Combined Blockade of Integrin-α4β1 Plus Cytokines SDF-1α or IL-1β Potently Inhibits Tumor Inflammation and Growth

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Abstract

Tumor-associated macrophages promote tumor growth by stimulating angiogenesis and suppressing antitumor immunity. Thus, therapeutics that inhibit macrophage recruitment to tumors can provide new avenues for cancer therapy. In this study, we showed how chemotactants stromal cell-derived growth factor 1 alpha (SDF-1α) and interleukin 1 beta (IL-1β) collaborate with myeloid cell integrin-α4β1 to promote tumor inflammation and growth. We found that SDF-1α and IL-1β are highly expressed in the microenvironments of murine lung, pancreatic, and breast tumors; surprisingly, SDF-1α was expressed only by tumor cells, whereas IL-1β was produced only by tumor-derived granulocytes and macrophages. In vivo, both factors directly recruited proangiogenic macrophages to tissues, whereas antagonists of both factors suppressed tumor inflammation, angiogenesis, and growth. Signals induced by IL-1β and SDF-1α promoted the interaction of talin and paxillin with the cytoplasmic tails of integrin-α4β1, thereby stimulating myeloid cell adhesion to endothelium in vitro and in vivo. Inhibition of integrin-α4β1, SDF-1α, or IL-1β was sufficient to block tumor inflammation and growth, and the combined blockade of these molecules greatly accentuated these effects. Furthermore, antagonists of integrin-α4β1 inhibited chemotherapy-induced tumor inflammation and acted synergistically with chemotherapeutic agents to suppress tumor inflammation and growth. These results show that targeting myeloid cell recruitment mechanisms can be an effective approach to suppress tumor progression. Cancer Res; 71(22): 6965–73. ©2011 AACR.

Introduction

Solid tumors trigger intrinsic inflammatory responses that promote tumorigenesis and metastasis (1, 2). CD11b+ myeloid cells, which include granulocytes, macrophages, and myeloid-derived suppressor cells, progressively accumulate in primary tumor sites, where they promote tumor angiogenesis, invasion, and progression (3–5). Several studies have shown correlations between tumor-associated macrophage (TAM) abundance and unfavorable tumor outcomes (6, 7).

TAMs secrete proangiogenic cytokines and growth factors, including VEGF-A, TNFα, interleukin (IL)-6, IL-8, and basic fibroblast growth factor (bFGF; refs. 3, 4, 8), as well as matrix metalloproteinase 9 (MMP-9), which can promote angiogenesis by releasing proangiogenic factors such as VEGF-A from the extracellular matrix (9–11). These cells also express IL-10 and TGFβ, which suppress antitumor immune responses (12–15). A number of tumor-derived chemotactants, including stromal cell-derived growth factor 1 alpha (SDF-1α), colony-derived stimulating factor (CSF; ref. 1), IL-1β, and monocyte chemoattractant protein-1 (MCP-1), can recruit angiogenesis-promoting myeloid cells into tumors and stimulate tumor invasiveness (5, 8, 12, 16). As tumor inflammation develops in most growing tumors and in response to chemotherapeutic interventions, leading to relapses in tumor growth (9, 17), agents that block tumor inflammation promise to be useful therapeutics for solid tumors.

We recently found that integrin-α4β1 on the surface of immune cells promotes endothelial progenitor and myeloid cell recruitment to tumors (18, 19). Antagonists of integrin-α4β1 disrupt the interaction between α4β1 and its ligand vascular cell adhesion protein 1 (VCAM-1), thereby inhibiting immune cell adhesion to the endothelium (18–20). Chemokine-induced signaling promotes increased integrin affinity and avidity, and clustering of integrins at the cytoplasmic membrane, leading to leukocyte arrest on inflamed endothelium (20, 21). In this study, we identified new roles for tumor-derived inflammatory factors in the activation of

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myeloid cell integrin-α4β1 during myeloid cell recruitment to the tumor microenvironment. Combinatorial administration of antagonists of integrin-α4 and tumor-derived chemotactic agents potently suppressed tumor inflammation and growth in tumor models.

