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IL-4 suppresses Very Late Antigen-4 expression which is required for therapeutic Th1 T cell trafficking into tumors^{1,2}

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Abstract

Murine CD4⁺ T cells cultured under Type-1 polarizing conditions selectively express significantly higher levels of the VLA-4 and VLA-6 integrins when compared to T cells cultured under Type-2 or non-polarizing (Type-0) conditions. This difference appears due to the action of IL-4, since loss of VLA-4/-6 expression on Th cells was prevented by inclusion of neutralizing anti-IL-4 mAb during the initial culture period. We also observed that CD4⁺ T cells deficient in Stat6, a critical component of the IL-4R signaling cascade, retained high levels of VLA-4 and VLA-6 expression, regardless of IL-4 status in culture conditions. When applied to committed Th1 cells, rIL-4 readily inhibited VLA-4 and VLA-6 expression to levels observed for Th2 cells, without altering the Type-1 functional status of these cells. Conversely, low levels of VLA-4/-6 expressed by committed Th2 cells could not be resurrected by culture in the presence of the Th1-kines IL-12p70 and IFN- γ . Predictably, among the Th populations evaluated, Th1 cells alone adhered efficiently to, and were co-stimulated by, plate-bound VCAM-1 and laminin in a VLA-4- or VLA-6-dependent manner, respectively. Finally, adoptive-transferred Th1 (but not Th2) cells developed from OT-II mice were uniquely competent to traffic into OVA⁺ M05 melanoma lesions *in vivo*, thereby enhancing the therapeutic benefits associated with co-transferred OVA-specific Tc1 (OT-I) cells. These data suggest that treatment strategies capable of sustaining/enhancing VLA-4/-6 expression on Th1 effector cells may yield improved clinical efficacy in the cancer setting.

Keywords

VLA-4; VLA-6; IL-4; T helper-1; melanoma

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INTRODUCTION

Primed CD4⁺ T cells may be subdivided into functional subsets based on their patterns of cytokine secretion in response to cognate antigen. Th1 cells produce IFN- γ and IL-2, and activate macrophages and cytotoxic T lymphocytes to promote cell-mediated immunity against intracellular pathogens and transformed cells, while Th2 cells produce IL-4, IL-5, and IL-13 and play important roles in the regulation of humoral and innate immunity against extracellular pathogens (1,2). Other CD4⁺ T cell functional subsets include Th17 cells, that secrete IL-17 and promote inflammatory responses involving activated macrophages and neutrophils (3,4), and Th3/Treg cells, that produce IL-10 and/or TGF- β while serving as “suppressor” T cells (5,6).

One important variable in the maintenance of functionally-polarized immune responses appears to be the acquisition of specific homing receptors that direct the localization of the different types of effector T cells within relevant sites of antigenic surveillance/restimulation (7,8). In this regard, signals delivered by distinct cytokines may imprint developing T cell subsets with specific homing patterns by “setting” expression levels of certain adhesion molecules and chemokine receptors (8,9). For example, the preferential expression of P- and E-selectin ligands (10) and the chemokine receptors CXCR3 and CCR5 (11) on Th1 T cells, at least in part, explains their selective recruitment into sites of inflammation. However, despite the key role(s) that integrins play in leukocyte recruitment and function, their regulated expression by distinct, polarized Th cell subsets has remained understudied.

We have recently demonstrated that Very Late Antigen (VLA)-4 (a heterodimer of the $\alpha 4$ and $\beta 1$ integrins) is preferentially expressed by Type-1 CD8⁺ cells (Tc1) when compared to Type-2 CD8⁺ T cells (Tc2), providing Tc1 cells with a differential ability to infiltrate into tumor lesions after adoptive T cell transfer (12,13). Since integrins expressed by CD4⁺ T cells are similarly known to play critical role(s) in regulating inflammation in peripheral tissues (including tumors), we sought to determine whether certain integrins (including VLA-4) were also preferentially expressed by Type-1 CD4⁺ T cells (Th1 cells) and associated with the ability of these cells to trafficking into tumor lesions *in vivo*. In this report, we show for the first time that VLA-4 (CD49d/CD29) and VLA-6 (CD49f/CD29) are both preferentially expressed on murine Th1 cells, but not on Th2 cells, and that these integrins are crucial to the migration of Type-1 CD4⁺ T cells into tumor lesions.

