

# Fine-tuning Tumor Immunity with Integrin Trans-regulation

Joseph M. Cantor, David M. Rose, Marina Slepak, and Mark H. Ginsberg

## Abstract

Inefficient T-cell homing to tissues limits adoptive T-cell immunotherapy of solid tumors.  $\alpha\text{L}\beta\text{2}$  and  $\alpha\text{4}\beta\text{1}$  integrins mediate trafficking of T cells into tissues via engagement of ICAM-1 and VCAM-1, respectively. Inhibiting protein kinase A (PKA)-mediated phosphorylation of  $\alpha\text{4}$  integrin in cells results in an increase in  $\alpha\text{L}\beta\text{2}$ -mediated migration on mixed ICAM-1-VCAM-1 substrates *in vitro*, a phenomenon termed "integrin trans-regulation." Here, we created an  $\alpha\text{4(S988A)}$ -bearing mouse, which precludes PKA-mediated  $\alpha\text{4}$  phosphorylation, to examine the effect of integrin trans-regulation *in vivo*. The

$\alpha\text{4(S988A)}$  mouse exhibited a dramatic and selective increase in migration of lymphocytes, but not myeloid cells, to sites of inflammation. Importantly, we found that the  $\alpha\text{4(S988A)}$  mice exhibited a marked increase in T-cell entry into and reduced growth of B16 melanomas, consistent with antitumor roles of infiltrating T cells and progrowth functions of tumor-associated macrophages. Thus, increased  $\alpha\text{4}$  trans-regulation of  $\alpha\text{L}\beta\text{2}$  integrin function biases leukocyte emigration toward lymphocytes relative to myeloid cells and enhances tumor immunity. *Cancer Immunol Res*; 3(6); 661–7. ©2015 AACR.

## Introduction

Different classes of leukocytes have opposing effects on the growth of tumors. Lymphocytes, particularly T cells, are critical components of the host defense that limit tumorigenesis (1). In sharp contrast, myeloid cells contribute cytokines that promote both tumor growth and angiogenesis (2, 3).  $\alpha\text{4}$  integrins play an important role in lymphocyte entry into sites of tissue injury (4, 5) in part because they markedly potentiate cell migration via signaling mediated by binding of paxillin to the  $\alpha\text{4}$  cytoplasmic tail (6). Protein kinase A (PKA)-mediated phosphorylation of the  $\alpha\text{4}$  integrin tail at Ser<sup>988</sup> inhibits paxillin binding in migrating cells (7); consequently, the  $\alpha\text{4(S988A)}$  mutation stabilizes the  $\alpha\text{4}$ -paxillin interaction and increases  $\alpha\text{4}$  integrin signaling. The increased signaling downstream of  $\alpha\text{4(S988A)}$  enhances integrin  $\alpha\text{L}\beta\text{2(LFA-1)}$ -mediated migration of cells, a phenomenon termed integrin trans-regulation (8).

Here, we examined the biologic consequences of blockade of  $\alpha\text{4}$  phosphorylation by generating  $\alpha\text{4(S988A)}$  mutant mice and found that these mice manifest a dramatic increase in recruitment of lymphocytes, but not myeloid cells, to an inflammatory site. We found that the  $\alpha\text{4(S988A)}$  mutation markedly increased the abundance of T cells, but not myeloid cells, in heterotopic B16 melanomas. Increased lymphocyte homing is needed for efforts to develop adoptive immunotherapies for solid tumors (9–14).

Department of Medicine, University of California, San Diego, La Jolla, California.

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

**Corresponding Authors:** J.M. Cantor, University of California–San Diego School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093. Phone: 858-822-6507; Fax: 858-822-6458; E-mail: jmcantor@ucsd.edu; or Mark H. Ginsberg, Phone: 858-822-6432, mhginsberg@ucsd.edu

**doi:** 10.1158/2326-6066.CIR-13-0226

©2015 American Association for Cancer Research.

The potential therapeutic value of this form of integrin trans-regulation was established by the finding that subcutaneously implanted melanomas grew more slowly in  $\alpha\text{4(S988A)}$  mice associated with markedly enhanced recruitment of T cells, but not myeloid cells, to the tumors. Thus, increasing integrin trans-regulation by blocking  $\alpha\text{4}$  integrin phosphorylation selectively recruits lymphocytes, but not myeloid cells, thereby reducing tumor growth. This finding suggests that modulation of integrin trans-regulation may be useful in overcoming a limitation of adoptive cellular immunotherapy of solid tumors.

## Materials and Methods

### Mice

$\alpha\text{4(S988A)}$  mice were generated as described in Supplementary Material of ref. 16. These mice were bred to create homozygous germline knock-ins and backcrossed to BL6 for >8 generations for all experiments, unless otherwise noted. For assessment of T-cell cytotoxic function,  $\alpha\text{4(S988A)}$  mice were crossed with the OT-1 strain. *Rag1*<sup>-/-</sup> mice were used as recipients for *in vivo* competitive migration experiments. All mice were housed at the University of California San Diego animal facility, and all experiments were approved by the Institutional Animal Care and Use Committee.

### Hematologic analysis

Blood from adult (10–20-week-old)  $\alpha\text{4(S988A)}$  and  $\alpha\text{4[wild-type(wt)]}$  mice was collected into tubes containing EDTA. Cell counts were obtained using an MS9-automated cell counter by the University of California San Diego Animal Care Program Diagnostic Laboratory, whose staff also manually performed differential counts on DiffQuick-stained smears.

