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Combined blockade of integrin $\alpha 4 \beta 1$ plus cytokines SDF-1 α or IL-1 β potently inhibits tumor inflammation and growth

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Abstract

Tumor-associated macrophages promote tumor growth by stimulating angiogenesis and suppressing anti-tumor immunity. Thus, therapeutics that inhibit macrophage recruitment to tumors may provide new avenues for cancer therapy. Here we show how the chemoattractants SDF-1 α and IL-1 β collaborate with myeloid cell integrin $\alpha 4 \beta 1$ to promote tumor inflammation and growth. We found that SDF-1 α and IL-1 β are highly expressed in the microenvironments of murine lung, pancreatic and breast tumors; surprisingly, SDF-1 α was expressed only by tumor cells, while IL-1 β was produced only by tumor-derived granulocytes and macrophages. In vivo, both factors directly recruited pro-angiogenic macrophages to tissues, while antagonists of both factors suppressed tumor inflammation, angiogenesis and growth. Signals induced by IL-1 β and SDF-1 α promoted the interaction of talin and paxillin with the cytoplasmic tails of integrin $\alpha 4 \beta 1$, thereby stimulating myeloid cell adhesion to endothelium in vitro and in vivo. While inhibiting integrin $\alpha 4 \beta 1$, SDF-1 α or IL-1 β was sufficient to block tumor inflammation and growth, the combined blockade of these molecules greatly accentuated these effects. Furthermore, antagonists of integrin $\alpha 4 \beta 1$ inhibited chemotherapy-induced tumor inflammation and synergized with chemotherapeutic agents to suppress tumor inflammation and growth. These results demonstrate that targeting myeloid cell recruitment mechanisms can be an effective approach to suppress tumor progression.

Keywords

tumor inflammation; chemoattractants; integrin $\alpha 4 \beta 1$; integrin activation; tumor progression

Introduction

Solid tumors trigger intrinsic inflammatory responses that promote tumorigenesis and metastasis (1–2). CD11b+ myeloid cells, which include granulocytes, macrophages, and myeloid derived suppressors cells progressively accumulate in primary tumor sites, where they promote tumor angiogenesis, invasion and progression (3–5). Several studies have

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shown correlations between tumor-associated macrophage (TAM) abundance and unfavorable tumor outcomes (6–7).

TAMs secrete pro-angiogenic cytokines and growth factors, including VEGF-A, TNF α , IL-6, IL-8 and bFGF (3–4,8), as well as MMP-9, which can promote angiogenesis by releasing pro-angiogenic factors such as VEGF-A from the extracellular matrix (9–11). These cells also express IL-10 and TGF- β , which suppress anti-tumor immune responses (12–15). A number of tumor-derived chemoattractants, including stromal derived growth factor 1 alpha (SDF-1 α), colony-derived stimulating factor (CSF-1) interleukin-1 beta (IL-1 β), 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 $\alpha 4\beta 1$ on the surface of immune cells promotes endothelial progenitor and myeloid cell recruitment to tumors (18–19). Antagonists of integrin $\alpha 4\beta 1$ disrupt the interaction between $\alpha 4\beta 1$ and its ligand 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 identify new roles for tumor-derived inflammatory factors in the activation of myeloid cell integrin $\alpha 4\beta 1$ during myeloid cell recruitment to the tumor microenvironment. Combinatorial administration of antagonists of integrin $\alpha 4$ and tumor-derived chemoattractants or chemotherapeutic agents potentially suppressed tumor inflammation and growth in tumor models.

Materials and Methods

Animals

PyMT+ mice (22) and integrin $\alpha 4Y991A$ mice (23) were derived as described. C57BL/6 mice were obtained from Charles River and C57BL/6-Tg(ACTB-EGFP) mice were 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 1h at 37°C in 10ml of Hanks 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 Bioscience), followed by CD11b-APC (M1/70, eBioscience) and Gr1-FITC (RB6-8C5, eBioscience). To exclude dead cells, 2.5 μ g/ml propidium iodide (PI) was added before data acquisition by FACs Calibur (BD Bioscience). Myeloid cells from human buffy coats, murine 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). qPCR was performed using primers for *SDF-1 α* , *IL-1 β* , *TNF- α* , *IL-8* and *IL-6* from Qiagen (QuantiTect Primer Assay). qPCR for *VegfA* expression was performed with primers: sense: GCTGTGCAGGCTGCTCTAAC anti-sense: CGCATGATCTGCATGGTGAT. Transcript levels were normalized to *GAPDH*. SDF-1 α and IL-1 β protein levels were determined in

lysates of cultured cells, whole tumors or from tumor derived CD11b⁺/– cells using Quantikine mouse SDF-1 α and IL-1 β kits (R&D Systems).

Cell biological and biochemical assays

Adhesion and integrin activation assays were previously described (24). For immunoprecipitations, BM monocytic cells from WT or α 4Y991A mice were treated with basal or conditioned medium for 30min at 37°C. Cells were lysed in Tris-buffered saline containing 1%CHAPS, 20 mM β -glycerophosphate, 1mM Na₃VO₄, 5mM NaF, 100ng/ml microcystin-LR, and protease inhibitor cocktail. Cell lysates were immunoprecipitated with 5 μ g of rat anti- α 4 β 1 (PS/2) antibody overnight, followed by addition of 25 μ l of protein G-conjugated Dynabeads for 3h with rotation. Protein precipitates were electrophoresed and immunoblotted with anti-integrin α 4 (C-20, Santa Cruz Biotechnology), anti-paxillin (H-114, Santa Cruz Biotechnology), or anti-talin (Clone TD77, Chemicon) antibodies. Immune complexes were visualized using an enhanced chemiluminescence detection kit (Pierce).

