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Fine-Tuning Tumor Immunity With Integrin Trans-regulation

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Abstract

Inefficient T-cell homing to tissues limits adoptive T-cell immunotherapy of solid tumors. α L β 2 and α 4 β 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 α 4 integrin in cells results in an increase in α L β 2-mediated migration on mixed ICAM-1-VCAM-1 substrates *in vitro*, a phenomenon termed “integrin trans-regulation.” Here we created an α 4(S988A)-bearing mouse, which precludes PKA-mediated α 4 phosphorylation, to examine the effect of integrin trans-regulation *in vivo*. The α 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 α 4(S988A) mice exhibited a marked increase in T cell entry into and reduced growth of B16 melanomas, consistent with anti-tumor roles of infiltrating T cells and pro-growth functions of tumor-associated macrophages. Thus, increased α 4 trans-regulation of α L β 2 integrin function biases leukocyte emigration towards lymphocytes relative to myeloid cells and enhances tumor immunity.

Keywords

α 4 Integrin; Transregulation; Immunotherapy; Migration; T-cell

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). α 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 α 4 cytoplasmic domain (tail) (6). Protein Kinase A (PKA)-mediated phosphorylation of the α 4 integrin tail at Ser⁹⁸⁸ inhibits paxillin binding in migrating cells (7); consequently the α 4(S988A) mutation stabilizes the α 4-paxillin interaction and increases α 4 integrin signaling. The increased signaling downstream of α 4(S988A) enhances integrin α L β 2(LFA-1)-mediated migration of cells, a phenomenon termed integrin trans-regulation (8).

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Here we examined the biological consequences of blockade of $\alpha 4$ phosphorylation by generating $\alpha 4(S988A)$ mutant mice and find that these mice manifest a dramatic increase in recruitment of lymphocytes, but not myeloid cells, to an inflammatory site. We found that the $\alpha 4(S988A)$ 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 immuno-therapies for solid tumors (9–14). The potential therapeutic value of this form of integrin trans-regulation was established by the finding that subcutaneously implanted melanomas grew more slowly in $\alpha 4(S988A)$ mice associated with markedly enhanced recruitment of T cells but not myeloid cells to the tumors. Thus increasing integrin trans-regulation by blocking $\alpha 4$ 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.

Methods

Mice

$\alpha 4(S988A)$ mice were generated as described in previous supplementary material (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, $\alpha 4(S988A)$ mice were crossed with the OT-1 strain. *Rag1*^{-/-} 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 (IACUC).

Hematological analysis

Blood from adult (10–20 wk-old) $\alpha 4(S988A)$ and $\alpha 4$ (wildtype) 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 who also manually performed differential counts on DiffQuick–stained smears.

Peritonitis model

Adult (10–20 weeks old) $\alpha 4(S988A)$ and control $\alpha 4$ (wt) mice were injected intraperitoneally with 4% (wt/vol) thioglycollate (Sigma-Aldrich) and sacrificed at various time points for peritoneal lavage. Cells (1×10^5) were adhered to glass slides with a Cytospin4 instrument (ThermoShandon) and stained with DiffQuick 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 the competitive peritonitis assay, peritonitis was elicited as described above, but in *Rag1*^{-/-} mice. 24h 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 lavage were stained using antibodies against CD3, CD8, and Ly5.1. The ratio of $\alpha 4(S988A)$ to Ly5.1+ $\alpha 4$ (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 (BM) cell suspensions were prepared by flushing femur and tibia bones from adult $\alpha 4$ (S988A) and $\alpha 4$ (wildtype) mice. Single-cell suspensions from BM, 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 (11-26)), 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 $\alpha 4$ (wildtype) mice (F5 backcross to BL6) were injected i.p. with 100 μ g TNP-KLH (Biosearch) emulsified in 250 μ l CFA (T cell-dependent antigen). Blood serum was collected by centrifugation of tail vein bleed (100–200 μ l with 1–2 mM EDTA solution as an anticoagulant) before (pre-immune) and at 1, 2, and 3 wk after immunization. TNP-specific antibody concentrations in blood sera were assessed by direct ELISA with TNP-OVA as the coating antigen and AP-conjugated polyclonal anti-mouse IgG (Sigma), polyclonal anti-mouse IgM (Sigma), or anti-mouse IgG₃ (Clone R40-82, BD Biosciences) 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) 3.0 μ 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 min at room temperature. Cells (2.0×10^5) were added to the top chamber in complete medium (10% FBS). Complete medium containing 15 ng/ml Stromal-derived factor-1 α (SDF-1 α ; R&D Systems) added to the lower chamber. To observe macrophage migration, 20 ng/ml of both SDF-1 and MCP-1 was necessary. After a 4h 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) cells were expanded in culture in complete medium (DMEM supplemented with 10% FBS, ℓ -glutamine, β ME, and pen/strep antibiotics). B16 cells (3×10^5) were injected subcutaneously in the right hind flank 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 calculated using the formula: $(\text{length} \times \text{width}^2)/2$. Mice were sacrificed on day 15 and tumors excised and weighed. To analyze tumor-infiltrating leukocytes (TIL), tumors were digested with collagenase (Sigma) for 20–30 min. at 37C and further processed to a single-cell suspension using a 7ml tissue