### Peritonitis model

Adult (10–20-week-old)  $\alpha\text{4(S988A)}$  and control  $\alpha\text{4(wt)}$  mice were injected i.p. with 4% (weight/vol) thioglycollate (Sigma-Aldrich) and sacrificed at various time points for peritoneal lavages. Cells ( $1 \times 10^5$ ) were adhered to glass slides with a

Cytospin4 instrument (ThermoShandon) and stained with Diff-Quick to differentiate cell types by light microscopy. The percentages of T, B1, and B2 cells were assessed by flow cytometry using fluorochrome-conjugated anti-CD3, anti-B220, and anti-Mac-1 antibodies. For competitive peritonitis assay, peritonitis was elicited as described above, but in *Rag1*<sup>-/-</sup> mice. Twenty-four hours after thioglycollate injection, mice received an i.v. injection of a mixture of splenocytes from adult  $\alpha$ 4(S988A) or control Ly5.1  $\alpha$ 4(wt). Splenocyte suspensions and peritoneal lavages were stained using antibodies against CD3, CD8, and Ly5.1. The ratio of  $\alpha$ 4(S988A) to Ly5.1<sup>+</sup>  $\alpha$ 4(wt) splenocytes was compared between spleen and peritoneum as a measure of enrichment for  $\alpha$ 4(S988A) cells in the inflammatory site.

### Lymphoid compartments

Bone marrow cell suspensions were prepared by flushing femur and tibia bones from adult  $\alpha$ 4(S988A) and  $\alpha$ 4(wt) mice. Single-cell suspensions from bone marrow, spleen, and thymus were treated with ACK lysis buffer, counted, and stained with fluorochrome-conjugated antibodies (eBioscience) against mouse B220 (RA3-6B2), IgM (II/41), IgD (11-26c), CD21/35 (7G6), CD23 (B3B4), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7) at optimized concentrations. Cells were washed and analyzed by flow cytometry. For integrin expression analysis, blood was collected by tail bleed, treated with ACK lysis buffer, and stained with anti-CD3, anti- $\alpha$ 4, anti- $\alpha$ L $\beta$ 2 integrin, or isotype control fluorochrome-conjugated antibodies before flow cytometry.

### Humoral immune response

For antigen-specific antibody responses,  $\alpha$ 4(S988A) and  $\alpha$ 4(wt) mice (F5 backcross to BL6) were injected i.p. with 100  $\mu$ g trinitrophenol-keyhole limpet hemocyanin (TNP-KLH; Biosearch) emulsified in 250  $\mu$ L complete Freund's adjuvant (T-cell-dependent antigen). Blood serum was collected by centrifugation of tail vein bleed (100–200  $\mu$ L with 1–2 mmol/L EDTA solution as an anticoagulant) before (pre-immune) and at 1, 2, and 3 weeks after immunization. TNP-specific antibody concentrations in blood sera were assessed by direct ELISA with trinitrophenol-ovalbumin (TNP-OVA) as the coating antigen and alkaline phosphatase-conjugated polyclonal anti-mouse IgG (Sigma) as the detection antibody.

### Migration assay

Resting B or T cells were purified from spleens of adult  $\alpha$ 4(S988A) and control  $\alpha$ 4(wt) mice by negative depletion. Macrophages were differentiated from  $\alpha$ 4(S988A) or control  $\alpha$ 4(wt) bone marrow by culture in 30% L292 supernatant for one week. *In vitro* migration was assessed following a modified Boyden Chamber assay (8). Briefly, transwells (Costar) with 3.0- $\mu$ m polycarbonate membrane inserts were coated with VCAM-1 and/or ICAM-1 Fc fusion proteins in carbonate buffer, pH 8.0. Transwell membranes were blocked in PBS, 2% BSA 30 minutes at room temperature. Cells ( $2.0 \times 10^5$ ) were added to the top chamber in complete medium (10% FBS). Complete medium containing 15 ng/mL stromal-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ; R&D Systems) was added to the lower chamber. To observe macrophage migration, 20 ng/mL of both SDF-1 and MCP-1 was necessary. After a 4-hour incubation at 37°C (overnight for macrophages), cells in the lower chamber and underside of transwell were harvested and counted by hemacytometer.

### B16 melanoma model

B16 (f1 subclone) or Lewis lung carcinoma (LLC) cells were expanded in culture in complete medium (DMEM supplemented with 10% FBS, L-glutamine,  $\beta$ ME, and pen/strep antibiotics). B16 cells ( $3 \times 10^5$ ) or LLC cells ( $1 \times 10^6$ ) were injected s.c. into the right hind flanks of adult  $\alpha$ 4(S988A) or control  $\alpha$ 4(wt) mice. When tumors became visible, length and width were measured daily with calipers. Tumors were assumed to be ellipsoid, and volume was calculated using the formula: (length  $\times$  width<sup>2</sup>)/2. Mice were sacrificed on day 15, and tumors were excised and weighed. To analyze tumor-infiltrating leukocytes (TIL), tumors were digested with collagenase (Sigma) for 20 to 30 minutes at 37°C and further processed to a single-cell suspension using a 7-mL tissue grinder (Kontes) and counted. Fluorochrome-conjugated antibodies were used to stain for tumor-infiltrating CD45<sup>+</sup> leukocytes and identify CD4<sup>+</sup> and CD8<sup>+</sup> T cells (CD3<sup>+</sup>), as well as CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cell (Treg) and NK1.1<sup>+</sup> natural killer (NK) cells. Total subset numbers were calculated by multiplying the total cell number with percentage of CD45<sup>+</sup> and percentage of each subset. For lymphoid cell depletion, we injected anti-CD8 antibody (53-6.7, 100  $\mu$ g) or a combination of anti-CD4, anti-CD8, and anti-NK1.1 antibodies (150  $\mu$ g each), compared with isotype control antibody i.p. 2 days before and 5 days after B16 tumor-cell inoculation. Splenocytes harvested on day 15 were stained with antibodies specific for CD3, CD4, and CD8 to determine efficiency of T-cell depletion.

