Preferential Expression of Very Late Antigen-4 on Type 1 CTL Cells Plays a Critical Role in Trafficking into Central Nervous System Tumors

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Abstract

We have previously shown preferential tumor-homing and therapeutic efficacy of adoptively transferred type 1 CTL (Tc1) when compared with type 2 CTL (Tc2) in mice bearing intracranial ovalbumin-transfected melanoma (M05). Further characterizing the expression of a panel of homing receptors on Tc1 and Tc2 cells, we found that very late antigen (VLA)-4 (a heterodimer of CD49d and CD29), but none of other receptors evaluated, was expressed at significantly higher levels on Tc1 cells than on Tc2 cells. Although CD49d (α_4 integrin) can form heterodimers with both β_1 (CD29) and β_7 integrins, $\alpha_4\beta_7$ complexes were not expressed by either Tc1 or Tc2 cells, suggesting that CD49d is solely expressed in VLA-4 complexes. VLA-4 expression on Tc2 cells was down-regulated in an interleukin (IL)-4 dose-dependent manner but not by other type 2 cytokines, such as IL-10 and IL-13, suggesting that IL-4 uniquely down-regulates VLA-4 expression on these cells. In accordance with the differential expression of VLA-4 on Tc1 versus Tc2 cells, Tc1 cells alone were competent to adhere to plate-bound VCAM-1-Ig fusion protein. Finally, the efficient trafficking of Tc1 cells into intracranial M05 lesions *in vivo* was efficiently blocked by administration of monoclonal antibodies against CD49d or VCAM-1 or small interfering RNA–mediated silencing of CD49d on Tc1 cells. Collectively, these data support the critical role of VLA-4 in the effective intracranial tumor homing of adoptive-transferred, antigen-specific Tc1 cells and suggest that more effective vaccine and/ or *ex vivo* T-cell activation regimens may be developed by promoting the generation of VLA-4+ antitumor Tc1 cells. [Cancer Res 2007;67(13):6451–8]

Introduction

Recent clinical studies have shown that superior clinical efficacy may be obtained through adoptive transfer of tumor-reactive autologous T cells, whereas active vaccination protocols rely on intact immune reactivity in cancer-bearing hosts, which is often impaired (reviewed in ref. 1). When T-cell receptor (TCR) transgenic CD8+ T cells polarized toward a type 1 (Tc1) or type 2 (Tc2) cytokine profile *in vitro* were investigated in tumor therapy models, interleukin (IL)-4–producing Tc2 cells exhibited less efficient antitumor efficacy than IFN-γ–producing Tc1 cells (2, 3). In our previous study with mice bearing intracranial M05 melanoma, we showed that adoptively transferred ovalbumin (OVA)-specific Tc1 cells were superior to Tc2 cells in trafficking into the intracranial tumor lesions and in mediating potent therapeutic responses (4). These observations are consistent with previous reports by others that Tc2 (versus Tc1) cells are poorly recruited into sites of inflammation caused by viral infection or autoimmunity (5, 6), which may reflect differential expression of integrins/addressins and/or chemokine receptors by these T-cell populations. For example, the P-selectin and E-selectin ligands and the chemokine receptors CXCR3 and CCR5, associated with trafficking into sites of inflammation, are preferentially expressed on type 1 compared with type 2 T cells (7).

More recently, certain integrins, such as very late antigen (VLA)-1, VLA-2, and VLA-6, have also been identified as type 1–associated molecules that are implicated in the specific recruitment of type 1 cells (8–10). In this report, we show for the first time that VLA-4 (CD49d/CD29) is preferentially expressed on Tc1 cells, but not on Tc2 cells, and that this integrin plays a critical role in the effective traffic of therapeutic Tc1 cells into central nervous system (CNS) tumors.

Materials and Methods

Mice. C57BL/6 mice (5–9 weeks of age) and OVA$_{257-264}$–specific TCR transgenic OT-1 mice (RAG-1$^{-/-}$ C57BL/6 background) were purchased from Taconic and maintained in a pathogen-free animal facility at the University of Pittsburgh Cancer Institute. All animal work was done in accordance with Institutional Animal Care and Use Committee–approved protocol.

Reagents. Recombinant murine (rm) IL-12 was purchased from Cell Sciences. rmIL-4, rmIL-10, rmIL-13, and recombinant human IL-2 (rhIL-2) were purchased from PeproTech. Mouse VCAM-1-Ig fusion protein, purified anti-CD49d monoclonal antibody (mAb; R1-2), purified anti-VCAM-1 mAb (MVCAM.A), and purified isotype control rat IgG2a (54447) were purchased from R&D Systems. Purified anti-CD49d mAb (PS/2) was purchased from Southern Biotech. Purified isotype control rat IgG2b (RTK4530) was purchased from BioLegend. Purified mAbs against IL-12 (C15.6), IFN-γ (R4-6A2), IL-4 (11B11), CD8, CD49a, and CD49c; FITC-conjugated mAbs recognizing CD29 (HMβ1), CD11a, CD54, CD25, CD62L, or IFN-γ; FITC-anti-hamster IgG polyclonal antibody; phycoerythrin (PE)-conjugated mAbs against CD49d, CD49b, CD49e, CD49f, $\alpha_4\beta_7$ integrin (LPAM-1), and IL-4; and

PE-anti-rat IgG polyclonal antibody were all purchased from BD PharMingen. PE-OVA-tetramer was purchased from Beckman Coulter. $OVA_{257-264}$ peptide (>95% pure) was synthesized by *N*-(9-fluorenyl)methoxycarbonyl chemistry in the University of Pittsburgh Cancer Institute Peptide Synthesis Facility.