**Materials and Methods**

**Animals**

PyMT+ mice (22) and integrin-α4Y991A mice (23) were derived as described previously. C57BL/6 mice were obtained from Charles River and C57BL/6-Tg(ACTB-EGFP) mice were procured from Jackson Laboratories.

**Isolation and quantification of myeloid cells from tissues by flow cytometry**

To quantify myeloid cells, tissues were excised, minced, and digested for 1 hour at 37°C in 10 mL Hank's Balanced Salt Solution (HBSS; Gibco) containing 1 mg/mL collagenase type IV (Sigma), 10 mg/mL hyaluronidase type V (Sigma), and 20 units/mL DNase type IV (Sigma). Cells were incubated in FC-blocking reagent (BD Biosciences), followed by CD11b-APC (M1/70; ebioscience) and Gr1-FITC (RB6-8C5; ebioscience). To exclude dead cells, 2.5 μg/mL propidium iodide (PI) was added before data acquisition by FACS Calibur (BD Biosciences). Myeloid cells from human buffy coats, murine basement membrane (BM), or enzymatically digested tumor tissue were purified by anti-CD11b magnetic bead affinity chromatography (Miltenyi Biotec).

**Gene and protein expression**

Total RNA was isolated from tissue or cells using ISOGEN (Nippon Gene). Quantitative PCR (qPCR) was carried out using primers for SDF-1α, IL-1β, TNFα, IL-8, and IL-6 from Qiagen (QuantItect Primer Assay). qPCR for VegFA expression was carried out with the following primers: sense, GCTGTGACGGCTGCTTAC; and anti-sense, CGCATGAT-CTGATGTTGAT. Transcript levels were normalized to GAPDH. SDF-1α and IL-1β protein levels were determined in lysates of cultured cells, whole tumors, or from tumor-derived CD11b+/- cells using Quantikine mouse SDF-1α and IL-1β kits (R&D Systems).

**Cell biological and biochemical assays**

Adhesion and integrin-activation assays have been described previously (24). For immunoprecipitations, BM monocytic cells from wild-type or α4Y991A mice were treated with basal or conditioned medium for 30 minutes at 37°C. Cells were lysed in Tris-buffered saline containing 1% CHAPS, 0.5% Triton X-100, 0.2% sodium deoxycholate, 20 mM NaCl, 100 mM Na2HPO4, 50 mM MgCl2, 1% FBS, 100 μg/mL leupeptin, 3 μg/mL pepstatin, 2 μg/mL aprotinin, 10 μg/mL phenylmethylsulfonyl fluoride, 50 μg/mL trypsin inhibitor, 1 mM Pefabloc, 1 mM benzamidine, 1 μg/mL RNase A, and 1 μg/mL protease and phosphatase inhibitor cocktails (Sigma). To evaluate hypoxia in treated tumors, mice received an i.p. injection of the hypoxia indicator pimonidazole hydrochloride (50 mg/kg body weight), with saline (n = 6) or 1.25 mg/kg SDF-1α inhibitor (AMD3100; Sigma-Aldrich; n = 7) daily for 9 days, or with anti-IL-1β or AMD3100 plus function-blocking anti-α4 integrin antibody (200 μg PS2×25 g body weight) on days 3 and 5.

In addition, 5 × 105 LLC cells were subcutaneously implanted into C57BL/6 mice. One week later, animals were treated once per week by i.p. injection with 80 mg/kg gemcitabine and every third day with 200 μg/mouse PS2, or both together for 2 weeks. Alternatively, 1 × 106 Panc02 pancreatic ductal adenocarcinoma cells were orthotopically implanted in the tail of the pancreas. Two weeks later, animals were treated once per week by i.p. injection with 80 mg/kg gemcitabine, every third day with 200 μg/mouse PS2 antibody, or both together for 3 weeks.