MATERIALS AND METHODS

Mice

C57BL/6 mice, BALB/c mice (5-9 weeks of age), OVA₃₂₃₋₃₃₉-specific TCR transgenic OT-II mice (RAG-1^{-/-} C57BL/6 background), Stat6^{-/-} mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). OVA₂₅₇₋₂₆₄-specific TCR transgenic OT-I mice (RAG-1^{-/-} C57BL/6 background) were purchased from Taconic (Germantown, NY). Mice were maintained in a specific-pathogen-free animal facility at the University of Pittsburgh Cancer Institute. All animal work was done in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Reagents

Reagents used in this study included: rmIL-12 (Cell Sciences, Canton, MA), rhIL-2, rmIL-4, rmIL-6 and mTGF- β (all from Peprotech, Rocky Hill, NJ), mouse VCAM-1-Ig fusion protein (R&D Systems, Minneapolis, MN), purified anti-CD49d mAb (PS/2; Southern Biotech, Birmingham, AL), purified anti-CD49f mAb (Go H3; GeneTex, San Antonio, TX), and purified isotype-control rat IgG2b (RTK4530; BioLegend, San Diego, CA). Additional

antibodies (all from BD-Pharmingen, San Diego, CA) included purified anti-mIL-12 mAb C15.6, anti-mIFN- γ mAbs R4-6A2 and XMG1.2, anti-mIL-4 mAb 11B11, anti-mCD3 mAb 145-2C11, anti-mCD29 mAb Ha2/5, fluorescein isothiocyanate (FITC)-conjugated anti-CD29 mAb HM β 1, FITC-anti-IFN- γ mAb, FITC-anti-CD69 mAb, FITC-anti-CD25 mAb, FITC-anti-CD54 mAb, FITC-anti-CD11a mAb and Phycoerythrin (PE)-conjugated anti-CD49d mAb, PE-anti-CD49f, PE-anti-IL-4 mAb, PE-anti-IL-17 mAb, PE-anti-CD44 mAb and PE-anti- α 4 β 3 integrin mAb. The OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉ peptides (>95% pure) were synthesized by N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry by the University of Pittsburgh Cancer Institute (UPCI) Peptide Synthesis Facility and confirmed for identity by the UPCI Protein Sequencing Facility (both Shared Resources).

Generation of functionally-polarized CD4⁺ T cells

Th1 and Th2 cells were induced from MACS-separated naïve CD4⁺ splenic T cells isolated from wild-type (or Stat6^{-/-}) mice as described previously (14). Briefly, to generate Th1 cells, CD4⁺ T cells were stimulated with 5 μ g/ml soluble anti-CD3 mAb in the presence of irradiated (3000 rad) C57BL/6 spleen cells as feeder cells, rhIL-2 (100 U/ml), rmIL-12 (4 ng/ml), rmIFN- γ (4 ng/ml) and anti-IL-4 mAb (10 μ g/ml). Th2 cells were induced in anti-CD3/feeder cultures supplemented with rhIL-2 (100 U/ml), rmIL-4 (50 ng/ml), two anti-IFN- γ mAbs (R4-6A2 and XMG1.2; 10 μ g/ml each), and anti-IL-12 mAb (C15.6; 10 μ g/ml). Where indicated, in some experiments, T cells were generated in the presence of rhIL-2 (100 U/ml) with or without various cytokines (rmIL-4, rmIL-12, rmIFN- γ) or neutralizing mAbs (anti-IL-4 mAb or anti-IL-4 mAb + anti-IL-12 mAb + anti-IFN- γ mAbs). rmIL-4-treated Th1 cells (Th1^{IL-4}) were generated by culturing day 6 Th1 cells with rmIL-4 (10 ng/ml) and rhIL-2 (100 U/ml) for 6 days.

For the adoptive-transfer experiments, polarized Th1 and Th2 cells were generated from OT-II mice using specific peptide (OVA₃₂₃₋₃₃₉; 5 μ g/ml) stimulation instead of anti-CD3 mAb. OT-I-derived Tc1 cells were developed as previously described (12). In all experiments, rhIL-2 was maintained for the entire culture period. On days 6, 8 and 12 after primary *in vitro* antigenic stimulation, T cells were harvested and then restimulated with 5 μ g/ml of plate-bound anti-CD3 mAb for 6 hrs in the presence of 10 μ g/ml of brefeldin A (Sigma) over the final 2 hours of the incubation period. These cells were then analyzed for IFN- γ (for Th1) vs. IL-4 (for Th2) production by intracellular staining as monitored using flow cytometry (Supplemental Fig.1) in order to confirm their functional polarization status.