### Analysis of clonal expansion *in vivo*

To analyze clonal expansion on a polyclonal T-cell receptor (TCR) background,  $\alpha$ 4(S988A) and wt mice were immunized with a combination of 100  $\mu$ g poly I:C (Invivogen), 50  $\mu$ g anti-CD40 antibody (Biolegend), and 500  $\mu$ g ovalbumin protein (Sigma) in PBS i.p. Six days later, mice were sacrificed and spleen cells were stained using anti-CD8<sup>+</sup> antibody and an H-2K<sup>b</sup>-SIINFEKL(PE) tetramer (Coulter).

### Assessment of CD8 T-cell cytotoxic function

To generate functional CTLs, splenocytes from  $\alpha$ 4(S988A) and wt-OT-1 mice were cultured with 1  $\mu$ g/mL SIINFEKL and 100 U/mL IL2 (NCI) for 6 days. Degranulation as a measure of cytotoxicity was measured as exposure of CD107a (LAMP-1) on the outer cell membrane. On day 6 following SIINFEKL stimulation, CTLs were harvested, counted, and cultured with SIINFEKL-pulsed (1  $\mu$ g/mL for 1–2 hours) or -unpulsed splenocytes as targets in the presence of anti-LAMP-1-PE antibodies (eBioscience) for 2.5 hours at 37°C. Effector:target cultures were stained with anti-CD8 antibodies and analyzed by flow cytometry. For target lysis *in vitro*, CTLs were generated as above and cultured with an approximately 50/50 mixture of peptide-pulsed (CFSElo) and -unpulsed (CFSEhi) splenocyte targets overnight. Specific lysis is the percentage decrease in the percentage of the peptide-pulsed peak between CTL-containing and no-CTL control cultures.

### Statistical analysis

The two-tailed *t* test was used for statistical comparison between groups in all experiments, except where otherwise noted. A value of *P* < 0.05 was considered statistically significant.

## Results and Discussion

### The $\alpha 4(S988A)$ mutation selectively increases lymphocyte migration to an inflammatory site

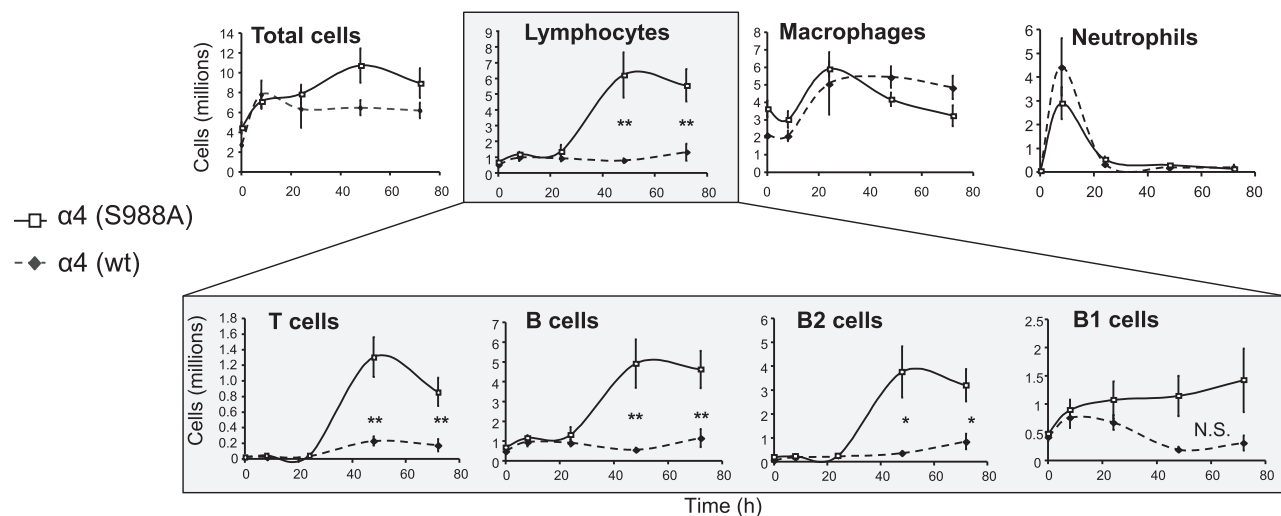
We used homologous recombination to generate  $\alpha 4(S988A)$  mice (Supplementary Fig. S1) and compared them to wt C57BL/6 mice as controls. We observed no statistically significant differences in formed elements of the blood (Supplementary Fig. S2A) or in lymphocyte numbers (with the possible exception of increased Pro-B cells in the bone marrow) in primary or secondary lymphoid tissue (Supplementary Fig. S2B) in  $\alpha 4(S988A)$  mice. Humoral immune responses to a T-dependent antigen were also similar between  $\alpha 4(S988A)$  and wt mice (Supplementary Fig. S2C).

Because the  $\alpha 4(S988A)$  mutation inhibits migration on substrates containing purified  $\alpha 4$  integrin ligands (7), we hypothesized that  $\alpha 4(S988A)$  mice might exhibit a similar defect in leukocyte migration *in vivo*. We therefore used a thioglycollate-induced peritonitis model to test the effect of this mutation on leukocyte entry into an inflammatory site. To our great surprise, we observed a sharp increase in the number of lymphocytes infiltrating the peritoneum (Fig. 1), whereas myeloid cell infiltration was unaffected. We stained the peritoneal lavage with antibodies to identify B1, B2, and T lymphocytes and found that both B2 and T cells exhibited significantly (>3–4-fold) greater numbers in the  $\alpha 4(S988A)$  mice compared with that in the controls (Fig. 1). In contrast, the numbers of B1 cells, a resident peritoneal population (15), showed no statistically significant differences from controls, indicating that the  $\alpha 4(S988A)$  mutation specifically affects influx of lymphocytes. An *in vivo* competitive migration assay (Supplementary Fig. S3) using a mix of wt (congenically marked) and  $\alpha 4(S988A)$  splenocytes confirmed that the increased migration of  $\alpha 4(S988A)$  T cells is intrinsic to leukocytes and not merely due to effects of  $\alpha 4$  phosphorylation in endothelial cells (16) or other changes in the inflammatory environment (e.g., production of chemokines by resident cells)

in these mice. Therefore, interfering with  $\alpha 4$  integrin phosphorylation selectively increased homing of lymphocytes to a site of inflammation but had no obvious effect on myeloid cells.