In other studies, C57BL/6 mice were subcutaneously implanted with 5 × 106 LLC cells and were treated every third day with subcutaneous injections of ELN476063 (3 mg/kg body weight), an integrin-α4 small-molecule inhibitor. Alternatively, 6-week-old PyMT+/− female mice (with spontaneous breast tumors), were treated by subcutaneous injection with ELN476063 (3 mg/kg body weight) or saline every third day for 3 weeks (n = 10).

Further, 5 × 105 LLC cells were subcutaneously implanted into GFP− BM-transplanted C57BL/6 mice. Tumor size was assessed regularly with Vernier calipers using the formula \( (\pi^2 \times d \times 0.5) \). When tumors reached a size of 500 mm³, OXi4503, a prodrug derivative of the vascular disrupting agent combretastatin-A4 phosphate (CA4P), was administered i.p. at a dose of 100 mg/kg. Anti-integrin-α4–blocking antibody (PS2) was administered i.p. at a dose of 200 μg per mouse. Mice were sacrificed after 3 days, and tumors were analyzed for CD11b+ Gr1+ cells, CD31+ vessels, volume, and hypoxia.

To evaluate hypoxia in treated tumors, mice received an i.p. injection of the hypoxia indicator pimonidazole hydrochloride (60 mg/kg; Millipore) 90 minutes before euthanasia. Tumors and organs were removed and immediately fixed in 10% buffered formalin and then placed in 70% ethanol, or frozen were visualized using an enhanced chemiluminescence detection kit (Pierce).

**In vivo assays**

Growth factor-reduced Matrigel (BD Biosciences) containing 400 ng/400 μL SDF-1α, IL-1β (R&D Systems), or saline was injected subcutaneously into C57Bl6 mice (n = 6) transplanted with BM from ACTB-EGFP mice. After 7, 14, or 21 days, Matrigel plugs were excised, cryopreserved, sectioned, and immunostained for the presence of myeloid cells and endothelial cells.

Mycoplasma-negative Lewis lung carcinoma (LLC) cells were cultured in antibiotic- and fungicide-free Dulbecco’s Modified Eagle Medium (DMEM) containing 10% serum. C57Bl6 mice were subcutaneously implanted with 1 × 106 LLC cells and treated on days 3 and 5 with intraperitoneal (i.p.) injections of function-blocking anti-IL-1β antibodies (MAB401; R&D Systems; n = 16) or isotype-matched control antibodies rat IgG1 (n = 14; 100 μg/25 g body weight), with saline (n = 6) or 1.25 mg/kg SDF-1α inhibitor (AMD3100; Sigma-Aldrich; n = 7) daily for 9 days, or with anti-IL-1β or AMD3100 plus function-blocking anti-α4 integrin antibody (200 μg PS2×25 g body weight) on days 3 and 5.

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on dry ice in Tissue-Tek OCT Compound (Miles Inc.) and kept protected from light at −70°C.

Cryopreserved tumors were sectioned and immunostained for CD11b⁺ myeloid cells and tumor cells from LLC tumors that express Ly6C, Ly6G, F4/80, or CD14. B, chemotactic gene expression in cultured LLC cells and tumors (n = 3). C, chemotactic gene expression in CD11b⁺ myeloid cells and tumor cells freshly isolated from d14 subcutaneous LLC tumors (n = 3). D, chemotactic gene expression in CD11b⁺ Gr1⁻/neg granulocytic cells and CD11b⁺Gr1⁺/neg macrophages isolated from d14 subcutaneous LLC tumors (n = 3). E, protein expression of SDF-1α and IL-1β in cultured LLC and Panc02 cells (n = 3). F, protein expression of SDF-1α and IL-1β in CD11b⁺ myeloid cells and tumor cells from d14 subcutaneous LLC tumors (n = 3). G, CD11b protein and Il-1b and Sdf-1a mRNA expression in tumors over time (n = 3), P < 0.01 compared with basal media. H, mice were reconstituted with GFP⁺ bone marrow and stimulated with growth factor–depleted Matrigel saturated with saline, IL-1β, or SDF-1α for 3 weeks (n = 6). Representative images of d21 Matrigel sections showing endothelial cells (red, CD31), nuclei (blue, DAPI), and GFP⁺ BM cells (green). Graphs: quantification of GFP⁺ cells and CD31⁺ vessels; *, P < 0.05 compared with saline. Scale bar, 40 μm. Error bars represent SEM. Statistical significance was determined by the 2-tailed Student t test.

