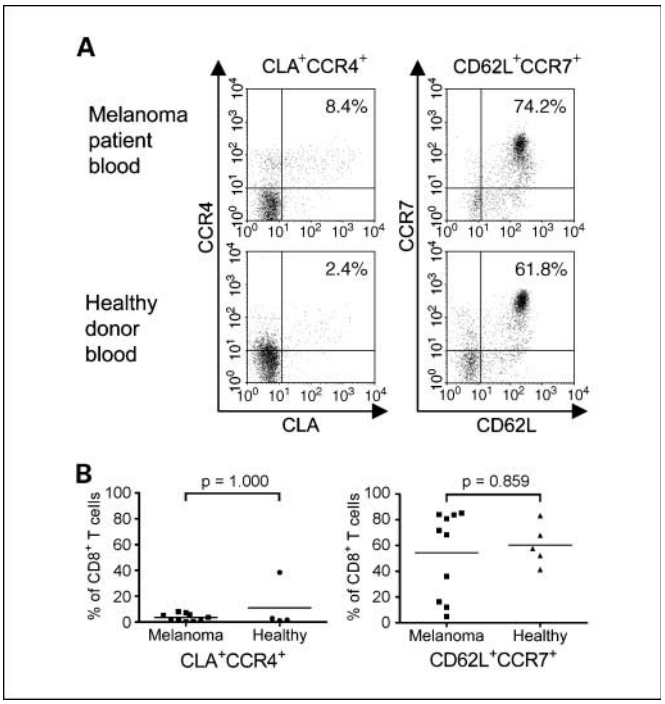

Fig. 3. Homing receptor expression on peripheral blood $CD8^+$ T cells is similar in melanoma and healthy patients. Skin-homing ($CLA^+/CCR4^+$) or lymph node–homing ($CCR7^+/CD62L^+$) receptor expression was detected by flow cytometry on $CD8^+$ T cells from melanoma patient blood (n = 10) as compared with T cells isolated from healthy donor blood (n = 5). Analysis using the Mann-Whitney test showed no significant difference between patient and healthy donor samples. Black bar, mean.

Discussion

The fundamental goal of cancer immunotherapy is the induction of a tumor-specific cytotoxic immune response. The current paradigm of antitumor immunity implies a sequence of events in which antigen-presenting cells from the tissue site of a tumor carry tumor antigen to local draining lymph nodes and promote the education and expansion of tumor antigen-specific cytotoxic effector T cells (34). Effector cells leave the lymph node and then traffic to the site(s) of the tumor where they carry out their functions and control tumor growth. This process may be augmented by the local presentation of antigen and further activation and proliferation of T cells within the local microenvironment.

With regard to malignant melanoma, several groups have documented immunotherapy protocols that successfully augment the development and activation of melanoma antigen–specific $CD8^+$ effector T cells. Clinical responses, however, have been infrequent, suggesting that antigen recognition by itself is not sufficient to limit tumor growth and implying that an additional level of control exists (35). Both the activation phase and the effector phase of the adaptive immune response require the migration of cells (both nascent antigen-presenting cells and effector T cells) from the blood into the site of the tumor and are thus dependent on vascular adhesion receptors to mediate recruitment of cells from the circulation to the vascular wall and to mediate transmigration. Investigators in this field, however, have not focused on recruitment of effector cells to tumor as a critical element of protocol design. In this report, we document a significant defect in the expression of the vascular

adhesion receptors E-selectin, P-selectin, and ICAM-1 in melanoma metastases. The impact of this local adhesion receptor immunodeficiency is to create a block in trafficking of effector T cells to tumor. Among 65 metastatic melanoma tissue samples examined, we noted that only a small fraction had tumor-associated lymphocytes located within the borders of the developing tumor. A large majority of lymphocytes in these samples were found at the tumor margin or within the adjacent normal tissue. As these samples were recovered from patients with advancing metastatic disease, it is clear that the observed immune response was not effective at controlling growth of tumor. Indeed, our analysis, although limited to metastatic melanoma, indicates that tumor-associated T cells from these patients were not enriched for a population of antitumor T cells but seem to be a nonspecific accumulation reflective of the peripheral blood pool. Although analysis of surface protein expression on cultured cells may not accurately reflect true expression *in situ*, the limited volumes of tissue available made use of such methods necessary. In support, the explant method used here does not result in alteration of these surface antigens on T cells isolated from normal skin, where direct comparison can be made to T cells *in situ* or recovered by short-term digestion or physical disruption protocols (30).