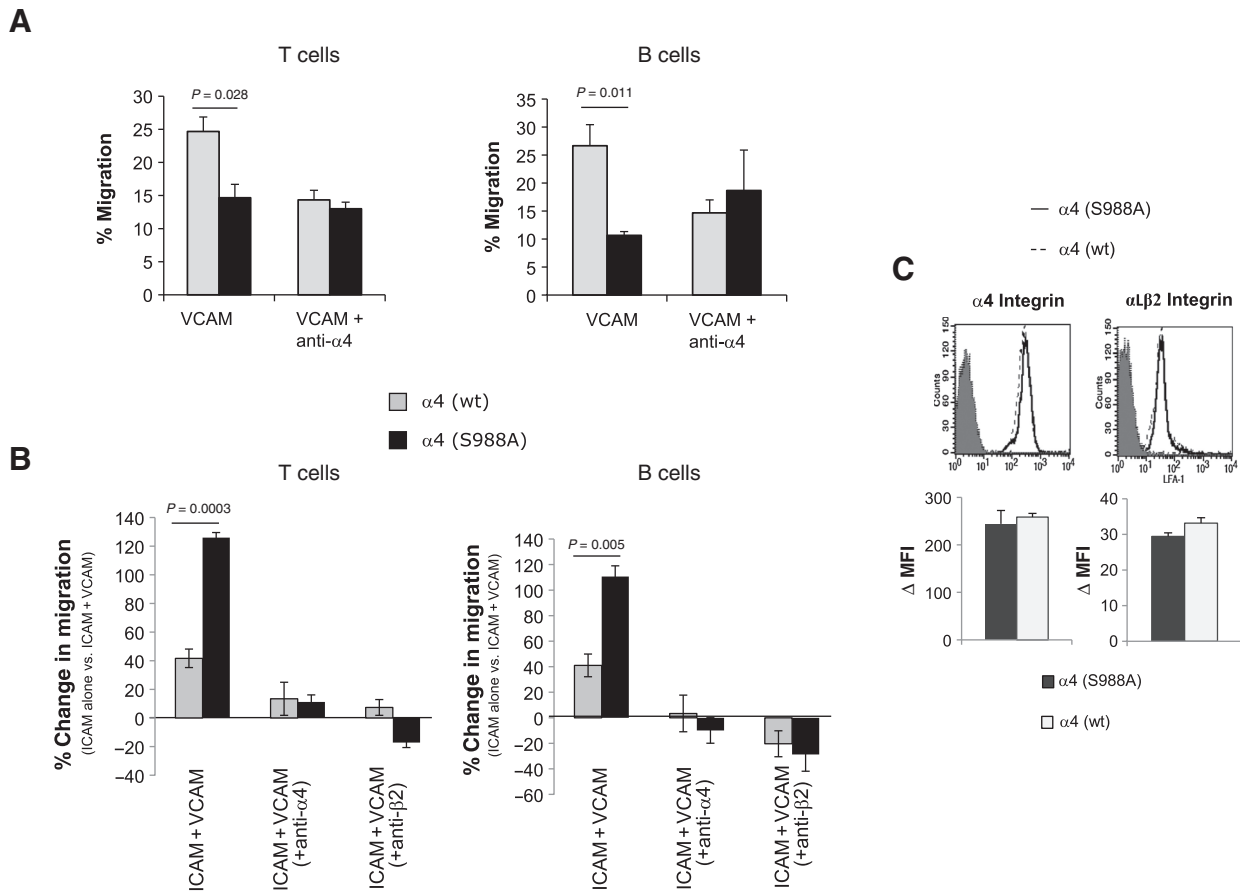
### Integrin trans-regulation explains increased migration of $\alpha 4(S988A)$ lymphocytes

Our initial expectation that the  $\alpha 4(S988A)$  mutation would decrease homing was based on results from *in vitro* migration on purified  $\alpha 4$  integrin ligands. *In vivo* migration to an inflammatory site is complex and involves several classes of integrins (17). We previously reported that the presence of small amounts of  $\alpha 4$  integrin ligands (e.g., VCAM-1) increases the migration of Jurkat T cells on  $\alpha L\beta 2$  integrin substrates (e.g., ICAM-1) *in vitro* (8). Paxillin binding to  $\alpha 4$  is required for this effect, and enforced paxillin- $\alpha 4$  association enhanced the trans-regulation of  $\alpha L\beta 2$  by  $\alpha 4$  integrins through increasing the activation of FAK and Pyk2 kinases (8, 18). The  $\alpha 4(S988A)$  mutation that blocks PKA phosphorylation of the  $\alpha 4$  cytoplasmic tail could therefore enhance  $\alpha L\beta 2$ -dependent migration. The *in vivo* peritonitis experiment requires migration on mixed substrates and is dependent on both  $\alpha 4$  and  $\beta 2$  integrins (17), conditions in which the  $\alpha 4(S988A)$  mutation could increase migration of lymphocytes (8). To explore this possibility in a controlled setting, we purified B and T cells from  $\alpha 4(S988A)$  mice and measured their ability to migrate *in vitro* on purified  $\alpha 4$  ligand (VCAM-1), purified  $\beta 2$  ligand (ICAM-1), or mixed substrates (VCAM-1 + ICAM-1) in response to the chemokine SDF-1 $\alpha$ . Using a modified Boyden chamber assay, we confirmed that  $\alpha 4(S988A)$  lymphocytes exhibited reduced migration on a purified  $\alpha 4$  integrin ligand: VCAM-1 (Fig. 2A). In contrast, when plated on substrates containing predominantly ICAM-1 and small amounts of VCAM-1, both B and T cells from  $\alpha 4(S988A)$  mice displayed enhanced migration that was dependent on both  $\alpha 4$  and  $\beta 2$  integrins (Fig. 2B; Supplementary Fig. S4). This observation cannot be explained by differences in surface integrin expression levels, as T cells from  $\alpha 4(S988A)$  and