Generation of OVA-specific CD8$^+$ effector T cells. Tc1 and Tc2 cells for adoptive transfer were induced from magnetic-activated cell sorting–separated naive CD8$^+$ splenic T cells isolated from OT-1 mice. Purified CD8$^+$ cells were stimulated with 5 μg/mL $OVA_{257-264}$ peptide in the presence of irradiated (3,000 rad) C57BL/6 spleen cells as feeder cells,
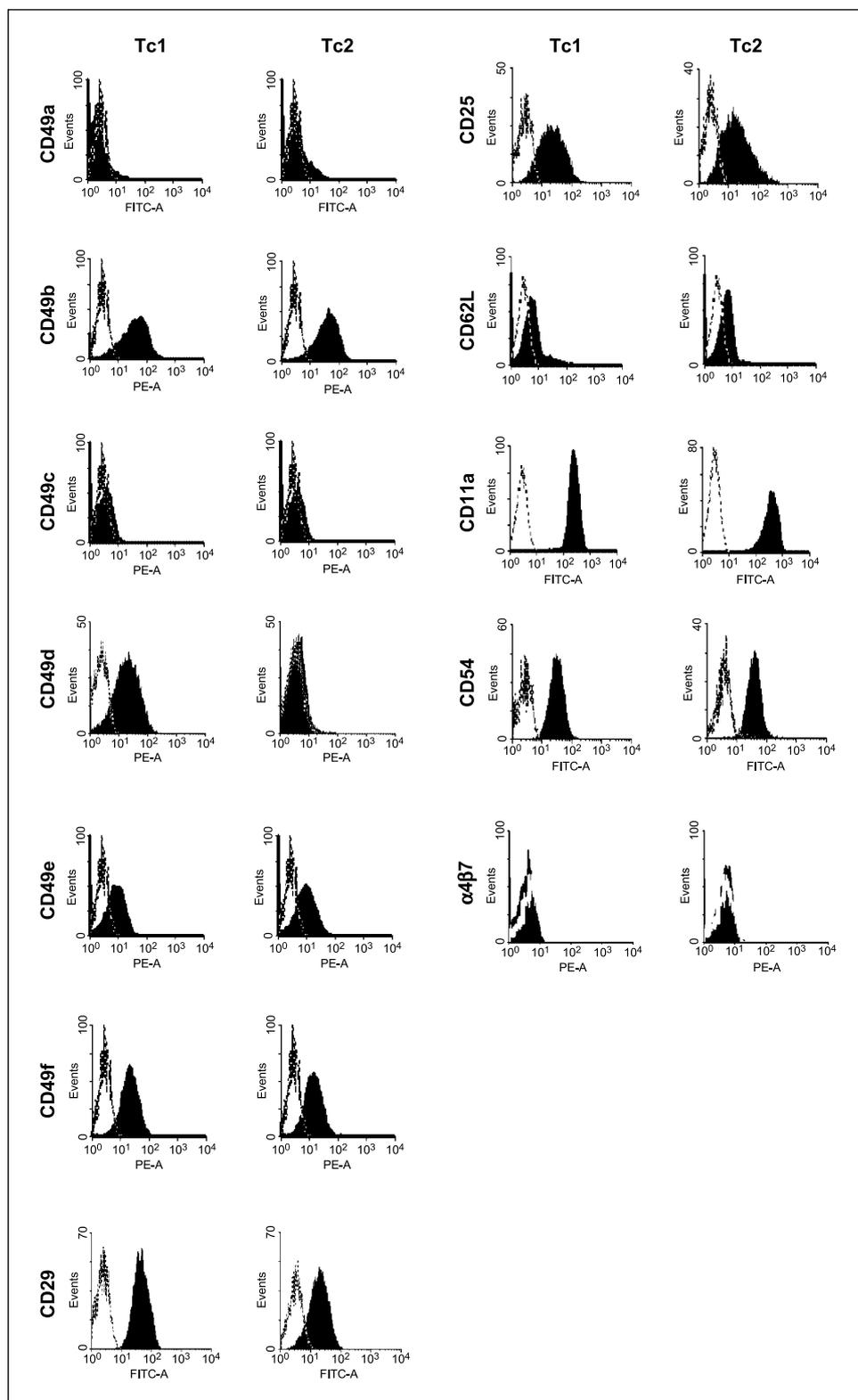

Figure 1. *Ex vivo*–generated Tc1 cells preferentially express VLA-4. Tc1 and Tc2 cells were induced from naive CD8$^+$ cells as described in Materials and Methods. On day 12, Tc1 and Tc2 cells were analyzed by flow cytometry for the expression of CD11a (LFA-1 α chain), CD54 (ICAM-1), CD49a (α_1 integrin), CD49b (α_2 integrin), CD49c (α_3 integrin), CD49d (α_4 integrin), CD49e (α_5 integrin), CD49f (α_6 integrin), CD29 (β_1 integrin), CD25, CD62L, and $\alpha_4\beta_7$ integrin (LPAM-1). Data are representative of three independent experiments with similar results.