The patients providing samples for this study were not part of a single treatment protocol and, as noted above, have quite variable therapeutic histories. Comparison between blood and tissue results was also limited because matched blood and tissue sets were not available. Whereas we were not able to discern a difference in the pattern of distribution of tumor-associated T cells or expression of vascular adhesion receptors between treated and untreated patients, or among the variously treated patients, the numbers of individuals with comparable specific therapeutic histories were small. Given the results of this study, it may be valuable to consider such effects in the design of future trials.

As noted, lymphocyte infiltration of melanoma, as well as other solid tumors, has been correlated with tumor regression and improved prognosis (15–20). For example, expression of CXC chemokine receptor 3 by $CD8^+CD45RO^+$ T cells was significantly associated with enhanced survival in patients with advanced melanoma (36). ICAM-1, VCAM-1, and neural cell adhesion molecule-1 (CD56) expression has been reported to be reduced in conjunctival melanoma (37) and P-selectin has been reported to be expressed at lower levels in melanoma compared with benign melanocytic tumors (38). Expression of vascular adhesion protein 1 has also been correlated with survival in melanoma (39). Similar findings have been described in mammary carcinoma (40) as well as gastric and lung carcinoma (41). A study of colon carcinoma reported high expression of ICAM-1, VCAM-1, and E-selectin in colon carcinoma, although inspection of the micrographs in this report suggests that the areas of increased expression lie at or outside the tumor margins (42), which is similar to the findings presented here. A more recent study of patients with colorectal tumors showed that disease-free survival was strongly correlated with an increased ratio of T cells within the tumor relative to the tumor margins (43). Studies examining regulation of adhesion molecule expression in tumor tissue have shown that the expression of ICAM-1, VCAM-1, and E-selectin is altered by vascular endothelial growth factor and tumor fibroblast growth