**Figure 1.**

Leukocyte migration in an  $\alpha 4(S988A)$  mouse. Adult  $\alpha 4(S988A)$  or wt mice were injected with 1 mL of thioglycollate medium i.p. Mice were sacrificed at various time points, and leukocytes from peritoneal lavages were enumerated and identified by cyto-spin and DiffQuick staining. Peritoneal lavages were also stained with antibodies to identify T cells (CD3<sup>+</sup>), total B cells (B220<sup>+</sup>), B2 cells (B220<sup>+</sup>CD11b<sup>-</sup>), and B1 cells (B220<sup>+</sup>CD11b<sup>+</sup>). Error bars, SEM of 5 mice for each group. \*,  $P < 0.03$ ; \*\*,  $P < 0.02$ ; N.S., not statistically significant.

**Figure 2.**

Increased integrin trans-regulation in  $\alpha 4$ (S988A) lymphocytes. B and T cells were purified from spleens of  $\alpha 4$ (S988A) or control  $\alpha 4$ (wt) mice. Chemotactic migration toward SDF-1 $\alpha$  (15 ng/mL) was assessed using a modified Boyden Chamber assay in wells coated with (A) VCAM-1 alone (2  $\mu$ g/mL) or (B) ICAM-1 (5  $\mu$ g/mL)  $\pm$  VCAM-1 (0.02  $\mu$ g/mL). For anti-integrin antibody-blocking studies, cells were treated with 10  $\mu$ g/mL of either anti- $\alpha 4$  or anti- $\beta 2$  integrin before the assay. B, percentage of increase in migration on ICAM-1 + VCAM-1 compared with ICAM-1 alone  $100 \times [(\text{ICAM} + \text{VCAM migration} - \text{ICAM migration}) / \text{ICAM migration}]$ . Error bars, SEM of 4 mice for each group. C, surface integrin expression levels on circulating  $\alpha 4$ (S988A) T cells. Blood leukocytes from  $\alpha 4$ (S988A) and  $\alpha 4$ (wt) control adult C57BL/6 mice ( $n = 3$  per group) were stained with antibodies specific for T cells (CD3),  $\alpha 4$  integrins (CD49d), and  $\alpha L\beta 2$  integrin heterodimer and analyzed by flow cytometry. Representative histograms show  $\alpha 4$  or  $\alpha L\beta 2$  staining (solid and dotted lines) compared with nonspecific isotype control staining (filled peak). Bar graphs summarize staining from 3 mice per group; no statistically significant differences were observed.  $\Delta$  MFI, change in mean fluorescence intensity.

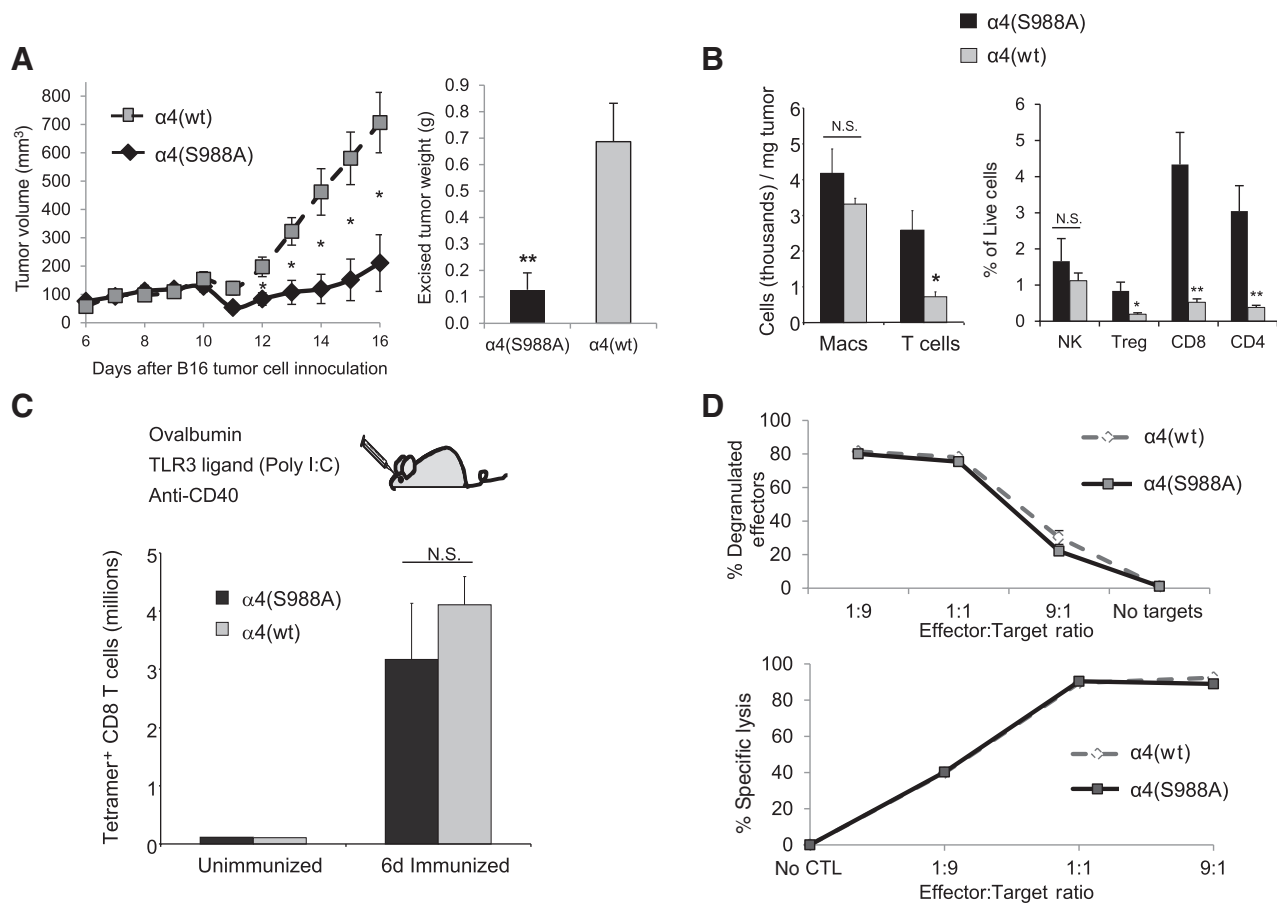
$\alpha 4$ (wt) mice show no difference in staining for  $\alpha 4$  or  $\alpha L\beta 2$  integrins (Fig. 2C). These data indicate that the  $\alpha 4$ (S988A) mutation provides an increase in  $\beta 2$  integrin-dependent migration, i.e., integrin trans-regulation in primary lymphocytes.