Figure 2. Kinetics of CD49d/CD29 expression on Tc1 and Tc2 cells. Naive T cells were stimulated *in vitro* under the Tc1- or Tc2-polarizing condition as described in Materials and Methods. *A, right,* flow cytometric analysis of CD49d (α_4 integrin) and CD29 (β_1 integrin) on Tc1 and Tc2 cells on days 4 and 13 following the primary antigen challenge; *left,* time course of CD49d expression on Tc1 and Tc2 cells *in vitro*. The CD49d expression on Tc1 cells was significantly higher than Tc2 ($P = 0.0002$, 0.0006, and 0.0002 on days 7, 10, and 13, respectively). Data represent mean ± SD from three independent experiments done. *B,* on day 6 following each round of weekly stimulation, Tc1 and Tc2 cells were evaluated for CD49d/CD29 expression. Primary effector CD8^{+} T cells were generated with one round of stimulation and cultured for 10 d before flow cytometric evaluation. Data are representative of three independent experiments done.
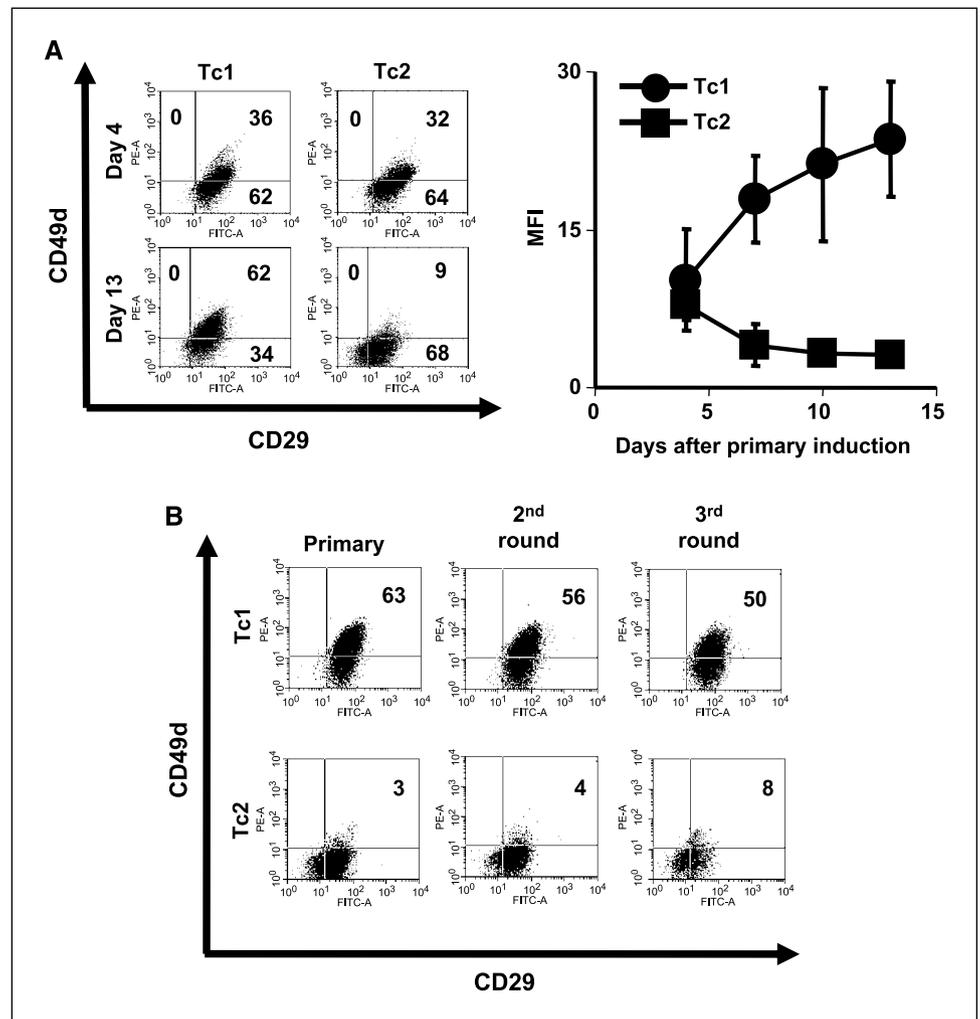
2 ng/mL rmIL-12, 1 μg/mL anti-IL-4 mAb, and 100 units/mL rhIL-2 for Tc1 development. Tc2 cells were generated from the same CD8^{+} cell precursors stimulated with 5 μg/mL OVA$_{257-264}$ peptide-pulsed feeder cells in the presence of 100 ng/mL rmIL-4, 10 μg/mL of two anti-IFN-γ mAbs (R4-6A2 and XMG1.2), 10 μg/mL anti-IL-12 mAb (C15.6), and 100 units/mL IL-2. For evaluation of long-term cultured Tc1 and Tc2 cells, cells were restimulated weekly with OVA peptide under Tc1- or Tc2-polarizing condition described above. In some experiments, T cells were generated in the presence of 100 units/mL rhIL-2 over a range of rmIL-4, rmIL-10, or rmIL-13 doses (0, 0.1, 1, 10, and 100 ng/mL). When indicated, in other experiments, T cells were generated in the presence of 100 units/mL rhIL-2 with IL-12 and IFN-γ or with anti-IL-4 mAb. After 48 h, cells were restimulated under the same conditions used for primary induction. When used, rmIL-12 and rmIL-4 were added only for the initial 6 days of culture and then removed. rhIL-2 was maintained for the entire culture period. At day 10 after stimulation, T cells were harvested to measure specific IFN-γ and IL-4 production using ELISAs and intracellular staining was monitored by flow cytometry as described elsewhere (11) to confirm functional polarization status (Supplementary Fig. S1). In some experiments, specific CTL activity was determined in standard 4-h ^{51}Cr release assays against M05 or B16 melanoma target cells as described previously (11).

Costimulation of Tc cells with VCAM-1-Ig. Aliquots of Tc1 or Tc2 cells suspended in 0.5% bovine serum albumin (BSA) in serum-free RPMI 1640 were added to individual wells in 96-well plates (2×10^5 per well) coated with a low dose (0.5 μg/mL) of anti-CD3 mAb together with either 10 μg/mL of VCAM-1-Ig or control human-Ig. After incubation for 12 h, supernatants were collected and IFN-γ production was measured by specific ELISA. The results represent mean ± SD of duplicate samples.

Cell adhesion assay. Ninety-six–well ELISA plates (Corning) were coated with 10 μg/mL of mouse VCAM-1-Ig or control human-Ig dissolved in 50 μL PBS for 3 h at 37°C. After washing twice with PBS, plates were blocked by incubation with 1% BSA in 50 μL PBS for 1 h at 37°C. Plates were then washed twice and used for assays. Tc1 and Tc2 cells were harvested at days 10 to 14, washed twice with PBS, and suspended in binding buffer (0.5% BSA, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$ in PBS) and then added to the plate. For blocking experiments, cells resuspended in binding buffer were pretreated with 20 μg/mL of anti-CD49d mAbs (R1-2 or PS/2) for 15 min at 37°C and then added to the plate. Plates were centrifuged at 500 rpm for 1 min and cells were allowed to adhere for 30 min at room temperature with gentle shaking. The plate was then gently washed thrice using binding buffer, and the number of adherent cells was enumerated by flow cytometry.