Cell adhesion assay

T cell adhesion to immobilized VCAM-1-Ig was assessed as described previously (12). Briefly, 96-well ELISA plates were coated with 10 μ g/ml of mouse VCAM-1-Ig, mouse laminin, mouse fibronectin, control-human Ig or heat-denatured BSA. T cells were harvested at day 9 of culture, suspended in binding buffer (0.5% BSA, 2 mM CaCl₂, 2 mM MgCl₂ in PBS) and then added to the plate. For blocking experiments, cells resuspended in binding buffer were pre-treated with 20 μ g/ml of anti-CD49d mAb (PS/2) or 20 μ g/ml anti-CD29 mAb (Ha2/5), or two different doses of anti-CD49f mAb (Go H3) (20 μ g/ml or 10 μ g/ml) for 15 min at 37°, before being added to microwells. Plates were then centrifuged at 500 rpm for 1 min and cells allowed to secure their adhesions for 30 min at 23° with gentle shaking. Plates were then gently washed 3 times using binding buffer and the number of adherent cells enumerated by flow cytometry. % Adhesion was calculated as follows; % Adhesion = [(Number T cells adherent to VCAM-1-Ig) – (Number of T cells to adhere to heat-denatured BSA)]/Total number of input T cells.

In vitro costimulation of T cells with VCAM-1-Ig or laminin

Aliquots of cultured day 12 Th1 or Th2 cells suspended in serum-free RPMI containing 0.5% BSA were added to 96-well plates (2 $\times$ 10⁵/well) that had been pre-coated with anti-CD3 mAb

(1 µg/ml or 8 µg/ml for co-immobilization with VCAM-1-Ig, laminin, respectively) together with either 5 µg/ml of VCAM-1-Ig, 50 µg/ml of laminin or an equivalent dose of heat-denatured BSA. After incubation for 24h, supernatants were collected and IFN- γ production measured by specific ELISA (BD-Pharmingen), with results reported as mean $\pm$ SD of triplicate determinations.

Adoptive T cell therapy of mice bearing s.c. M05 melanoma

C57BL/6 mice received s.c. injections of 1×10^6 M05 (OVA-transfected B16 melanoma) cells in their right flank on day 0. On day 7, tumor-bearing mice received i.v. tail-vein injections with $2-5 \times 10^7$ Th1 or Th2 cells developed from OT-II mice, with or without, 6×10^6 Tc1 cells developed from OT-I mice. Treated animals were monitored daily for any therapy-associated toxicity and for tumor size (in mm²). Mice were sacrificed when tumors exceeded 400 mm², if their lesions became ulcerated or if an animal displayed any signs of distress. Data are reported as percent survival over time and are displayed in Kaplan-Meier plots.

Trafficking of adoptively-transferred T cells into s.c. M05 tumors

To investigate the *in vivo* trafficking of T cells into M05 tumor bearing mice, cultured day 9 Th1 and Th2 cells generated from OT-II mice were labeled *in vitro* with 0.4 µM of Carboxyfluorescein Succinimidyl Ester (CFSE, Vybrant CFDA SE Cell Tracer kit; Molecular Probes) per the manufacturer's protocol. Seven days after tumor inoculation, mice received i.v. injections with 2×10^7 CFSE-labeled Th1 or Th2 cells. For VLA-4 or VLA-6 inhibition experiments, 50 µg/ml of anti-CD49d mAb (PS/2), anti-CD49f mAb (Go H3) or control rat IgG was used to pre-treat T cells prior to i.v. infusion. Additionally, the PS/2 (250 µg) or GoH3 (150 µg) mAbs or equivalent quantities of control rat IgG were administered i.p. 4h prior to adoptive T cell transfers. To examine TIL, M05 tumors were resected, minced and then enzymatically-digested using a cocktail of 1% collagenase, 1% hyaluronidase and 0.1% DNAase for 40 min at 23° with gentle stirring. The resulting cell suspension was filtered through cell strainers (BD-Biosciences, San Jose, CA) and the flow through overlaid on an equal volume of lympholyte-M (Cedarlane Laboratories, NC) prior to centrifugation for 20 min at $800 \times g$. An enriched population of tumor-infiltrating lymphocytes (TIL) was then recovered at the gradient interface. Lung-infiltrating lymphocytes were similarly isolated by enzymatical digestion, and along with splenocytes, used as controls, where indicated. Enriched lymphocytes were counterstained with PE-anti-mouse CD8 mAb and the frequency of CD8⁺CFSE⁺ T cells determined using flow cytometry.

Statistical analysis

All inter-group comparisons 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 ≤ 0.05 were considered significant.