#### Increased integrin trans-regulation reduces tumor growth by increasing T-cell homing

Specifically increasing lymphocyte entry into an inflammatory site might offer therapeutic benefit during an immune response to a tumor. T-cell migration to solid tumors is important for tumor immunity (1), whereas tumor infiltration by macrophages may promote tumor growth through increased angiogenesis and suppressed immunity (2, 3). In adoptive immunotherapy (11–14, 19), naïve T cells are modified and activated *in vitro*; however, T-cell homing is a critical limiting variable in solid tumor adoptive immunotherapy (9, 10). Based on our results with the peritonitis model, we hypothesized that  $\alpha 4$ (S988A) mice may have increased

ability to resist tumors due to selective migration of lymphocytes to the tumor site. We tested this idea using a melanoma model (20), in which B16 melanoma cells are injected s.c., and tumor size is measured by weighing excised tumors 15 days later.  $\alpha 4$  (S988A) mice had approximately 5-fold smaller tumors than wt BL6 controls (Fig. 3A), indicating that blocking  $\alpha 4$  integrin phosphorylation on Ser988 increased tumor protection. This observation was not unique to the B16 melanoma, as  $\alpha 4$  (S988A) mice also displayed increased resistance to the growth of Lewis lung carcinoma (Supplementary Fig. S5).

We next asked whether the  $\alpha 4$ (S988A) resistance to tumors was associated with increased T-cell homing. Indeed, B16 tumors in  $\alpha 4$ (S988A) mice had greater concentrations of T cells than those grown in wt mice (Fig. 3B, left). This finding was in striking contrast with a similar number of macrophages found in tumors from mice of both genotypes, supporting the idea that the decreased tumor growth is a result of selective homing of lymphocytes versus macrophages in the  $\alpha 4$ (S988A) mice. Among

**Figure 3.**

Blocking  $\alpha 4$  integrin phosphorylation increases tumor-infiltrating T cells and reduces tumor growth in mice. A, Reduced tumor growth in  $\alpha 4$  (S988A) mice. B16 melanoma cells ( $3 \times 10^5$ ) were injected into  $\alpha 4$ (S988A) or control  $\alpha 4$ (wt) mice. Tumor area was measured daily and converted to an ellipsoid volume: (length  $\times$  width<sup>2</sup>)/2. Fifteen days later, tumors were excised and weighed. B,  $\alpha 4$ (S988A) T cells migrate more efficiently to a tumor site. B16 tumors were grown as in A. On day 15, mice were sacrificed, and excised tumors were weighed, digested with collagenase, and stained for (CD45<sup>+</sup>) CD11b<sup>+</sup> macrophages, CD3<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, Foxp3<sup>+</sup> (CD4<sup>+</sup>) Treg, and NK1.1<sup>+</sup> NK cells. Error bars, SEM of  $\geq 10$  mice per group. \*,  $P < 0.015$ ; \*\*,  $P < 0.002$ . C, T-cell clonal expansion.  $\alpha 4$ (S988A) and  $\alpha 4$ (wt) control littermate mice were immunized with ovalbumin protein, anti-CD40, and Poly I:C, i.p. in PBS. Six days later, splenocytes were isolated and stained with anti-CD8 antibody and an H-2k<sup>b</sup>-SIINFEKL tetramer. D, T-cell cytotoxic function. Splenocytes of 8- to 12-week-old OT-1<sup>+</sup>  $\alpha 4$ (S988A) or OT-1<sup>+</sup>  $\alpha 4$ (wt) control littermate mice were differentiated to CTLs *in vitro* with SIINFEKL (1  $\mu$ g/mL) and IL2 for 5 days and cultured for 2 hours at the indicated effector-to-target ratios (E:T) in the presence of anti-LAMP-1 antibody, followed by staining for CD8 and flow cytometric analysis. For target lysis, CTLs were cultured with SIINFEKL-pulsed or -unpulsed targets that had been differentially labeled with carboxyfluorescein diacetate succinimidyl ester. Specific killing was detected by flow cytometry as the decrease in the percentage of specific targets. No statistically significant differences were noted, with the exception of the 9:1 E:T ratio, at which the  $\alpha 4$ (S988A)-specific killing was 3.7% lower than that for  $\alpha 4$ (wt) CTLs ( $P < 0.005$ ).  $n = 3$  mice per group. Experiment was performed twice.