In vivo **homing of Tc1 and Tc2 cells into intracranial M05 tumors.** Using a Hamilton syringe, 5×10^4 M05 (OVA-transfected B16 melanoma) cells were stereotactically injected through an entry site at the bregma, 3 mm to the right of sagittal suture and 4 mm below the surface of the skull of anesthetized mice using a stereotactic frame (Kopf). On day 12, mice received an i.v. injection with 5×10^6 to 8×10^6 of cultured day 9 to 12 Tc1 or Tc2 cells. For VLA-4 inhibition experiments, 150 μg/mouse of anti-CD49d mAb (PS/2 or R1-2) or an isotype control rat IgG2b mAb (RTK4530) was added to the cell mix before i.v. infusion. For VCAM-1 inhibition experiments, 200 μg/mouse of an anti-VCAM-1 mAb (MVCAM.A) or an

isotype-matched, control rat IgG2a mAb (54447) was injected i.v. 2 h before Tc1 cell transfer. For experiments with small interfering RNA (siRNA) silencing, 5×10^6 of either nontargeting control siRNA–transfected or CD49d siRNA–transfected Tc1 cells were adoptive transferred. Twenty-four hours later, the ratio of migrated Tc cells was assessed by flow cytometry as described previously (4).

siRNAs and nucleofection. Tc1 cells cultured for 9 days were resuspended ($5 \times 10^7/100\ \mu L$) in Nucleofector solution (Mouse T Cell Nucleofector kit, Amaxa). siRNA ($2\ \mu g$) was added and gently mixed. The cell-siRNA mixture was then transferred to an electroporation cuvette and placed in the Nucleofector I device (Amaxa). Nucleofection of the cells was accomplished using the X-01 program. Cells were then transferred to 4 mL of prewarmed medium and incubated for 30 h at 37°C before used for the assay. The siRNA sequences specific for mouse CD49d #67813 [sense, 5′-GGAUGUUUGGACAAUCUAUTT-3′; antisense, 5′-AUAGAUUGUCCAAA-CAUCCTT-3′] and a nontargeting siRNA #4 (Ambion, Inc.) were synthesized and annealed by the manufacturer (Ambion).

Statistical analyses. All intergroup comparisons of means were assessed with one-sided, equal variance t tests. Before testing, percentage data were logit transformed and all other data were log transformed. P values of ≤0.05 were considered significant.

Results

VLA-4 (CD49d/CD29) is preferentially expressed by Tc1 versus Tc2 cells. To delineate mechanisms underlying the efficient CNS tumor homing of Tc1 cells (4), we investigated expression of a panel of homing receptors on Tc1 and Tc2 cells generated *ex vivo* from naive OT-1 splenocytes (Fig. 1). Among the receptors evaluated, we observed that CD49d (α_4 integrin), which forms $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrin heterodimers at the cell surface (12), was preferentially expressed on Tc1 compared with Tc2 cells. Interestingly, no other surface markers evaluated, including CD29 (β_1 integrin), exhibited differential staining patterns on Tc1 versus Tc2 cells.

Other activation markers, such as CD25 and CD62L, exhibited similar levels of expression on Tc1 and Tc2 cells. This suggests that the difference of VLA-4 expression on Tc1 versus Tc2 is unlikely to represent a generic difference in activation status between Tc1 and Tc2 cells. Interestingly, although CD29 (β_1 integrin) can pair with at least six different α integrin chains on T cells, Tc1 and Tc2 cells displayed similar levels of each of the other α integrins (CD49a, CD49b, CD49c, CD49e, and CD49f), indicating that VLA-4 is a unique integrin associated with committed Tc1 cells. Although the CD49d (α_4 integrin) can be expressed in both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers at the cell surface (12, 13), $\alpha_4\beta_7$ complexes were not expressed on either Tc1 or Tc2 cells, suggesting that CD49d solely forms VLA-4 heterodimers on Tc1 cells.

VLA-4 serves as a novel marker dissociating Tc1 versus Tc2 cells. To understand how VLA-4 expression is regulated during differentiation of Tc1 and Tc2 cells, we next examined the time course of CD49d/CD29 expression on Tc1 and Tc2 cells. Although naive CD8+ cells showed little expression of CD49d and very low levels of CD29 (data not shown), VLA-4 expression initially increased on both cultured Tc1 and Tc2 cells by day 4 (Fig. 2A). Thereafter, however, CD49d expression was consistently up-regulated/maintained on Tc1 cells, whereas a marked decrease in CD49d expression was observed on Tc2 cells. Consequently, mature Tc1 cells express higher levels of CD49d when compared
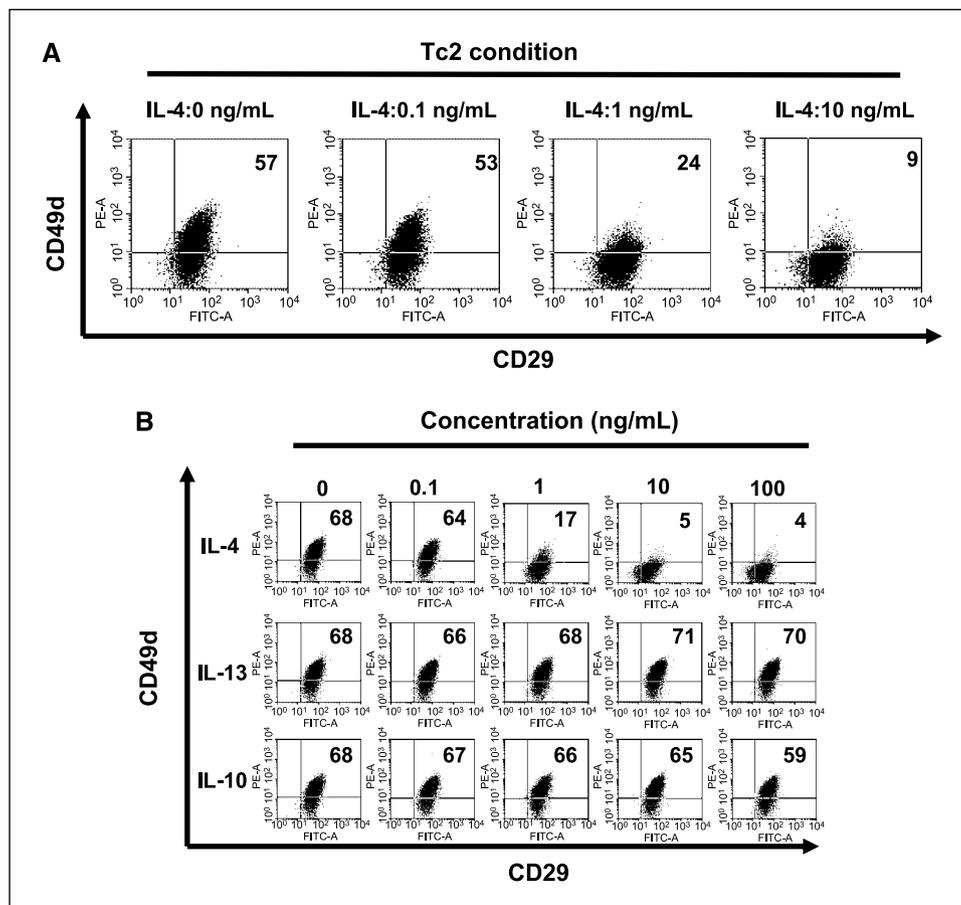