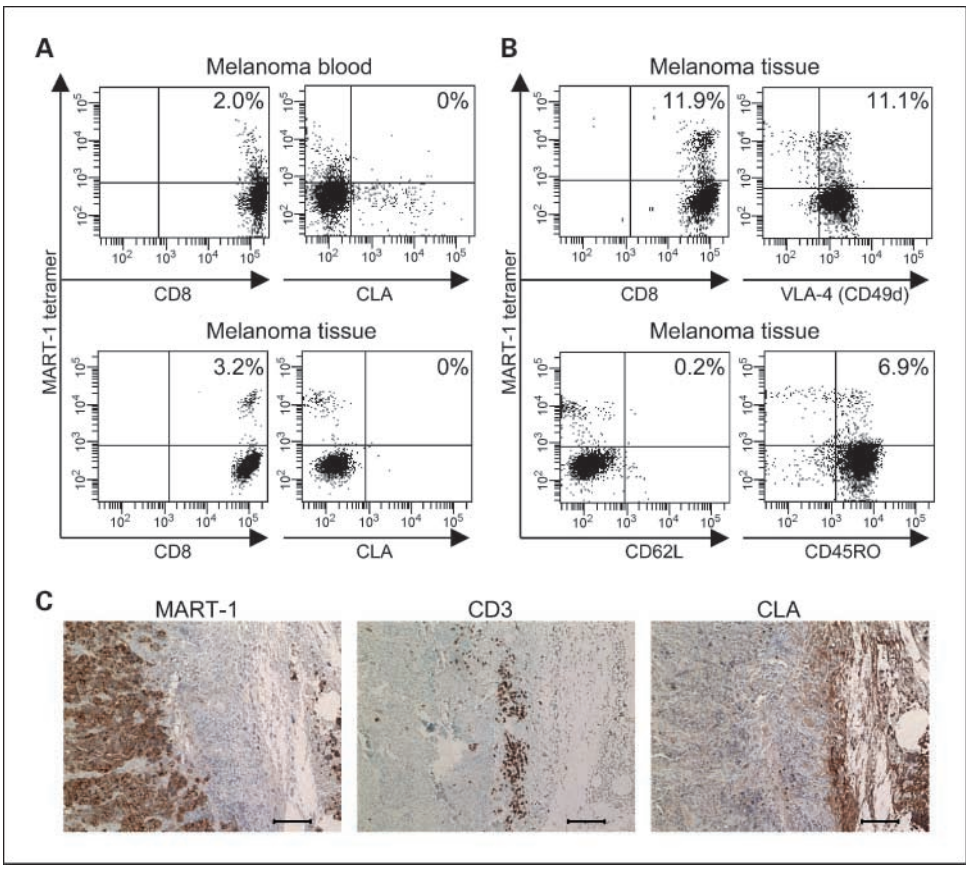

Fig. 4. CLA expression is low on tumor-specific $CD8^+$ T cells. *A,* four of eleven HLA-A2 positive patients had measurable (>0.5%) $CD3^+CD8^+$ T cells reactive with melanoma antigen-specific tetramer (blood, $n = 3$; tissue, $n = 1$). Top, blood from a patient with mesenteric metastases. Bottom, T cells were isolated from a paratracheal metastasis by 1-wk explant culture and stained as indicated. *B,* T cells were isolated from 2-wk explant cultures established from a paratracheal mass. Isolated $CD3^+CD8^+$ MART-1 tetramer – reactive T cells were stained for VLA-4, CD62L, and CD45RO ($n = 1$). *C,* immunohistochemical staining confirmed that MART-1 – positive tumor-infiltrating $CD3^+CD8^+$ cells do not express CLA. Representative images of an axillary mass. Cells were gated on $CD8^+$ T cells. Bar, 120 μm.

factor and, therefore, may be connected with tumor angiogenesis (44–46). Thermal stress associated with fever has also been proposed to regulate lymphocyte trafficking via interleukin 6 (47). Although provocative, interpretation of these findings awaits further investigation of the regulation of adhesion receptors on tumor vasculature.

Dysregulation of leukocyte trafficking is clearly not the only mechanism limiting immune activity against metastatic melanoma. Other documented mechanisms of immune dysfunction include down-regulation of MHC class I antigen on metastatic tumor cells (48) and a tumor microenvironment that promotes T-cell anergy (49, 50). Inhibition of T cells may also occur through inhibitory ligands expressed on the tumor cells such as PD-L1 (51–53) or through metabolic factors such as indoleamine 2,3-dioxygenase (54, 55). The distribution of innate immune cells also seems to be modulated to restrict the antitumor response. Mature dendritic cells have been reported to be absent from melanoma tissue but have been noted to accumulate in the adjacent tissues in a fashion similar to the findings of T cells in this report (56). Similarly, in breast cancer only immature dendritic cells have been observed within tumor whereas mature dendritic cells were located in peritumoral areas (57). Regulatory T cells have also been shown to accumulate in and around metastatic tumors and may actively suppress the recruitment and functions of cytotoxic effector T cells (58, 59). Whereas these latter observations may also reflect dysregulation of leukocyte trafficking, the lack of clear boundaries between tumor and normal tissue in many of these samples makes interpretation of immunohistochemical stains more difficult than in the melanoma samples we examined.

In summary, we have shown that although significant numbers of T cells may be associated with melanoma metastases, these cells preferentially accumulate in the tissue immediately surrounding the tumor. Furthermore, melanoma-specific $CD8^+$ cells in the peripheral blood and in metastatic tissue show low levels of specific homing receptor expression, comparable with the bulk population of circulating T cells, indicating a lack of selection for specialized homing phenotypes. Most importantly, we show that the vasculature of melanoma metastases shows low expression of both the vascular selectins and ICAM-1. Taken together, these results indicate that a block to efficient recruitment of T cells exists within melanoma metastases and may represent a significant limitation on the effectiveness of therapeutic interventions designed to augment antitumor immunity.

Acknowledgments

We thank Sandy King for technical support, David Panka for providing us with control specimens and technical advice for the tetramer staining, Marianne Boes and Rachael Clark for review of the manuscript, and Bryan Vander Lugt for fruitful discussions.

We thank Dr. Michael Atkins, Amanda Youmans, and Lucy Mandato at Beth Israel Deaconess Medical Center, and the Tissue Bank/Pathology Core of the Dana-Farber/Harvard Cancer Center Specialized Program in Research Excellence in Skin Cancer for assistance with the acquisition of metastatic melanoma tissue.

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Clin Cancer Res 2007;13:2549-2556.