Additional details of methods can be found in Supplementary Materials.

Results

**IL-1β and SDF-1α recruit proangiogenic monocytic cells to the tumor microenvironment**

To determine whether specific inflammatory factors recruit myeloid cells to tumors, we first characterized the tumor-associated myeloid cell population in LLC tumors. CD11b⁺ myeloid cells extensively invaded LLC tumors and accounted for up to one quarter of the tumor cell population (Supplementary Fig. S1; ref. 24). Tumor-derived...
myeloid cells included 81.7% CD11b<sup>+</sup>Gr1<sup>lo/neg</sup>Ly6G<sup>-</sup>-monocytic cells and 18.3% CD11b<sup>+</sup>Gr1<sup>hi</sup>Ly6G<sup>+</sup> granulocytic cells (Supplementary Fig. S1). The majority of myeloid cells in tumors were CD11b<sup>+</sup>CD14<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup>macrophages (Fig. 1A). All tumor-derived myeloid cells expressed the adhesion receptor CD49d, integrin-α4β1 (Supplementary Fig. S2A and S2B).

To determine which myeloid cell chemoattractants are expressed in tumors, we isolated tumor cells and tumor-derived CD11b<sup>+</sup> myeloid cells from LLC, Panc02 pancreatic carcinomas and PyMT spontaneous breast tumors in vitro and in vivo. Lung, pancreatic, and breast tumor cells expressed high levels of Sdf1<sub>a</sub> mRNA whether cultured in vitro or in vivo, whereas only CD11b<sup>+</sup> myeloid cells from tumors expressed high levels of IL-1β mRNA (Fig. 1B and C; Supplementary Fig. S3A and S3B). Although CD11b<sup>+</sup>Gr1<sup>lo</sup>F4/80<sup>+</sup>macrophages expressed high levels of IL-1β, IL-6, TNFα, and VEGF-A (Fig. 1D; Supplementary Fig. S3C). Tumor cells expressed SDF-1α but not IL-1β protein in vitro and in vivo, whereas CD11b<sup>+</sup> myeloid cells isolated from tumors expressed IL-1β but not SDF-1α protein (Fig. 1E and F; Supplementary Fig. S3D). Notably, the recruitment of CD11b<sup>+</sup> myeloid cells to tumors paralleled the overall expression of IL-1β and SDF-1α in the tumor (Fig. 1G; Supplementary Fig. S3E), indicating functional relationships between expression of these factors and myeloid cell recruitment.

Both SDF-1α and IL-1β can promote hematopoietic cell trafficking and tumor inflammation (25, 26). To compare the roles of IL-1β and SDF-1α in myeloid cell recruitment to tumors and subsequent angiogenesis, we transplanted ACTB-EGFP bone marrow into wild-type mice and, 4 weeks later, implanted growth factor-depleted Matrigel containing saline, IL-1β, or SDF-1α into mice. We then evaluated the...
number of GFP<sup>+</sup> bone marrow-derived cells and blood vessels in Matrigel plugs (Fig. 1H). SDF-1α and IL-1β both stimulated recruitment of GFP<sup>+</sup> bone marrow-derived cells as well as new blood vessel growth (Fig. 1H). These findings indicate that both SDF-1α and IL-1β directly recruit bone marrow-derived cells and stimulate angiogenesis. Because tumor cells express SDF-1α, and myeloid cells express IL-1β, results of this study indicate that both tumor and myeloid cells promote tumor inflammation.