lymphoid cells, tumors in  $\alpha 4$ (S988A) mice had significantly greater numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, and regulatory T cells; NK-cell abundance showed a modest, but statistically insignificant, increase compared with that of controls (Fig. 3B, right). The increased density of tumor-infiltrating T cells seen in  $\alpha 4$ (S988A) mice could be due to subtle, unrelated immunologic changes in this strain such as greater clonal expansion of tumor-specific T cells; however, expansion of antigen-specific T cells (SIINFEKL-tetramer<sup>+</sup>) was similar between  $\alpha 4$ (S988A) and  $\alpha 4$ (wt) mice in response to immunization with ovalbumin (Fig. 3C). Furthermore, the cytotoxic function of  $\alpha 4$ (S988A) CD8<sup>+</sup> T cells was nearly identical to that of wt (Fig. 3D) cells as measured by degranulation of  $\alpha 4$ (S988A) OT-1 CD8<sup>+</sup> T cells and specific lysis of SIINFEKL-pulsed target cells. Thus, we concluded that the reduction in tumor growth observed in  $\alpha 4$ (S988A) mice is largely

ascribable to increased lymphoid-cell homing. As an additional test of this conclusion, we used antibodies to deplete the majority of CD4 T, CD8 T, and NK cells. Depleting these multiple lymphoid subsets reversed the reduction in tumor growth in  $\alpha 4$ (S988A) relative to wt mice (Supplementary Fig. S6A). In contrast, partial depletion of CD8 T cells did not abolish the reduced tumor growth in  $\alpha 4$ (S988A) mice (Supplementary Fig. S6B). Thus, the capacity of the  $\alpha 4$ (S988A) mutation to increase the recruitment of multiple lymphocyte subsets is responsible for its ability to enhance tumor resistance.

The differential requirement of  $\beta 2$  integrins for migration of lymphocytes or myeloid cells *in vivo* can account for the remarkable leukocyte specificity of this form of trans-regulation. Whereas  $\alpha$ L $\beta$ 2 plays a major primary role in the migration of T cells to inflammatory sites (17, 21–23), macrophage migration to

inflamed peritoneum is not dependent on  $\beta 2$  integrins and is reported to be solely dependent on  $\alpha 4\beta 1$  (24, 25). Thus, trans-regulation of migration would be absent in monocytes and macrophages because  $\beta 2$  integrins are not utilized. Indeed, we did not observe increased migration of macrophages from  $\alpha 4$ (S988A) or  $\alpha 4$ (wt) mice (Supplementary Fig. S7) on mixed ICAM-1-VCAM-1 substrates.

The finding that  $\alpha 4\beta 1$  trans-regulation of  $\alpha L\beta 2$  integrin-mediated migration promotes tumor immunity has important therapeutic implications. Inhibiting focal adhesion kinase (FAK) can suppress such trans-regulation (8), a finding that sounds a cautionary note in the use of FAK inhibitors in tumor therapy (26–28) and suggests that the effect of these agents on lymphocyte trafficking to tumors should be evaluated. Homing of infused lymphocytes is currently a rate-limiting step in applying T-cell immunotherapy to solid tumor cancers (9). Because lymphocytes are modified *ex vivo* before adoptive transfer for immunotherapy (11–14), the opportunity exists to simultaneously optimize their homing capacity by increasing integrin trans-regulation.  $\alpha 4$  phosphorylation is type I-PKA-dependent (29); thus, increased trans-regulation could be induced by introduction of a dominant  $\alpha 4$ (S988A) integrin subunit, a cell permeating type I-specific A-kinase anchor protein (AKAP) peptide, or genetically encoded type I-specific PKA inhibitor (29, 30). Increased integrin trans-regulation might also have utilities beyond tumor immunotherapy. Migration and survival of long-lived plasma cells in the bone marrow appear to be dependent on  $\alpha 4$  and  $\alpha L\beta 2$  integrins (31–35) and could involve integrin trans-regulation. Thus,

enhanced integrin trans-regulation can offer a new approach to selectively potentiate  $\beta 2$  integrin-mediated homing of lymphocytes and plasma cells.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** J.M. Cantor, D.M. Rose, M.H. Ginsberg  
**Development of methodology:** D.M. Rose, M.H. Ginsberg  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.M. Cantor, D.M. Rose, M. Slepak  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.M. Cantor, D.M. Rose, M.H. Ginsberg  
**Writing, review, and/or revision of the manuscript:** J.M. Cantor, D.M. Rose, M.H. Ginsberg  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Slepak  
**Study supervision:** M.H. Ginsberg

### Grant Support

This work was funded in part by NIH R01 HL 31950 and HL 117807. J.M. Cantor is funded by NIH K01-DK090416 and MS Society grant RG4981A1/T. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 20, 2013; revised December 11, 2014; accepted January 10, 2015; published OnlineFirst January 19, 2015.

