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. α4β2 and α4β1 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 α4 integrin in cells results in an increase in α4β2-mediated migration on mixed ICAM-1–VCAM-1 substrates in vitro, a phenomenon termed “integrin trans-regulation.” Here, we created an α4(S988A)-bearing mouse, which precludes PKA-mediated α4 phosphorylation, to examine the effect of integrin trans-regulation in vivo. The α4(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 α4(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 α4 trans-regulation of α4β2 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). α4 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 α4 cytoplasmic tail (6). Protein kinase A (PKA)–mediated phosphorylation of the α4 integrin tail at Ser988 inhibits paxillin binding in migrating cells (7); consequently, the α4(S988A) mutation stabilizes the α4–paxillin interaction and increases α4 integrin signaling. The increased signaling downstream of α4(S988A) enhances integrin α4β2(α4β1)–mediated migration of cells, a phenomenon termed integrin trans-regulation (8).

Here, we examined the biologic consequences of blockade of α4 phosphorylation by generating α4(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 α4(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).

Materials and Methods

Mice

α4(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, α4(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.

Hematologic analysis

Blood from adult (10–20-week-old) α4(S988A) and α4[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 facility, whose staff also manually performed differential counts on DiffQuick-stained smears.

Peritonitis model

Adult (10–20-week-old) α4(S988A) and control α4(wt) mice were injected i.p. with 4% (weight/vol) thioglycollate (Sigma-Aldrich) and sacrificed at various time points for peritoneal lavages. Cells (1 × 10⁶) were adhered to glass slides with a
Cytospin4 instrument (ThermoShandon) and stained with Diff-Quick to differentiate cell types by light microscopy. The percentage 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 α4(S988A) or control Ly5.1 α4(wt). Splenocyte suspensions and peritoneal lavages were stained using antibodies against CD3, CD8, and Ly5.1. The ratio of α4(S988A) to Ly5.1<sup>+</sup> α4(wt) splenocytes was compared between spleen and peritoneum as a measure of enrichment for α4(S988A) cells in the inflammatory site.

**Lymphoid compartments**

Bone marrow cell suspensions were prepared by flushing femur and tibia bones from adult α4(S988A) and α4(wt) mice. Single-cell suspensions from bone marrow, spleen, and thymus were treated with ACK lysis buffer, counted, and stained with fluorochrome-conjugated antibodies against mouse B220 (RA3-6B2), IgM (II/41), IgD (11-26c), CD21/35 (7G6), CD23 (B384), 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 in 250 μL complete Freund’s adjuvant (TNP-ova) as the coating antigen and alkaline phosphatase 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.

**Humoral immune response**

For antigen-specific antibody responses, α4(S988A) and α4(wt) mice (F5 backcross to BL6) were injected i.p. with 100 μg trinitrophenol-keyhole limpet hemocyanin (TNP-KLH; Biosearch) emulsified in 250 μL complete Freund’s adjuvant (T-cell–dependent antigen). Blood serum was collected by centrifugation of tail vein bleed (100–200 μL with 1–2 mmol/L EDTA solution as an anticoagulant) before (preimmune) 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 α4 (S988A) and control α4(wt) mice by negative depletion. Macrophages were differentiated from α4(S988A) or control α4(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-μm 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 × 10<sup>5</sup>) were added to the top chamber in complete medium (10% FBS). Complete medium containing 15 ng/mL stromal-derived factor-1α (SDF-1α; 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, 1-glutamine, βME, and pen/strep antibiotics). B16 cells (3 × 10<sup>5</sup>) or LLC cells (1 × 10<sup>5</sup>) were injected s.c. into the right hind flanks of adult α4(S988A) or control α4(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 × 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 CD4<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 CD4<sup>+</sup> and percentage of each subset. For lymphoid cell depletion, we injected anti-CD8 antibody (53-6.7, 100 μg) or a combination of anti-CD4, anti-CD8, and anti-NK1.1 antibodies (150 μg 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, α4(S988A) and wt mice were immunized with a combination of 100 μg poly I:C (Inviron), 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 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 α4(S988A) and wt–OT-1 mice were cultured with 1 μg/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 μg/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. Effectortarget 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 α4(S988A) mutation selectively increases lymphocyte migration to an inflammatory site

We used homologous recombination to generate α4(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 α4(S988A) mice. Humoral immune responses to a T-dependent antigen were also similar between α4(S988A) and wt mice (Supplementary Fig. S2C).