Blockade of IL-1β or SDF-1α inhibits tumor inflammation and growth

To determine whether SDF-1α and IL-1β recruit myeloid cells during LLC tumor progression, mice bearing LLC tumors were treated for 10 days with antagonists of SDF-1α or IL-1β; tumor inflammation and growth was analyzed. Anti-IL-1β significantly suppressed tumor growth (Fig. 2A) as well as myeloid cell recruitment and angiogenesis (Fig. 2B). These results indicate that inhibition of myeloid cell recruitment by IL-1β antagonists decreased tumor blood vessel formation. Similarly, blockade of SDF-1α signaling with the CXCR4 antagonist AMD3100 inhibited LLC tumor growth (Fig. 2C), myeloid cell recruitment, and angiogenesis (Fig. 2D). Both inhibitors reduced expression levels of SDF-1α, IL-1β, IL-6, TNFα, and VEGF-A in tumors (Fig. 2E), of IL-1β, IL-6, and VEGF-A in tumor-derived macrophages (Fig. 2F) and of IL-1β in tumor-derived CD11b<sup>+</sup> Gr1hi granulocytes (Supplementary Fig. S4). Taken together, these findings indicate that IL-1β and SDF-1α recruit tumor-promoting myeloid cells to the tumor microenvironment and that inhibition of these inflammatory factors blocks myeloid cell recruitment to tumors, thereby inhibiting angiogenesis and resulting in suppression of tumor growth.

Inflammatory factors induce integrin-α4β1 activation and clustering

Immune cell extravasation depends upon activation of integrins by chemoattractant-stimulated adhesion of cells to vascular endothelium. We investigated whether specific tumor-derived inflammatory factors promote integrin-dependent myeloid cell adhesion to the endothelium. Fluorescence-labeled murine myeloid cells exhibited increased adhesion to vascular endothelium after a brief (30-minute)

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**Figure 3.** Tumor-derived factors promote integrin-α4β1 activation and myeloid cell adhesion to endothelium. A, adhesion of chemoattractant-treated myeloid cells [fluorescence units (F.U.)] to endothelium in the absence or presence of control IgG, anti-α4, anti-αM, anti-α5, and anti-αv integrin antibodies (n = 3) or after transfection with integrin-α4 and control siRNAs; *P < 0.01 compared with IgG. B, adhesion to endothelium of chemoattractant-stimulated wild-type (WT) and MyD88<sup>−/−</sup> myeloid cells and WT myeloid cells in the absence or presence of pertussis toxin (PTX) or AMD3100 (n = 3). *P < 0.01 compared with WT. C, histogram of human myeloid cells stained to detect active (HUTS21; top) and total (PAC10; bottom) human integrin-β1 in the absence (unstimulated; gray-filled) or presence (stimulated; black line) of SDF-1α, IL-1β, or Mn2<sup>+</sup>; a positive control activator. IgG-negative control appears as a thin line (n = 5). D, images: clustering of integrin-α4 – green, arrowheads) in human myeloid cells incubated with SDF-1α, IL-1β-, or human serum albumin (HSA)-coated microspheres and counterstained for DNA (blue). Graph: percentage of integrin-α4β1 clustering per myeloid cell (n > 15). *P < 0.001 compared with HSA. E, percentage of integrin-α4β1 clustering of WT and MyD88<sup>−/−</sup>, PTX-, and AMD3100-treated WT myeloid cells after incubation with SDF-1α or IL-1β-coated microspheres (n > 15). *P < 0.001 compared with WT. Scale bar, 5 μm. Error bars represent SEM. EC, endothelial cells.
stimulation by purified SDF-1α, IL-1β, or LLC tumor cell-conditioned medium (TCM, which contains largely SDF-1α, TNFα, and VEGF-A; Fig. 3A). This adhesion event was mediated by integrin-α4, as antagonists of integrin-α4 and siRNA-mediated knockdown of integrin-α4 expression blocked myeloid cell adhesion to endothelium, whereas antagonists of other myeloid cell integrins, including αv, α5, and αM, had no effects (Fig. 3A; Supplementary Fig. S5A and S5B).