Figure 3. IL-4 down-regulates VLA-4 expression on Tc2 cells in a dose-dependent manner. *A,* OT-1 mouse–derived naive CD8+ T cells were stimulated *in vitro* under the Tc2-polarizing condition using either 0, 0.1, 1, or 10 ng/mL of rmIL-4 in the presence of 100 units/mL rmIL-2, anti-IFN-γ mAbs, and anti-IL-12 mAb. CD49d/CD29 expression was evaluated on day 10 of culture. *B,* OT-1 mouse–derived naive CD8+ T cells were stimulated *in vitro* with increasing doses of rmIL-4, IL-10, or IL-13 (0, 0.1, 1, 10, and 100 ng/mL) in the presence of rmIL-2 as described in Materials and Methods. Cells were harvested on day 10 of culture and VLA-4 (CD49d/CD29) expression was assessed by flow cytometry. Data are representative of three independent experiments done.

with mature Tc2 cells by day 13 (i.e., CD49d was found to be expressed on >60% of Tc1 cells but on <10% of Tc2 cells; Fig. 2*A*). In contrast, CD29 was expressed in a majority of both Tc1 and Tc2 cells (Fig. 1*A*).

We next examined if the differential expression of VLA-4 is maintained after long-term cultured Tc1 and Tc2 cells. As shown in Fig. 2*B*, differential expression pattern of VLA-4 was sustained even in third-round stimulated Tc1 and Tc2 cells, suggesting that VLA-4 could serve as a novel stable marker that distinguish Tc1 versus Tc2 cells at least *in vitro*.

IL-4 down-regulates CD49d expression on murine CD8^{+} T cells. To elucidate putative mechanisms underlying differential VLA-4 expression on Tc1 versus Tc2 cells, CD49d/CD29 expression on OT-1 mouse–derived CD8^{+} T cells was evaluated under Tc2-polarizing cytokine conditions using a range of rmIL-4 doses (0, 0.1, 1, or 10 ng/mL; Fig. 3*A*). In the presence of fixed doses of anti-IFN-γ and anti-IL-12 mAbs, rmIL-4 promoted the down-regulation of CD49d expression in a dose-dependent manner (Fig. 3*A*). Moreover, even in the absence of anti-IFN-γ mAbs or anti-IL-12 mAb, IL-4 down-regulated CD49d expression on OT-1 mouse–derived CD8^{+} T cells (Fig. 3*B*), whereas other Th2 cytokines, such as IL-10 and IL-13, failed to down-regulate CD49d expression even at high concentrations (10–100 ng/mL), suggesting a novel and specific role for IL-4 in negatively regulating VLA-4 expression on CD8^{+} effector T cells (Fig. 3*B*).

VLA-4 on Tc1 cells mediates adhesion and costimulatory signaling through VCAM-1. To examine the functional significance of differentially expressed VLA-4 on Tc subsets, Tc1 and Tc2 cells were tested for their ability to adhere to plate-bound VCAM-1-Ig fusion protein. In line with the differential expression of VLA-4 on Tc1 and Tc2 cells, Tc1 cells showed specific adhesion to plate-coated VCAM-1-Ig (63.3 ± 7.1% of the total plated cells adhered), whereas Tc2 cells showed only background levels of adhesion (7.25 ± 1.25%; Fig. 4*A*). Furthermore, pretreatment of Tc1 cells with the anti-VLA-4α mAbs (PS/2 or R1-2) significantly blocked their ability to adhere to VCAM-1-Ig–coated plates compared with Tc1 cells pretreated with isotype control IgG (Fig. 4*B*).

With regard to the inability of Tc2 cells to adhere to VCAM-1-Ig–coated plates, it has been reported that type 2 T cells can exhibit functional defects in integrin receptors (11, 14), such as the defects in activation-induced alterations in affinity/avidity of integrins (15), rather than the lack of receptor expression. However, given that early (day 4) Tc2 cells expressing significant levels of VLA-4 are competent to adhere to immobilized VCAM-1 (Supplementary Fig. S2), the failure of long-term cultured Tc2 cells to respond to VCAM-1 is most likely due to the deficiency in VLA-4 expression rather than to impaired VLA-4 activation on Tc2 cells.