RESULTS

The VLA-4 (CD49d/CD29) and VLA-6 (CD49f/CD29) integrins are preferentially expressed by Th1 versus Th2 cells

In vitro cultured Th1 and Th2 cells developed from wild-type C57BL/6 mice were evaluated for expression of various activation and adhesion molecules by flow cytometry. We noted that the VLA-4 (CD49d/CD29) and VLA-6 (CD49f/CD29) integrins were preferentially expressed by Th1 vs. Th2 cells (Fig. 1A). In contrast, other activation markers (CD25, CD44, CD69) and adhesion molecules (CD11a, CD54, $\alpha 4\beta 3$ integrin) were expressed equitably by both Th1 and Th2 cells (Supplementary Fig. 2). To better understand how VLA-4 and VLA-6 expression may be differentially regulated during the *in vitro* differentiation of Th1 and Th2 cells, we next

examined the expression kinetics of these β 1-integrins by Th1 vs. Th2 cells over their first 13 days in culture. Although both Th1 and Th2 cells initially displayed similar levels of VLA-4 (CD49d/CD29) and VLA-6 (CD49f/CD29) expression (through day 4 of polarizing cultures), thereafter, both molecules were consistently up-regulated/maintained on Th1 cells, while being coordinately down-modulated on Th2 cells (Fig. 1B, 1C).

Since our initial experiments employed heterogeneous T cell populations activated using anti-CD3 mAb, we next evaluated whether these results could be generalized to OVA-specific Th1 and Th2 cells developed from OT-II (OVA-specific TCR transgenic) mice. As depicted in Supplemental Fig. 3, both VLA-4 and VLA-6 were preferentially expressed by Th1 vs. Th2 cells generated from OT-II mice, regardless of whether the “cognate” antigen used *in vitro* was provided in the form of OVA₃₂₃₋₃₃₉ peptide-pulsed syngenic APC or immobilized, plate-bound anti-CD3. Since either antigen format yielded similar results, we believe that the differential expression of VLA-4 and VLA-6 by Th1 vs. Th2 cells was most likely due to the impact of specific cytokines added or neutralized by antibodies employed during the *in vitro* polarization culture period.

IL-4 negatively regulates VLA-4 and VLA-6 expression on Th2 cells

To determine why VLA-4 and VLA-6 exhibited differential expression patterns on Th1 vs. Th2 cells, we next investigated the potential regulatory influence of the soluble factors employed in the polarizing cultures. CD4⁺ T cells cultured under neutral conditions (rIL-2 without other factors) displayed low levels of VLA-4 and VLA-6 expression that were comparable to those observed for Th2 cells (Fig. 2). Notably, the inclusion of neutralizing anti-IL-4 mAb prevented loss of VLA-4 and VLA-6 expression on “Th0” cells (Fig. 2A, 2B). This suggested the functional dominance of endogenous IL-4 production on CD4⁺ T cell expression of these β 1-integrins. The addition of IL-12, but not IFN- γ to Th0 culture conditions resulted in partial maintenance of CD49f and CD29 (but not CD49d) expression on CD4⁺ T cells, suggesting that VLA-4 and VLA-6 expression are not regulated via absolutely identical mechanisms and that these Type-1 associated cytokines are not operationally dominant in determining levels of VLA-4 (and to a lesser extent VLA-6) expression on activated CD4⁺ T cells. This was further evidenced under neutral culture conditions depleted of IL-4, where the co-addition of anti-IL-12 and anti-IFN- γ mAbs did not result in any additional modulation of VLA-4 (or VLA-6) expression by CD4⁺ T cells (when compared to anti-IL-4 mAb only controls, Fig. 2A, 2B). CD4⁺ T cells cultured in the presence of neutralizing antibodies against IL-4, IL-12 and IFN- γ were poor producers of IFN- γ or IL-4, suggesting that up-regulation of VLA in CD4⁺ T cells occurs irrespective of polarization status (data not shown). We also observed that Th17 cells (producing IL-17 but not IFN- γ or IL-4) induced *in vitro* also expressed high levels of VLA-4 and VLA-6, suggesting that up-regulation of VLA-4/-6 is not a property unique to Th1 cells (data not shown). Given functional polarization biases in T cell responses that have been reported between various strains of mice (i.e. C57BL/6 are Th1-biased, while BALB/c mice are Th2-biased; ref. ¹⁵), we performed an analogous set of experiments using T cells isolated from BALB/c mice and observed similar results to those obtained in our B6 model (Supplemental Fig. 4).