grinder (Kontes) and counted. Fluorochrome-conjugated antibodies were used to stain for tumor-infiltrating CD45+ leukocytes and identify CD4+ and CD8+ T cells (CD3+), as well as CD4+Foxp3+ Treg and NK1.1+ NK cells. Total subset numbers were calculated by multiplying the total cell number with %CD45+ and % of each subset. For lymphoid cell depletion, we injected anti-CD8 antibody (53-6.7, 100µg) or a combination of anti-CD4, anti-CD8, and anti-NK1.1 antibodies (150 µg each), compared with isotype control antibody(s) 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 TCR background, α4(S988A) and wild type mice were immunized with a combination of 100 µg poly I:C (Invivogen), 50 µg anti-CD40 antibody (Biolegend), and 500 µg ovalbumin protein (Sigma) in PBS i.p. Six days later, mice were sacrificed and spleen cells stained using anti-CD8+ antibody and a H-2K^b-SIINFEKL(PE) tetramer (Coulter).

Assessment of CD8 T cell cytotoxic function

To generate functional CTL, splenocytes from α4(S988A) and wild type OT-1 mice were cultured with 1 µg/ml SIINFEKL and 100U/ml IL-2 (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, CTL were harvested, counted, and cultured with SIINFEKL pulsed (1 µg/ml for 1–2h) or unpulsed splenocytes as targets in the presence of anti-LAMP-1-PE antibodies (eBioscience) for 2.5 h at 37C. Effector/target cultures were stained with anti-CD8 antibodies and analyzed by flow cytometry. For target lysis *in vitro*, CTL were generated as above and cultured with a ~50/50 mixture of peptide-pulsed (CFSElo) and unpulsed (CFSEhi) splenocyte targets overnight. Specific lysis is the %decrease in the percentage of the peptide-pulsed peak between CTL-containing and no-CTL control cultures.

Statistical Analysis

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 α4(S988A) mutation selectively increases lymphocyte migration to an inflammatory site

We used homologous recombination to generate α4(S988A) mice (Supplemental, S1) and compared them to wild type C57BL/6 mice as controls. We observed no significant differences in formed elements of the blood (Supplemental, S2A) or in lymphocyte numbers (with the possible exception of increased Pro B cells in the bone marrow) in primary or secondary lymphoid tissue Supplemental, S2B) in α4(S988A) mice. Humoral immune responses to a T-dependent antigen were also similar between α4(S988A) and wild type mice (Supplemental, 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 $\alpha 4(S988A)$ mouse compared with controls (Fig. 1). In contrast, the numbers of B1 cells, a resident peritoneal population (15), showed no significant differences from controls, indicating that the $\alpha 4(S988A)$ mutation specifically affects influx of lymphocytes. An *in vivo* competitive migration assay (Supplemental, S3) using a mix of wild-type (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 Protein Kinase A 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 α . 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, Supplemental, S4). This observation cannot be explained by differences in surface integrin expression levels, as T cells from $\alpha 4(S988A)$ and $\alpha 4(wt)$ 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 subcutaneously and tumor size is measured by weighing excised tumors 15 days later. $\alpha 4(S988A)$ mice had ~5-fold smaller tumors than wild type BL6 controls (Fig. 3A), indicating that blocking $\alpha 4$ integrin phosphorylation on Ser988 increased tumor protection. This observation did not appear to be unique to the B16 model, as $\alpha 4(S988A)$ mice also displayed increased resistance to the growth of Lewis Lung Carcinoma (Supplemental, S5)