Because the VLA-4-VCAM-1 interaction is known to provide costimulation to T cells (12, 13), we next evaluated the costimulatory effects of VCAM-1 on Tc1 and Tc2 cells using VCAM-1-Ig or control human-Ig coimmobilized with a low dose of anti-CD3 mAb. As expected, Tc1 cells produced IFN-γ on stimulation with VCAM-1-Ig and anti-CD3 mAb (9.16-fold increase compared with Tc1 stimulated with control Ig and anti-CD3mAb; *P* = 0.0032), whereas Tc2 cells were poorly responsive (Fig. 4*C*). Together, these results suggested that VLA-4 expressed on Tc1, but not Tc2, cells acts not only as a functional adhesion molecule but also as a costimulatory molecule for Tc1 cell activation.
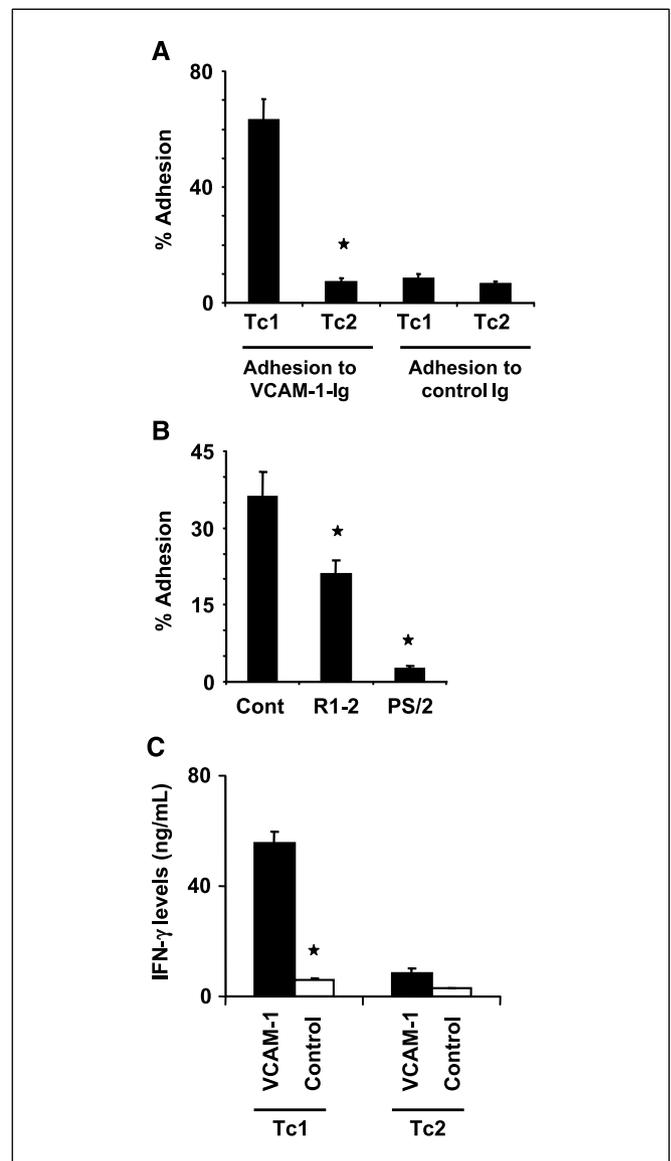

Figure 4. VLA-4 expressed by Tc1 plays a critical role in adhesion to VCAM-1 and VCAM-1–mediated costimulation *in vitro*. *A*, Tc1 and Tc2 cells were incubated in 96-well plates coated with either VCAM-1-Ig or control human-Ig for 30 min. The plates were then washed thrice with binding buffer, and the number of cells adherent to the bottom of the wells was counted by flow cytometry as described in Materials and Methods. *P* = 0.006, percentage adhesion of Tc1 versus Tc2 against VCAM-1-Ig. *B*, Tc1 cells were preincubated with either control rat IgG2b mAb or anti-VLA-4 mAb (R1-2 or PS/2) before being inoculated to VCAM-1–coated plates. *, *P* = 0.049, control mAb versus R1-2 pretreated groups; *, *P* = 0.0039, control versus PS/2 pretreated groups. *C*, Tc1 or Tc2 cells were plated to 96-well plates coated with anti-CD3 mAb together with either VCAM-1-Ig or control human-Ig. After incubation for 12 h, supernatants were collected and IFN-γ production by Tc1 and Tc2 cells was measured by ELISA. *, *P* = 0.0008, VCAM-1–stimulated versus control-stimulated Tc1 groups. *Columns*, mean of samples in duplicates; *bars*, SD. Data are representative of three independent experiments done.

***In vivo* blocking of CD49d abrogates intracranial tumor homing of Tc1 cells.** Finally, the significance of VLA-4 expression on Tc1 cells was determined in mice bearing intracranial M05 melanomas. Tumor-bearing mice received i.v. transfers of 8×10^6 Tc1 or Tc2 cells developed from OT-1 mice. One day later, brain-infiltrating lymphocytes (BIL) were isolated and analyzed by flow cytometry. Consistent with our previous study (4), a high

percentage (15%) of BILs gated for lymphocytes represented OVA-specific CD8^{+} T cells as identified by the H-2K[b]/OVA peptide-specific tetramer following Tc1 infusion, whereas very few (0.2%) CD8^{+}/tetramer^{+} events were identified in BILs following Tc2 infusion (Fig. 5A, *left*). This confirms our previous study (4) that Tc1 cells can efficiently traffic into intracranial M05 tumor sites. The total number of OVA-reactive CD8^{+} BILs isolated from mice receiving i.v. Tc1 cells was also remarkably higher than that observed for mice receiving Tc2 cells (Fig. 5A, *right*). Neither Tc1 nor Tc2 cells accumulated in spleen, cervical lymph node, and inguinal lymph node at high levels (OVA-tetramer–reactive cells were <0.6% of total lymphocytes in each organ in both Tc1 and Tc2 groups; data not shown).

We subsequently examined the role of VLA-4 on Tc1 cell trafficking into intracranial M05 tumors by coinjecting anti-VLA-4 mAb (R1-2 or PS/2) or isotype control mAb together with Tc1 cells in tumor-bearing mice. In accordance with our *in vitro* results, although both the R1-2 and PS/2 mAbs significantly inhibit Tc1 cell migration into intracranial M05 tumors, PS/2 was more effective than R1-2 [85% inhibition (PS/2) versus 37% inhibition (R1-2)] at comparable dosing (Fig. 5B). To further confirm the significance of VLA-4/VCAM-1–mediated Tc1 cell trafficking into intracranial M05 tumors, anti-VCAM-1 mAb was given i.v. 2 h before Tc1 cell transfer, with BIL analyzed at 24 h after the adoptive transfer of Tc1 cells. As shown in Fig. 5C, blocking of VCAM-1 significantly reduced the entry of Tc1 cells into intracranial M05 tumor lesions.

siRNA-mediated inhibition of CD49d reduces Tc1 cell homing to intracranial tumors. To further confirm the specific role of VLA-4 expression on Tc1 cells, we next used siRNA-mediated silencing of VLA-4 on Tc1 cells. Tc1 cells transfected with specific murine CD49d siRNA showed >50% reduction of CD49d expression compared with control Tc1 cells transfected with nontargeting control siRNA (Fig. 6A). Nontargeting control siRNA did not alter CD49d expression levels compared with Tc1 cells without transfection (data not shown). CD29 expression levels were found to be similar between all groups (Fig. 6A). We also confirmed that inhibition of CD49d expression was sustained up to 3 days (data not shown). These CD49d siRNA–transfected or nontargeting control siRNA–transfected Tc1 cells were subsequently adoptive transferred into intracranial M05 tumor-bearing mice, and the trafficking of these transferred cells into brain tumor lesion was counted 24 h later. As expected, inhibition of CD49d expression on Tc1 cells diminished the migration of these T cells into the intracranial M05 tumor lesion by >50% (Fig. 6B). Collectively, these data support the critical role of VLA-4/VCAM-1 in the ability of antigen-specific Tc1 cells to effectively home into CNS tumor sites, a prerequisite for effective therapy.