Because the α4(S988A) mutation inhibits migration on substrates containing purified α4 integrin ligands (7), we hypothesized that α4(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 the numbers of B1 cells, a resident lymphoid tissue (Supplementary Fig. S2B) in α4(S988A) mice. Therefore, interfering with α4 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 α4(S988A) lymphocytes

Our initial expectation that the α4(S988A) mutation would decrease homing was based on results from in vitro migration on purified α4 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 α4 integrin ligands (e.g., VCAM-1) increases the migration of Jurkat T cells on αLβ2 integrin substrates (e.g., ICAM-1) in vitro (8). Paxillin binding to α4 is required for this effect, and enforced paxillin–α4 association enhanced the trans-regulation of αLβ2 by α4 integrins through increasing the activation of FAK and Pyk2 kinases (8, 18). The α4(S988A) mutation that blocks PKA phosphorylation of the α4 cytoplasmic tail could therefore enhance αLβ2-dependent migration. The in vivo peritonitis experiment requires migration on mixed substrates and is dependent on both α4 and β2 integrins (17), conditions in which the α4(S988A) mutation could increase migration of lymphocytes (8). To explore this possibility in a controlled setting, we purified B and T cells from α4(S988A) mice and measured their ability to migrate in vitro on purified α4 ligand (VCAM-1), purified β2 ligand (ICAM-1), or mixed substrates (VCAM-1 + ICAM-1) in response to the chemokine SDF-1α. Using a modified Boyden chamber assay, we confirmed that α4(S988A) lymphocytes exhibited reduced migration on a purified α4 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 α4(S988A) mice displayed enhanced migration that was dependent on both α4 and β2 integrins (Fig. 2B; Supplementary Fig. S4). This observation cannot be explained by differences in surface integrin expression levels, as T cells from α4(S988A) and...
α4(wt) mice show no difference in staining for α4 or αLβ2 integrins (Fig. 2C). These data indicate that the α4(S988A) mutation provides an increase in β2 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 α4(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. α4(S988A) mice had approximately 5-fold smaller tumors than wt BL6 controls (Fig. 3A), indicating that blocking α4 integrin phosphorylation on Ser988 increased tumor protection. This observation was not unique to the B16 melanoma, as α4(S988A) mice also displayed increased resistance to the growth of Lewis lung carcinoma (Supplementary Fig. S5).

We next asked whether the α4(S988A) resistance to tumors was associated with increased T-cell homing. Indeed, B16 tumors in α4(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 α4(S988A) mice. Among
lymphoid cells, tumors in α4(S988A) mice had significantly greater numbers of CD4⁺, CD8⁺, 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 α4(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⁺) was similar between α4(S988A) and α4(wt) mice in response to immunization with ovalbumin (Fig. 3C). Furthermore, the cytotoxic function of α4(S988A) CD8⁺ T cells was nearly identical to that of wt (Fig. 3D) cells as measured by degranulation of α4(S988A) OT-1 CD8⁺ T cells and specific lysis of SIINFEKL-pulsed target cells. Thus, we concluded that the reduction in tumor growth observed in α4(S988A) mice is largely ascribable to increased lymphoid-cell homing. As an additional test of this conclusion, we used antibodies to deplete the major-ity of CD4 T, CD8 T, and NK cells. Depleting these multiple lymphoid subsets reversed the reduction in tumor growth in α4(S988A) relative to wt mice (Supplementary Fig. S6A). In contrast, partial depletion of CD8 T cells did not abolish the reduced tumor growth in α4(S988A) mice (Supplementary Fig. S6B). Thus, the capacity of the α4(S988A) mutation to increase the recruitment of multiple lymphocyte subsets is responsible for its ability to enhance tumor resistance.

The differential requirement of β2 integrins for migration of lymphocytes or myeloid cells in vivo can account for the remarkable leukocyte specificity of this form of trans-regulation. Whereas α4β2 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 β2 integrins and is reported to be solely dependent on α4β1 (24, 25). Thus, trans-regulation of migration would be absent in monocytes and macrophages because β2 integrins are not utilized. Indeed, we did not observe increased migration of macrophages from α4(S988A) or α4(wt) mice (Supplementary Fig. S7) on mixed ICAM-1–VCAM-1 substrates.

The finding that α4β1 trans-regulation of α4β2 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. α4 phosphorylation is type I–PKA–dependent (29); thus, increased trans-regulation could be induced by introduction of a dominant α4(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 α4 and α4β2 integrins (31–35) and could involve integrin trans-regulation. Thus, enhanced integrin trans-regulation can offer a new approach to selectively potentiate β2 integrin–mediated homing of lymphocytes and plasma cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was funded in part by NIH R01 HL 31950 and HL 117807. J.M. Cantor is funded by NIH R01–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.

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Published OnlineFirst January 19, 2015; DOI: 10.1158/2326-6066.CIR-13-0226


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

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