### References

- Talmadge JE, Donkor M, Scholar E. Inflammatory cell infiltration of tumors: Jekyll or Hyde. *Cancer Metastasis Rev* 2007;26:373–400.
- Porta C, Subhra Kumar B, Larghi P, Rubino L, Mancino A, Sica A. Tumor promotion by tumor-associated macrophages. *Adv Exp Med Biol* 2007;604:67–86.
- Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010;141:39–51.
- Rose DM, Han J, Ginsberg MH. Alpha4 integrins and the immune response. *Immunol Rev* 2002;186:118–24.
- Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;7:78–689.
- Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M, et al. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature* 1999;402:676–81.
- Goldfinger LE, Han J, Kiosses WB, Howe AK, Ginsberg MH. Spatial restriction of alpha4 integrin phosphorylation regulates lamellipodial stability and alpha4beta1-dependent cell migration. *J Cell Biol* 2003;162:731–41.
- Rose DM, Liu S, Woodside DG, Han J, Schlaepfer DD, Ginsberg MH. Paxillin binding to the alpha 4 integrin subunit stimulates LFA-1 (integrin alpha L beta 2)-dependent T cell migration by augmenting the activation of focal adhesion kinase/proline-rich tyrosine kinase-2. *J Immunol* 2003;170:5912–8.
- Ngo MC, Rooney CM, Howard JM, Heslop HE. Ex vivo gene transfer for improved adoptive immunotherapy of cancer. *Hum Mol Genet* 2011;20:R93–9.
- Parente-Pereira AC, Burnet J, Ellison D, Foster J, Davies DM, van der Stegen S, et al. Trafficking of CAR-engineered human T cells following regional or systemic adoptive transfer in SCID beige mice. *J Clin Immunol* 2011;31:710–8.
- Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 2011;118:4817–28.
- Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011;3:95ra73.
- Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 2012;119:2709–20.
- Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 2011;365:725–33.
- Fagarasan S, Watanabe N, Honjo T. Generation, expansion, migration and activation of mouse B1 cells. *Immunol Rev* 2000;176:205–215.
- Goldfinger LE, Tzima E, Stockton R, Kiosses WB, Kinbara K, Tkachenko E, et al. Localized alpha4 integrin phosphorylation directs shear stress-induced endothelial cell alignment. *Circ Res* 2008;103:177–85.
- Ulyanova T, Priestley GV, Banerjee ER, Papayannopoulou T. Unique and redundant roles of alpha4 and beta2 integrins in kinetics of recruitment of lymphoid vs myeloid cell subsets to the inflamed peritoneum revealed by studies of genetically deficient mice. *Exp Hematol* 2007;35:1256–65.
- Han J, Rose DM, Woodside DG, Goldfinger LE, Ginsberg MH. Integrin alpha 4 beta 1-dependent T cell migration requires both phosphorylation and dephosphorylation of the alpha 4 cytoplasmic domain to regulate the reversible binding of paxillin. *J Biol Chem* 2003;278:34845–53.
- Xu XJ, Zhao HZ, Tang YM. Efficacy and safety of adoptive immunotherapy using anti-CD19 chimeric antigen receptor transduced T-cells: a systematic review of phase I clinical trials. *Leuk Lymphoma* 2013;54:255–60.
- Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. *Curr Protoc Immunol* 2001;Chapter 20:Unit 20.1.

21. Engelhardt B. Molecular mechanisms involved in T cell migration across the blood-brain barrier. *J Neural Transm* 2006;113:477–85.
22. Koboziev I, Karlsson F, Ostanin DV, Gray L, Davidson M, Zhang S, et al. Role of LFA-1 in the activation and trafficking of T cells: implications in the induction of chronic colitis. *Inflamm Bowel Dis* 2012;18:2360–2370.
23. Takeichi T, Moczecius P, Deduchovas O, Salnikova O, Castro-Santa E, Buchler MW, et al. alphaL beta2 integrin is indispensable for CD8+ T-cell recruitment in experimental pancreatic and hepatocellular cancer. *Int J Cancer* 2012;130:2067–76.
24. Henderson RB, Hobbs JA, Mathies M, Hogg N. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood* 2003;102:328–35.
25. Imhof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol* 2004;4:432–44.
26. Beierle EA, Ma X, Stewart J, Nyberg C, Trujillo A, Cance WG, et al. Inhibition of focal adhesion kinase decreases tumor growth in human neuroblastoma. *Cell Cycle* 2010;9:1005–15.
27. Golubovskaya VM, Nyberg C, Zheng M, Kweh F, Magis A, Ostrov D, et al. A small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, targeting the y397 site of focal adhesion kinase decreases tumor growth. *J Med Chem* 2008;51:7405–16.
28. Hochwald SN, Nyberg C, Zheng M, Zheng D, Wood C, Massoll NA, et al. A novel small molecule inhibitor of FAK decreases growth of human pancreatic cancer. *Cell Cycle* 2009;8:2435–43.
29. Lim CJ, Han J, Yousefi N, Ma Y, Amieux PS, McKnight GS, et al. Alpha4 integrins are type I cAMP-dependent protein kinase-anchoring proteins. *Nat Cell Biol* 2007;9:415–21.
30. Burns-Hamuro LL, Ma Y, Kammerer S, Reineke U, Self C, Cook C, et al. Designing isoform-specific peptide disruptors of protein kinase A localization. *Proc Natl Acad Sci U S A* 2003;100:4072–7.
31. Berlin-Rufenach C, Otto F, Mathies M, Westermann J, Owen MJ, Hamann A, et al. Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *J Exp Med* 1999;189:1467–78.
32. Cyster JG. Homing of antibody secreting cells. *Immunol Rev* 2003;194:48–60.
33. Koni PA, Joshi SK, Temann UA, Olson D, Burkly L, Flavell RA. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J Exp Med* 2001;193:741–54.
34. Leuker CE, Labow M, Muller W, Wagner N. Neonatally induced inactivation of the vascular cell adhesion molecule 1 gene impairs B cell localization and T cell-dependent humoral immune response. *J Exp Med* 2001;193:755–68.
35. Minges Wols HA, Underhill GH, Kansas GS, Witte PL. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J Immunol* 2002;169:4213–21.