Discussion

The most significant finding in the current study is that differentially expressed VLA-4 on Tc1 versus Tc2 cells seems to play a fundamental role in the preferential trafficking of Tc1 cells into intracranial tumor lesions and the consequent superior efficacy associated with the adoptive transfer of tumor antigen-specific Tc1 effector cells (4).
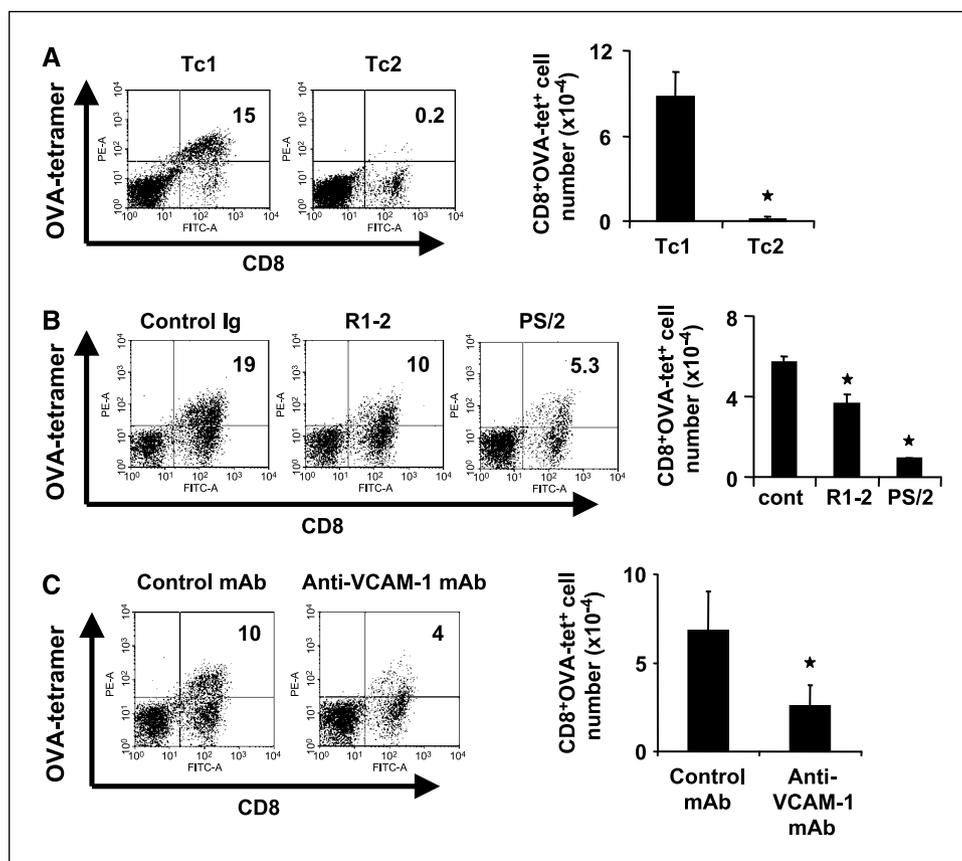

Figure 5. VLA-4 on Tc1 cells plays a critical role in their trafficking into brain tumor sites. BILs were harvested 24 h after the adoptive transfer of cells and stained with PE-H-2K[b]-OVA$_{257-264}$–specific tetramer and FITC-anti-CD8 mAb and then analyzed by flow cytometry to enumerate the percentage and total numbers of CD8^{+}/OVA-tetramer^{+} populations in the lymphocyte-gated populations. *A,* mice bearing day 12 M05 tumors in the brain received i.v. adoptive transfer of 8 × 10^{6} Tc1 cells or Tc2 cells. *,* *P* = 0.0018, Tc1 versus Tc2 transferred groups. *B,* M05-bearing mice received adoptive transfers of 8 × 10^{6} Tc1 cells and either 150 μg/mouse anti-VLA-4 mAb (R1-2 or PS/2) or isotype control rat IgG2b mAb. *,* *P* = 0.020, control-treated versus R1-2–treated groups; *,* *P* = 0.00016, control-treated versus PS/2-treated groups. *C,* M05-bearing mice received i.v. injections of 200 μg/mouse anti-VCAM-1 mAb or isotype control rat IgG2a mAb 2 h before Tc1 cell transfer. *,* *P* = 0.013, control mAb-treated versus anti-VCAM-1 mAb-treated groups. *A* to *C, columns,* mean of three mice; *bars,* SD. Data are representative of at least two experiments with similar results.
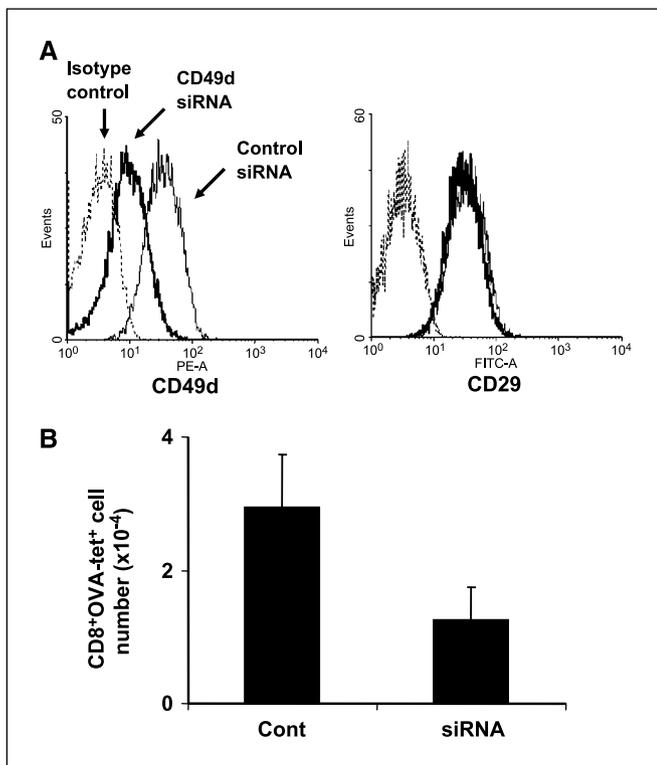
Figure 6. siRNA-mediated suppression of CD49d on Tc1 cells inhibited *in vivo* trafficking of Tc1 cells into intracranial M05 tumors. Tc1 cells were transfected *in vitro* with either CD49d-specific siRNA or nontargeting control siRNA. *A,* expression of VLA-4 (CD49d/CD29) on Tc1 cells was analyzed at 30 h after transfection. *Thin line,* negative control siRNA; *thick line,* CD49d siRNA; *dotted line,* isotype control. *B,* 5×10^6 cells/mouse of either CD49d-transfected or negative control siRNA–transfected Tc1 cells were adoptively transferred in M05-bearing mice. At 24 h after adoptive transfer, numbers of BILs per mouse were assessed as described for data in Fig. 5. *n* = 2/group. *Columns,* mean of two mice; *bars,* SD.