We, therefore, investigated the role of receptor-mediated signaling in integrin-α4–mediated cell adhesion. Myeloid cells from MyD88−/− mice, which are defective in TLR/IL-1β receptor signaling, failed to adhere to the endothelium in the presence of IL-1β, but adhered normally in the presence of SDF-1α (Fig. 3B), indicating that IL-1R–mediated signal transduction is required for IL-1β–induced adhesion. Similarly, pertussis toxin and AMD3100 inhibited SDF-1α–induced, but not IL-1β–induced, adhesion, indicating that CXCR4–mediated signal transduction is necessary for SDF-1α and TCM-induced cell adhesion to the endothelium (Fig. 3B), thus indicating that SDF-1α is a key active component in TCM (Fig. 3B). Together, these data indicate that diverse tumor-derived chemoattractants promote integrin-α4β1–mediated myeloid cell adhesion using nonoverlapping signaling pathways.

Chemokine signaling can promote rapid conformational changes in integrin-β chains that rapidly unfold integrin heterodimers and increase their affinity for ligand (20, 21, 27, 28). These changes can be detected by the binding of a monoclonal antibody, HUTS21, to newly revealed epitopes on the human β1-integrin subunit (21). To determine whether tumor-derived factors induce conformational changes in integrins of myeloid cells, we stimulated human myeloid cells for 10 minutes with 200 ng/mL SDF-1α, IL-1β, or Mn2+...
(a positive control) and conducted flow cytometry to detect binding of HUTS21 and P4C10, an antibody that recognizes β1 integrins regardless of conformation. SDF-1α and IL-1β increased HUTS21 binding but had no effect on P4C10 binding, indicating that SDF-1α and IL-1β can activate myeloid cell β1 integrins without affecting integrin expression (Fig. 3C).

Conformational changes in integrins can alter their avidity for ligand by inducing integrin clustering within the plane of the lipid bilayer. Therefore, we examined the effect of SDF-1α and IL-1β on integrin-α4β1 clustering. Both SDF-1α and IL-1β rapidly (within 5 minutes) stimulated integrin-α4 clustering in human and murine myeloid cells (Fig. 3D; Supplementary Fig. S6). Receptor-mediated signaling is required for IL-1β and SDF-1α-induced integrin-α4 clustering, as IL-1β–stimulated MyD88−/− cells and SDF-1α–stimulated wildtype cells treated with pertussis toxin or AMD3100 exhibited no integrin-α4 clustering (Fig. 3E). These results support the hypothesis that tumor-derived factors stimulate integrin-α4β1–mediated myeloid cell adhesion by increasing both the affinity and the avidity of integrin-α4β1.

Inflammatory factors regulate integrin-α4 activation by increasing integrin-α4 interactions with paxillin and talin

Activation of integrin-α4β1 depends on association of talin with an NPXY domain in β1 cytoplasmic tails, and its adhesion under flow depends on association of paxillin with tyrosine-991 within the integrin-α4 cytoplasmic tail (20, 23, 27–29). We, therefore, evaluated integrin-α4β1 co-clustering with paxillin and talin in myeloid cells from wild-type mice and from mice expressing an α4 Y991A mutation (α4Y991A), which disrupts the integrin-α4–paxillin binding site and partially disrupts α4β1 interactions with talin (23). IL-1β and SDF-1α rapidly (within 5 minutes) stimulated integrin-α4 clustering in wild-type but not α4Y991A cells (Fig. 4A). Inflammatory mediators induced co-clustering of integrin-α4β1 with paxillin and talin in wild-type but not α4Y991A myeloid cells (Fig. 4B and C), although integrin-α4 surface expression levels are identical in wild-type and α4Y991A cells (Supplementary Fig. S7). In addition, paxillin and talin co-immunoprecipitated with integrin-α4β1 in myeloid cells from wild-type but not α4Y991A mice upon stimulation with TCM (Fig. 4D). Importantly, α4Y991A...
myeloid cells failed to adhere in response to SDF-1α or IL-1β (Fig. 4E) and exhibited defective cell migration on the integrin-α4 ligand VCAM-1 but not on vitronectin, an integrin-αv ligand (Fig. 4F).