IL-4 suppresses VLA-4 and VLA-6 expression by committed Th1 cells without changing their polarized pattern of cytokine production

Since IL-4 appeared to represent a dominant suppressor of VLA-4/-6 expression by CD4⁺ T cells cultured under non-Type-1 polarizing conditions, we next determined whether IL-4 could down-regulate the expression of these VLA molecules on committed Th1 cells. We observed that the treatment of committed Th1 cells with IL-4 (i.e. Th1^{IL-4}) resulted in the dramatic down-regulation of both VLA-4 and VLA-6 expression (Fig. 3A). Interestingly, this phenotypic change occurred in the absence of any overt alteration in the Type-1 pattern of cytokine

production by these T cells, which remained committed to IFN- γ (but not IL-4) production (Fig. 3B). In contrast, the addition of Type-1 cytokines (IFN- γ , IL-12) could not resurrect expression of VLA-4 and VLA-6 expression on committed Th2 cells (Supplemental Fig. 5), suggesting the possible irreversible (IL-4-dependent) nature of VLA-4 and VLA-6 loss of expression on Th2 cells.

IL-4-mediated VLA down-regulation is Stat6-dependent

Stat6 is an important down-stream component of the IL-4R-mediated signaling pathway (16, 17). Therefore, we next determined whether ablation of IL-4R-signaling in STAT6(-/-) mice would yield T cells refractory to IL-4-induced silencing of VLA-4 and VLA-6 expression. We observed that Stat6-deficient CD4⁺ T cells cultured under Th1, Th2, or neutral conditions displayed comparable, high levels of VLA-4 and VLA-6 expression (Fig. 4). These data are consistent with IL-4/IL-4R-mediated signaling dictating the expression pattern of these integrins by polarized subsets of Th cells.

VLA-4 and VLA-6 provide costimulatory signals to Th1 cells and support differential adhesion of Th1 cells to solid-phase VCAM-1 and laminin

Despite the typical redundancy in integrin recognition of a range of physiologic ligands, VLA-4 and VLA-6 expressed on T cells are primarily thought to recognize VCAM-1 and laminin, respectively (18-20). To examine the functional significance of differentially expressed VLA-4 and VLA-6 on Th subsets, Th1 and Th2 cells were tested for their abilities to adhere to plate-bound VCAM-1-Ig fusion protein or laminin. Consistent with the differential expression of VLA-4 and VLA-6 on Th1 and Th2 cells, Th1 cells exhibited specific adhesion to plate-coated VCAM-1-Ig and laminin, whereas Th2 cells displayed only background levels of adhesion to these ligands (Fig. 5A). In contrast, both Th1 and Th2 cells (which express comparable levels of the VLA-5 and α V β 3 integrins, Supplemental Fig. 2) adhered equitably to plates coated with fibronectin (Fig. 5A). Pretreatment of Th1 cells with specific anti-VLA mAbs or anti-CD29 (but not control Ig) completely abrogated the ability of Th1 cells to adhere to relevant ligand-coated plates (Fig. 5B, C). Consistent with the comparable levels of VLA-4 and VLA-6 expression found on all Th subsets developed from STAT6^{-/-} mice, these T cells displayed a uniformly strong binding capacity to immobilized VCAM-1 and laminin (Supplemental Fig. 6A). Moreover, while wild-type CD4⁺ T cells cultured under neutral condition bound to VCAM-1 or laminin poorly, depletion of IL-4 from the culture media resulted in sustained VLA-4 and VLA-6 expression (Fig. 2) and the ability of these T cells to adhere to both immobilized VCAM-1 and laminin, respectively (Supplemental Fig. 6B).

Since the VLA-4-VCAM-1 and VLA-6-laminin interactions are known to provide costimulatory signals to T cells (18,20), we next evaluated the impact of providing both immobilized VCAM-1 or laminin along with a sub-mitogenic dose of anti-CD3 mAb on T cell production of cytokines. As expected, when compared with the anti-CD3 only treatment group, committed Th1 cells produced higher levels of IFN- γ , while committed Th2 cells failed to produce elevated levels of IL-4, upon co-stimulation with either VCAM-1-Ig or laminin (Fig. 5D).