We initially showed that mature Tc1 and Tc2 cells exhibit distinct VLA-4 expression profiles likely due to IL-4–mediated down-regulation of VLA-4 expression on Tc2 cells (Figs. 1–3). Interestingly, although we did not observe distinct CD49b expression levels in Tc1 versus Tc2 cells in the current study, IL-4 down-regulated VLA-2 (a heterodimer of CD49b and CD29)[7] as well as VLA-4 expression[7] on cultured Th1 versus Th2 cells, suggesting that a common signaling cascade may exist downstream of IL-4 receptor that negatively regulates VLA expression on T cells (8).

One may raise a question as to whether the absence of rIL-4 in primary inductions is sufficient to generate effector T cells with favorable characteristics for therapy of intracranial tumors. We have observed that blockade of IL-4 can augment CD8^{+} T-cell expression of CD49d and adhesion to plate-bound VCAM-1 (Supplementary Fig. S3*A* and *B*). On the other hand, although addition of IL-12 and IFN-γ in the priming culture did not enhance VLA-4 expression (data not shown), IL-12 and IFN-γ enhanced granzyme B and IFN-γ expression as well as CTL activity (Supplementary Fig. S3*C*–*E*). Together, these results suggest distinct roles of IL-4 blockade and type 1 cytokines (i.e., IFN-γ and IL-12) for generation of Tc1 effector cells favorable for anti-CNS tumor therapy (1).

The leukocyte integrin VLA-4 and its counterreceptor VCAM-1 have been previously identified as key mediators of T-cell entry into the CNS in multiple sclerosis and other CNS inflammatory conditions (16, 17). However, to date, few studies have addressed the role of VLA-4 in T-cell trafficking into CNS tumors. In the current study, we have documented a critical role for VLA-4/VCAM-1 in Tc1 cell trafficking into CNS tumors. In contrast to other CNS inflammatory conditions, the critical feature of CNS tumors is their ability to skew anti-CNS tumor immune responses toward type 2 and regulatory responses (18). Therefore, it is of critical significance to find VLA-4 expression on Tc1 cells as a critical factor that facilitates T-cell entry into CNS tumors. In addition, our current findings may have significant implications to CNS and basic immunology in the following respects: (*a*) differential expression of VLA-4 on Tc1 and Tc2 cells may explain the dominance of type 1 over type 2 response in CNS autoimmune pathology (17, 19) and (*b*) the critical role of IL-4 in down-regulation of VLA-4 on T cells, which may at least partially explain the *in vivo* efficiency of IL-4 in ameliorating CNS autoimmunity (19, 20).

To improve the magnitude and durability of antitumor immune response, adoptive-transferred cells need to home to lymphoid organs and subsequently undergo priming/restimulation efficiently (1). Further studies are clearly warranted to investigate the longitudinal trafficking/expansion of Tc1 in the peripheral lymphoid organs of tumor-bearing mice. Even in secondary lymphoid organs, we would anticipate that the differential expression of VLA-4 by Tc1 versus Tc2 cells may be sustained and provide costimulation to Tc1 cells via its interaction with ligands expressed by antigen-presenting cells (APC; ref. 21). In this context, Calzascia et al. (22) have shown that APCs derived from the brain tumor environment cross-present tumor antigens to Tc cells in the draining lymph node, thereby imprinting responder Tc cells with a VLA-4^{+} phenotype, which is important for their consequent entry into CNS tumors.

Although type 1 cells are known to migrate into sites of inflammation based on the selective expression of P-selectin and E-selectin ligands and chemokine receptors (CXCR3 and CCR5; ref. 7), selectins do not seem to be directly involved in T-cell trafficking into CNS autoimmune lesions (23, 24). Hence, we would suggest that, based on our current and previously published work (4), the recruitment of therapeutic Tc1 cells into intracranial M05 tumor sites is directed by the chemokine CXCL10/IP-10 (4) and the VLA-4/VCAM-1 interaction. Although additional factors that affect the preferential migration of Tc1 cells into CNS tumors will undoubtedly be identified in the future, our studies support the development of vaccine or *ex vivo* adoptive therapy protocols that expand antitumor, VLA-4^{+} Tc1 cells that may mediate beneficial clinical outcomes in patients with advanced-stage disease and/or brain tumors/metastases.

Acknowledgments

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[7] Our unpublished data.

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Preferential Expression of Very Late Antigen-4 on Type 1 CTL Cells Plays a Critical Role in Trafficking into Central Nervous System Tumors

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