**Combination of integrin-α4 antagonists with inflammatory inhibitors effectively blocks tumor inflammation and growth**

As SDF-1α and IL-1β can stimulate integrin-α4β1-mediated adhesion to endothelium in vitro, we asked whether these factors stimulate integrin-α4β1-mediated trafficking of myeloid cells into tumors in vivo. Fluorescence-labeled myeloid cells injected intravenously into mice rapidly accumulated in established tumors (Fig. 5A). In contrast, myeloid cells that were co-injected with antagonists of SDF-1α, IL-1β, or integrin-α4β1 failed to accumulate in tumors (Fig. 5A). Notably, combined blockade of SDF-1α and IL-1β reduced the recruitment of myeloid cells to tumors to the same extent as integrin-α4β1 blockade, supporting the idea that activation of integrin-α4β1 by both SDF-1α and IL-1β plays a critical role in tumor inflammation. Importantly, trafficking of myeloid cells to other organs was not affected (Fig. 5A). Our results show that tumor-derived SDF-1α and macrophage-derived IL-1β activate integrin-α4 and together promote myeloid cell recruitment to the tumor microenvironment.

To determine whether antagonists of integrin-α4, IL-1β, and SDF-1α in combination could have beneficial antitumor effects, we evaluated the effects of treating LLC tumors with the integrin-α4 function-blocking antibody PS2, the SDF-1α inhibitor AMD3100, and anti–IL-1β antibodies alone or in combination. Treatment with AMD3100, anti–IL-1β, or anti-α4 alone inhibited tumor growth, decreasing tumor burdens to approximately 50% that of untreated tumors (Fig. 5B). Combined administration of anti–IL-1β and AMD3100 further suppressed tumor growth, while combined blockade of integrin-α4 and SDF-1α or IL-1β reduced tumor growth by 80% (Fig. 5B). Combination therapies also strongly blocked tumor inflammation (Fig. 5C). These findings indicate that targeting inflammation by blocking integrin-α4 and inflammatory factors such as IL-1β or SDF-1α may provide therapeutic benefit in the treatment of solid tumors.

**Targeting integrin-α4 reduces tumor inflammation and growth in spontaneous tumor models**

To determine whether small-molecule integrin antagonists could block tumor inflammation and growth, we evaluated the effect of the small-molecule inhibitor of integrin-α4, ELN476063, on myeloid cell adhesion in vitro and on tumor inflammation in vivo. ELN476063 efficiently blocked IL-1β, SDF-1α, and TCM-stimulated myeloid cell adhesion to the integrin-α4 ligand VCAM-1 (Fig. 6A) and reduced LLC primary
tumor growth (Fig. 6B). ELN476063 significantly inhibited myeloid cell infiltration of LLC tumors and tumor angiogenesis (Fig. 6C).

We also evaluated the effects of α4 antagonists on spontaneous breast tumor growth by treating PyMT+/FVB mice with the ELN476063 integrin-α4 antagonist (from postnatal weeks 6 to 9). Treated mice exhibited reduced tumor burden, reduced macrophage content, and decreased blood vessel density (Fig. 6D). In support of these findings are our findings that spontaneous PyMT/α4Y991A breast tumors exhibited reduced tumor inflammation, growth, and progression (24). Together, our data indicate that blockade of integrin-α4 inhibits the recruitment of proangiogenic myeloid cells to the tumor microenvironment, thus impairing tumor progression.

Blockade of integrin-α4 in combination with chemotherapeutic agents

Radiation and chemotherapy can induce inflammatory responses that complicate cancer therapy (30–32). Blockade of inflammation may improve responsiveness or prevent resistance to therapeutic agents. Microtubule-inhibiting vascular disrupting agents (VDA) damage the tumor endothelium, resulting in decreased tumor vascularization and reduced tumor growth over the long term. However, these drugs rapidly induce extensive tumor inflammation (Fig. 7A–C). To determine whether inhibition of myeloid cell recruitment can suppress inflammation associated with cancer therapeutics, pre-established LLC tumors were treated with VDA OXi4503 in the presence and absence of an integrin-α4 antagonist. OXi4503 inhibited
short-term tumor growth (Fig. 7A), but stimulated inflammation (Fig. 7B and C) and new blood vessel formation in tumors (Fig. 7C). However, when OX4503 was combined with integrin-α4β1 antagonists, tumor inflammation, angiogenesis, and growth were strongly inhibited, and tumor necrosis was enhanced (Fig. 7D).