Blockade of VLA-4 prevents the preferential homing of Th1 cells into tumor lesions in vivo

We next sought to determine the physiologic significance of differential VLA-4 and VLA-6 expression by Th1 cells using an adoptive transfer approach applied to the M05 (B16.OVA) melanoma model. Mice bearing established day 7 s.c. melanomas received i.v. transfers of CFSE-labeled Th1 or Th2 cells developed from OT-II mice. Thirty-six hours later, tumor-infiltrating lymphocytes (TILs) were isolated and analyzed by flow cytometry. A high percentage (23%) of CFSE⁺ CD4⁺ TILs were identified from the cohort of animals receiving Th1 infusions, whereas, only very few CFSE⁺ CD4⁺ events (1%) were observed for the cohort

of mice receiving Th2 cells (Fig. 6A, B). The number of CFSE⁺ cells accumulating in the spleens and lungs of mice treated with Th1 or Th2 infusions was comparable (Fig. 6B), suggesting the tumor-specific relevance of VLA-4 and/or VLA-6 to tumor homing/infiltration. As shown in Fig. 6C, pretreatment of OT-II Th1 cells with anti-VLA-4 mAb significantly inhibited their infiltration into MO5 tumor lesions after adoptive transfer, but had little effect on their localization within the spleens of treated mice. While analogous experiments using the anti-VLA-6 mAb suggested a partial reduction in Th1 cell infiltration of the tumor (Fig. 6C), this difference did not reach statistical significance. These results were further confirmed in experiments in which anti-VLA-4 (PS/2), anti-VLA-6 (Go H3) or isotype control mAb were co-administered along with i.v. transferred Th1 cells in MO5-bearing mice (Fig. 6C).

Adoptively-transferred Th1, but not Th2, cells enhance the anti-tumor efficacy of Tc1 cells in the MO5 tumor model

We next assessed the therapeutic benefit of adoptively-transferred OT-II Th1 vs. Th2 cells applied to a day 7 established MO5 (B16.OVA) melanoma model. Intravenous transfer of (up to 4×10^7) anti-OVA Th1 or Th2 cells in the absence of OT-I Tc1 transfer did not prolong the survival of treated animals (Fig. 6D, **top**). However, when combined with the co-transfer of OT-I Tc1 cells, Th1 (but not Th2) cells prolonged the survival of tumor-bearing mice vs. animals with Tc1 cells alone ($p < 0.05$; Fig. 6D, **bottom**).

DISCUSSION

While the vast majority of cancer immunotherapy studies have focused on the generation of anti-tumor CD8⁺ T cells, recent studies support the critical functions of CD4⁺ Th cells in productive anti-tumor immunity (5,^{21,26}). In this regard, CD4⁺ T cells have shown to assist in the priming of naïve, tumor-specific CD8⁺ T cells by directly providing IL-2 (27-29) or via the indirect CD40/CD40L-dependent “licensing” of DC (30-32). Recent studies also support an important role of tumor-specific CD4⁺ T cells in facilitating and sustaining CD8⁺ T effector and memory cell functions (21-23). In the former case, recruitment of tumor-specific CD4⁺ T cells into tumor lesions is envisioned to result in changes within the tumor microenvironment that are permissive for CD8⁺ T effector cell recruitment (via loco-regional production of inflammatory chemokines) and survival/proliferation (via production of IL-2 among other cytokines).

These observations suggest that the efficacy associated with adoptive immunotherapy approaches employing anti-tumor CD8⁺ T cells would likely be improved by the co-transfer of tumor-specific CD4⁺ T cells. Based on the results obtained in the current study, Type-1 CD4⁺ T cells would likely be most beneficial in such approaches, at least in part, due to their preferred capacity to effectively traffic into tumor sites *in vivo*. The differential ability of Th1 (vs. Th2) cells to become TIL appears critically dependent upon their expression of the β 1-integrin VLA-4 and its interaction with VCAM-1 (expressed by activated vascular endothelial cells, such as those found in the tumor stroma; ref. 33). While there was some evidence for the ability of anti-VLA-6 mAb to limit tumor-infiltration by Th1 cells *in vivo*, which would appear to warrant prospective re-evaluation, this level of antagonism did not reach significance in the current studies. Regardless of the functional dominance of VLA-4 vs. VLA-6 in the ability of Th1 cells to migrate into MO5 tumors, these cells clearly facilitated the anti-tumor efficacy of co-transferred Tc1 cells, while exhibiting minimal anti-tumor impact when adoptively-transferred on their own.

Interestingly, while we observed that the Type-2 cytokine IL-4 was unable to alter the functional polarization of Th1 cells (based on cytokine profiling) *in vitro*, IL-4 was competent to suppress both VLA-4 and VLA-6 expression by Th1 cells via a STAT6-dependent mechanism. This finding suggests IL-4R/STAT6-signaling in committed Th1 cells may not

induce expression of GATA-3 or c-Maf, which serve as central regulators of Type-2 cytokine genes in Th2 cells (34,³⁵). We are currently evaluating this possibility.