We next asked whether $\alpha 4(S988A)$ resistance 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 wild type mice (Fig. 3B, left panel). This was in striking contrast to 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 vs. macrophages in the $\alpha 4(S988A)$ mice. Among lymphoid cells, tumors in $\alpha 4(S988A)$ mice had significantly greater numbers of CD4+, CD8+, and regulatory T cells; NK cell abundance showed a modest, but statistically insignificant, increase compared to controls (Fig. 3B, right panel). The increased density of tumor-infiltrating T cells seen in $\alpha 4(S988A)$ mice could be due to greater clonal expansion of tumor-specific T cells; however, expansion of antigen-specific T cells (SIINFEKL-tetramer+) 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+ T cells was nearly identical to that of wild type (Fig. 3D) cells as measured by degranulation of $\alpha 4(S988A)$ OT-1 CD8+ T cells and specific lysis of SIINFEKL-pulsed target cells. Thus, we conclude that the reduction in tumor growth observed in $\alpha 4(S988A)$ mice is largely ascribable to increased lymphoid cell homing. Intriguingly, we also observed smaller tumors in $\alpha 4(S988A)$ mice partially depleted of CD8 T cells; however, reduced tumor growth in $\alpha 4(S988A)$ mice could not be observed when the majority of CD4 T, CD8 T, and NK cells were depleted (Supplemental, S6). This finding suggests that increased migration of multiple lymphoid cell subsets can contribute to the tumor resistance in $\alpha 4(S988A)$ mice.

The differential requirement of $\beta 2$ integrins for 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 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 mononuclear leukocytes since $\beta 2$ integrins are not required. Indeed, we did not