To determine whether longer term combinations of chemotherapeutic agents and integrin-α4 inhibitors could synergize to reduce tumor growth and metastasis, we treated LLCs and orthotopic murine Panc02 pancreatic carcinomas with gemcitabine, anti–integrin-α4, or a combination of both for 3 weeks. Combination of gemcitabine and anti-α4β1 antibodies suppressed tumor growth, inflammation, angiogenesis, and metastasis to a much greater extent than either agent alone (Fig. 7E–I). Our findings thus indicate that suppression of myeloid cell trafficking with antagonists of integrin-α4 can prevent unwanted tumor inflammatory responses and suppress tumor growth.

Discussion

Our studies show that tumor-derived inflammatory factors recruit bone marrow-derived myeloid cells, including CD11b+Gr1loLy6G-monocytic cells and CD11b+Gr1hiLy6G+ granulocytic cells, to the tumor microenvironment by regulating the activation state of integrin-α4β1 on these cells. IL-1β and SDF-1α are commonly found in the tumor microenvironment (25, 26). In the tumors studied in this study, only tumor-associated myeloid cells expressed IL-1β, whereas only tumor cells expressed SDF-1α. These results indicate that SDF-1α (33) initially recruits the first myeloid cells to tumors and that these newly recruited myeloid cells secrete IL-1β to recruit additional waves of myeloid cells, thereby establishing a chronic cascade of inflammation in the tumor microenvironment. Some tumor cells express IL-1β, thereby promoting inflammatory cell recruitment, angiogenesis, tumor cell invasiveness, tumor growth, and tumor spread (34–38). Thus, limiting inflammatory recruitment to tumors may not only inhibit tumor angiogenesis but also directly limit tumor cell invasiveness and tumor growth. Our studies show that tumors can vary in their expression of myeloid cell chemotactants; however, targeting the downstream effector integrin-α4β1 is an effective strategy to inhibit tumor inflammation and progression.

In this study, we showed that tumor-derived factors, such as SDF-1α and IL-1β, activate integrin-α4β1 on myeloid cells, thereby increasing adhesion and recruitment to the tumor microenvironment. The chemokine SDF-1α and the proinflammatory cytokine IL-1β both increase integrin-α4β1 activity, clustering, and adhesion of primary myeloid cells to the endothelium. As blockade of receptor signaling abolished inside-out activation of integrin-α4β1, our findings indicate that myeloid cell trafficking is tightly controlled by receptor-mediated signal transduction.

Integrin-α4β1 activation is regulated by binding of the integrin-α4 cytoplasmic tail to the adaptor protein paxillin, which stabilizes integrin-α4β1–ligand binding (27, 28). In the present study, we observed that paxillin–integrin-α4 interactions are required for integrin clustering and for myeloid cell adhesion to the endothelium. Similarly, the adaptor molecule talin also interacts with integrin-α4β1 upon stimulation. Binding of talin to the integrin-β1 chain promotes conformational changes of integrin-α4β1 to its active state. Our data provide evidence that blockade of α4β1 with these adapter proteins affects the biological function of primary myeloid cells.

In conclusion, our studies indicate that integrin-α4β1 activation by inflammatory mediators plays a critical role in tumor inflammation. Tumor cells likely initiate recruitment of proangiogenic myeloid cells by expressing factors such as SDF-1α and that myeloid cells recruit further myeloid cells by expressing IL-1β. Our results indicate that blockade of integrin-α4 in combination with cytokine inhibitors or chemotherapeutic agents could provide substantial benefit in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Integrin-a4 Antagonists Inhibit Tumor Inflammation


Combined Blockade of Integrin-α4β1 Plus Cytokines SDF-1α or IL-1β Potently Inhibits Tumor Inflammation and Growth


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