Given that neutralization of endogenous IL-4 resulted in enhanced VLA-4 and VLA-6 expression by CD4⁺ T cells activated under neutral polarizing conditions, and that Th2 cells developed from STAT6^{-/-} mice express high levels of VLA-4/-6, these findings may suggest that therapeutic benefits would be anticipated from antagonizing the IL-4R/STAT6 signaling pathway in cancer patients. Under such conditions, one would expect improvements in Type-1 effector T cell function, recruitment of Th1 (and Tc1; ref. ¹²) into tumor lesions and corollary enhancement in anti-tumor efficacy.

The finding that antibodies directed against VLA-6 only modestly block Th1 cell recruitment into M05 tumors *in vivo* does not formally minimize the physiologic role of the VLA-6/laminin in Th1 cells. It remains possible that the VLA-6/laminin interaction plays an important role in retention of Th1 cells within laminin-rich tumor interstitium (36,³⁷) and/or that VLA-6 (along with VLA-4) may contribute key co-stimulatory signals to tumor-specific Th1 cells within the tumor microenvironment that may confer prolonged survival and effector function *in vivo*. Further studies in the current model, as well as alternate murine tumor models, will be needed in order to confirm or refute these possibilities and to determine the generality of these results and their translational utility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression kinetics of VLA-4 and VLA-6 on cultured Th1 and Th2 cells
 $CD4^+$ splenocytes were stimulated *in vitro* under the Th1- or Th2-polarizing condition, as described in the *Materials and Methods*. **A.**, Flow cytometric analysis of CD49d ($\alpha 4$ integrin), CD49f ($\alpha 6$ integrin), and CD29 ($\beta 1$ integrin) expression 12 days after initial T cell stimulation *in vitro*. **B.**, Time course of CD49d, CD49f, and CD29 expression on cultured Th1 and Th2 cells. In panels **A.** and **B.**, inset numbers reflect the percentage of $CD49^+CD29^+$ cells among total T cells analyzed. **C.**, Mean Fluorescence Intensity of CD49d, CD49f, and CD29 expression on cultured Th1 and Th2 cells. Th1 cells showed significantly higher expression of CD49d (* $P < 0.002$ after day 7), CD49f (* $P < 0.05$ after day 4) and CD29 (* $P < 0.05$ after day 4) than Th2 cells. The mean of three independent experiments is shown; Bars, S.D.

Figure 2. IL-4 down-regulates VLA-4 and VLA-6 expression on cultured CD4⁺ T cells
 C57BL/6-derived CD4⁺ T cells were stimulated with anti-CD3 mAb under Th1- or Th2-inducing cytokine conditions or under neutral condition in the presence of various cytokines (IL-4, IL-12, or IFN- γ) or antibodies (anti-IL-4 mAb or anti-IL-4 + anti-IL-12 + anti-IFN- γ mAbs). T cells were harvested on day 10 of culture and VLA-4 (CD49d; panel A.) or VLA-6 (CD49f; panel B.) expression assessed by flow cytometry. Inset numbers reflect the percentage of CD49⁺CD29⁺ cells among total T cells analyzed. Data are representative of three independent experiments performed.

Figure 3. IL-4 down-regulates VLA-4 and VLA-6 expression on committed Th1 cells without modulating their cytokine production profile

Th1 cells were induced from naive C57BL/6-derived splenic CD4⁺ T cells as described in *Materials and Methods*. Day 6 Th1 cells were treated with 10 ng/ml of IL-4 for an additional 6 days to generate IL-4-treated Th1 cells (Th1^{IL-4}). **A.**, Flow cytometric analysis of CD49d, CD49f and CD29 expression by Th1 vs. Th1^{IL-4} cells. *P = 0.0003, CD49d expression of Th1 versus Th1^{IL-4}; *P = 0.041, CD49f expression of Th1 versus Th1^{IL-4}. *P = 0.028, CD29 expression of Th1 versus Th1^{IL-4}. **B.**, Intracellular cytokine expression patterns of Th1 cells (day 6 and day 12), Th2 cells (day 6) and Th1^{IL-4} cells (day 12) after *in vitro* restimulation with 5 µg/ml of plate-bound anti-CD3 mAb for 6 hours. Inset numbers reflect the percentage of T cells in the indicated quadrant among total T cells analyzed. Data are reflective of that obtained in 3 independent experiments performed.