observe increased migration of monocytes/macrophages from $\alpha 4(S988A)$ or $\alpha 4(wt)$ mice (Supplemental, 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). Since 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-Protein Kinase A-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 Protein Kinase A 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 appears 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 offers a new approach to selectively potentiate $\beta 2$ integrin-mediated homing of lymphocytes and plasma cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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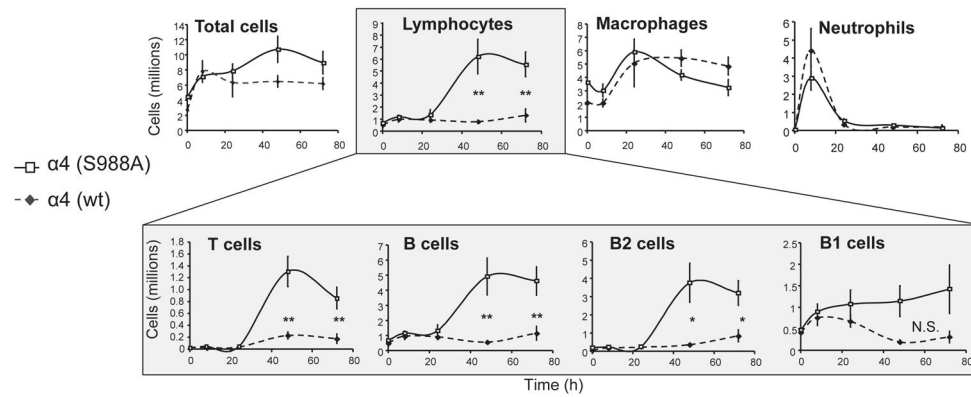
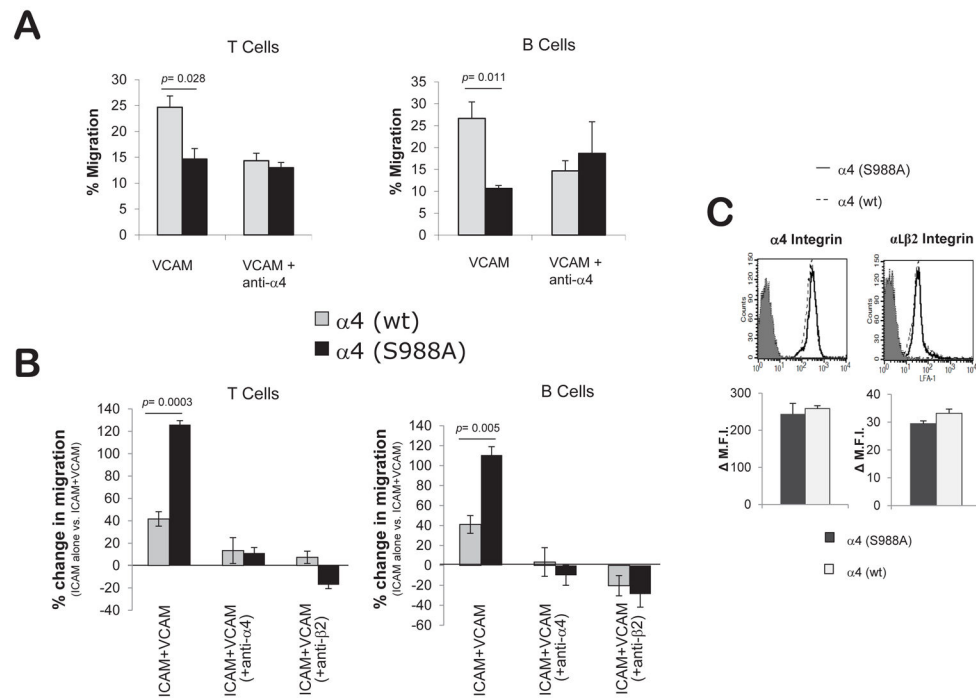
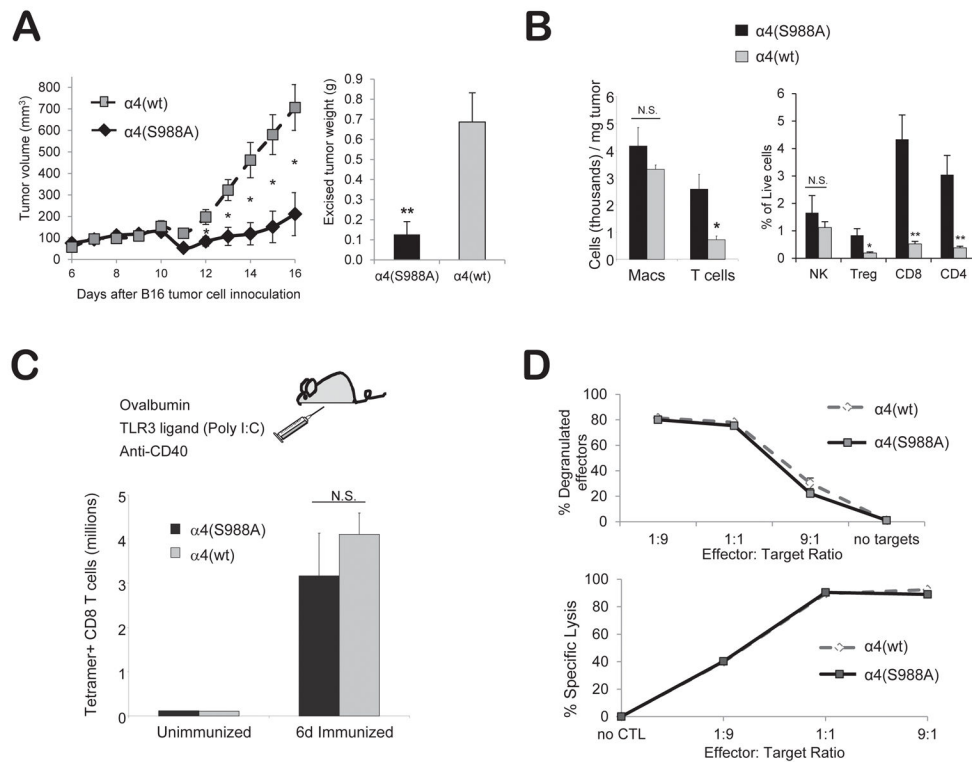


Figure 1. Leukocyte migration in the $\alpha 4$ (S988A) mouse. Adult $\alpha 4$ (S988A) or wild type mice were injected with 1 ml thioglycollate medium i.p. Mice were sacrificed at various time points and leukocytes from peritoneal lavage were enumerated and identified by cyto-spin and DiffQuick staining. Peritoneal lavage was also stained with antibodies to identify T-cells (CD3+), Total B-cells (B220+), B1 cells (B220+CD11b+), and B2 cells (B220+CD11b-). Error bars are S.E.M. of n=5 for each group. * $p < 0.03$, ** $p < 0.02$; N.S. = not statistically significant.

**Figure 2.**

Increased integrin trans-regulation in α 4(S988A) lymphocytes. B and T-cells were purified from spleens of α 4(S988A) or control α 4(wt) mice. Chemotactic migration towards SDF-1 α (15ng/ml) was assessed using a modified Boyden Chamber assay in (A) wells coated with VCAM-1 alone (2 ug/ml), or (B) ICAM-1 (5 ug/ml) +/- VCAM-1 (0.02 ug/ml). For anti-integrin antibody blocking studies, cells were treated with 10 μ g/ml of either anti- α 4 or anti- β 2 integrin prior to the assay. Part (B) is the % increase in migration on ICAM-1+VCAM-1 compared to ICAM-1 alone $100 * [(ICAM+VCAM \text{ migration} - ICAM \text{ migration}) / ICAM \text{ migration}]$. Error bars are S.E.M. of n=4 for each group. (C) *Surface integrin expression levels on circulating α 4(S988A) T-cells*. Blood leukocytes from α 4(S988A) and α 4(wt) control adult C57BL/6 mice (n=3 per group) were stained with antibodies specific for T-cells (CD3), α 4 integrins (CD49d), and α L β 2 integrin heterodimer and analyzed by flow cytometry. Representative histograms show α 4 or α L β 2 staining (solid and dotted lines) compared with non-specific isotype control staining (filled peak). Bar graphs summarize staining from n= 3 mice per group; No significant differences were observed. M.F.I = Change in Mean Fluorescence Intensity.

**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 (3×10^5) were injected into $\alpha 4$ (S988A) or control $\alpha 4$ (wt) mice. Tumor area was measured daily and converted to an ellipsoid volume: (length x width²)/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+) CD11b+ macrophages, CD3+ T cells, CD4+ T-cells, CD8+ T-cells, Foxp3+(CD4+) Treg, and NK1.1+ NK cells. Error bars are S.E.M. of n 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 stained with anti-CD8 antibody and an H-2K^b-SIINFEKL tetramer. **(D) T-cell cytotoxic function.** Splenocytes of 8–12 wk-old *OT-I*+ $\alpha 4$ (S988A) or *OT-I*+ $\alpha 4$ (wt) control littermate mice were differentiated to CTL in vitro with SIINFEKL (1 μ /ml) and IL-2 for 5 days and cultured for 2 h 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, CTL were cultured with SIINFEKL-pulsed or unpulsed targets that had been differentially labeled with CFSE. Specific killing was detected by flow cytometry as the decrease in the percentage of specific targets. No 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 $\alpha 4$ (wt) CTL ($p < .005$). n = 3 mice per group. Experiment was performed twice.