Figure 4. Stat6 is required for IL-4-induced down-modulation of VLA-4 and VLA-6 on CD4⁺ T cells

CD4⁺ splenic T cells harvested from wild-type and Stat6^{-/-} C57BL/6 mice were stimulated with anti-CD3 mAb and then cultured under Th1, Th2 or neutral cytokine polarizing conditions. The resultant T cells were analyzed by flow cytometry for expression of the VLA-4 and VLA-6 β 1-integrins on day 10 of culture. Inset numbers reflect the percentage of CD49⁺CD29⁺ cells among total T cells analyzed. Results are representative of 3 independent experiments performed.

Figure 5. VLA-4 and VLA-6 are preferentially expressed by Th1 cells and play critical roles in CD4⁺ T cell adhesion/co-stimulation mediated by VCAM-1, or laminin, respectively

A., Th1 and Th2 cells were incubated in 96-well plates coated with VCAM-1-Ig (VCAM-1), laminin (LN), fibronectin (FN), BSA, 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. * $P = 0.002$, percentage adhesion of Th1 versus Th2 against VCAM-1-Ig; * $P = 0.005$, Th1 versus Th2 against laminin. **B.**, Th1 cells were pre-incubated with either control rat IgG mAb or anti-α4 mAb (PS/2) before being inoculated to VCAM-1-coated plates. * $P = 0.006$, percentage adhesion of control mAb versus anti-VLA-4 mAb-pretreated Th1 cells. **C.**, Th1 cells were pre-incubated with PBS, control rat IgG mAb, anti-α6 mAb or anti-β1 mAb before being introduced to laminin-coated plates. * $P < 0.003$, compared to Control-Ig-treated cells. **D.**, Th1 or Th2 cells were added to 96-well plates coated with anti-CD3 mAb together with either VCAM-1 (top panel), laminin (LN) (bottom panel) or heat-denatured BSA. * $P = 0.016$, IFN-γ production from Th1 cells co-stimulated by BSA versus VCAM-1; * $P = 0.011$, IFN-γ production from Th1 cells co-stimulated by BSA versus Laminin. After incubation for 24h, supernatants were collected and IFN-γ vs. IL-4 production quantitated by specific ELISA. All data are representative of 3 independent experiments performed.

Figure 6. VLA-4 plays a critical role in the preferential trafficking of therapeutic Th1 cells into tumor sites

TILs were harvested 24h after the adoptive transfer of CFSE-labeled Th1 or Th2 cells, stained with PE-anti-CD4 mAb and then analyzed by flow cytometry to enumerate the percentage and total number of CD4⁺/CFSE⁺ cells among lymphocyte-gated populations. **A.**, Mice bearing established (day 10) M05 melanomas in the right flank received i.v. adoptive transfer of 2×10^7 CFSE-labeled Th1 or Th2 cells. Flow cytometric analysis of TILs isolated from Th1 or Th2-infused mice are shown. Inset numbers reflect the percentage of cells in a given quadrant among total T cells analyzed. **B.**, Total number of CD4⁺/CFSE⁺ cells in TIL and the spleen of mice receiving adoptive-transfer of Th1 vs. Th2 cells. * $P = 0.044$, Th1 vs. Th2 transferred groups. **C.**, M05-bearing mice received adoptive transfers of 2×10^7 Th1 cells and were co-administrated anti-VLA-4 mAb (anti- $\alpha 4$ mAb), anti-VLA-6 mAb (anti- $\alpha 6$ mAb) or control rat IgG. * $P = 0.0057$, control IgG- versus anti-VLA-4 mAb-treated groups. **D. top**, Mice bearing established (day 7) s.c. M05 melanomas received i.v. transfer of either 2×10^7 Th1 (Th1, circle) or Th2 (Th2, triangle) or they were left untreated (Control, square). **D. bottom**, Mice bearing day 7 s.c. M05 melanomas received i.v. transfer of either 2×10^7 Th1 (Th1 + Tc1, circle) or Th2 (Th2 + Tc1, triangle) in combination with 6×10^6 Tc1 cells, 6×10^6 Tc1 cells only (Tc1 only, diamond) or they were left untreated (Control, square). Symptom-free survival of mice was monitored over time. * $P = 0.024$ for the Th1 + Tc1-treated group versus the Th2 + Tc1 treated group; * $P = 0.026$ for the Th1 + Tc1-treated group versus the Tc1 only-treated group; * $P = 0.02$ for the Th1 + Tc1-treated group versus the non-treated group. All data are representative of 3 independent experiments performed.