In vivo assays

Growth Factor-reduced Matrigel (BD Bioscience) containing 400ng/400 μ l SDF-1 α , IL-1 β (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 LLC cells were cultured in antibiotic- and fungicide-free DMEM media containing 10% serum. C57Bl6 mice were subcutaneously implanted with 1 $\times$ 10⁶ LLC cells and treated on d3 and d5 with intraperitoneal injections of function-blocking anti-IL-1 β antibodies (MAB401, R&D Systems) (n=16) or isotype-matched control antibodies rat IgG1 (n=14) (100 μ g/25g body weight), with saline (n=6) or 1.25mg/kg SDF-1 α inhibitor (AMD3100, Sigma Aldrich) (n=7) daily for 9d, or with anti-IL-1 β or AMD3100 plus function blocking anti- α 4 integrin antibody (200 μ g PS2/25g body weight) on d3 and d5.

Additionally, 5 $\times$ 10⁵ LLC cells were subcutaneously implanted into C57BL/6 mice. One week later, animals were treated once per week by i.p. injection with 80mg/kg gemcitabine and every third day with 200 μ g/mouse PS2 or both together for two weeks. Alternatively, 1 $\times$ 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 80mg/kg gemcitabine, every third day with 200 μ g/mouse PS2 antibody or both together for three weeks.

In other studies, C57BL/6 mice were subcutaneously implanted with 5 $\times$ 10⁵ LLC cells and were treated every third day with subcutaneous injections of ELN476063 (3mg/kg body weight), an integrin α 4 small molecule inhibitor. Alternatively, 6 week old PyMT+ female mice (with spontaneous breast tumors), were treated by subcutaneous injection with ELN476063 (3mg/kg body weight), or saline every third day for three weeks (n=10).

Additionally, 5 $\times$ 10⁵ LLC cells were subcutaneously implanted into GFP+ BM transplanted C57BL/6 mice. Tumor size was assessed regularly with Vernier calipers by using the formula ($w^2 \times l \times 0.5$). When tumors reached 500mm³, OXi4503, a pro-drug derivative of the vascular disrupting agent combretastatin-A4 phosphate (CA4P), was administered intraperitoneally (i.p.) at a dose of 100mg/kg. Anti- α 4 integrin blocking antibody (PS2) was administered i.p. at a dose of 200 μ g/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 (60mg/kg) (Millipore) 90min before euthanasia. Tumors and organs were removed and immediately fixed in 10% buffered formalin and then placed in 70% ethanol, or frozen 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 using M1/70 (BD Bioscience), for F4/80+ using BM8 (eBioscience) and for CD31 using MEC13.3 (BD Bioscience). Slides were counterstained with Dapi (Invitrogen). Pixel density was quantified using Metamorph (Version 6.3r5, Molecular Devices).

Statistical Analysis

Statistical significance was assessed with Student's t-test or ANOVA using SigmaXL 6.02. A value of $P < 0.05$ was considered significant.

Additional detailed methods can be found in Supplementary Materials.

Results

IL-1 β and SDF-1 α recruit pro-angiogenic 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 Lewis Lung Carcinoma (LLC) tumors. CD11b+ myeloid cells extensively invaded LLC tumors and accounted up to one quarter of the tumor cell population (Supplementary Fig. 1; ref. 24). Tumor-derived myeloid cells included 81.7% CD11b+Gr1^{lo/neg}Ly6G⁻ monocytic cells and 18.3% CD11b+Gr1^{hi}Ly6G⁺ granulocytic cells (Supplementary Fig. 1). The majority of myeloid cells in tumors were CD11b+CD14+F480+Ly6C+ macrophages (Fig. 1A). All tumor-derived myeloid cells expressed the adhesion receptor CD49d, integrin $\alpha 4\beta 1$ (Supplementary Fig. 2A–B).

To determine which myeloid cell chemoattractants are expressed in tumors, we isolated tumor cells and tumor-derived CD11b+ myeloid cells from Lewis lung carcinomas (LLC), Panc02 pancreatic carcinomas and PyMT spontaneous breast tumors *in vitro* and *in vivo*. Lung, pancreatic and breast tumor cells expressed high levels of *Sdf1a* mRNA whether cultured *in vitro* or *in vivo*, while only CD11b+ myeloid cells from tumors expressed high levels of *Il1b* mRNA (Fig. 1B–C; Supplementary Fig. 3A–B). While CD11b+Gr1^{hi} granulocytes expressed slight amounts of IL-1 β and TNF α , CD11b+Gr1^{lo/neg}F4/80+ macrophages expressed high levels of IL-1 β , IL-6, TNF α and VEGF-A (Figure 1D; Supplementary Fig. 3C). Tumor cells expressed SDF-1 α but not IL-1 β protein *in vitro* and *in vivo*, while CD11b+ myeloid cells isolated from tumors expressed IL-1 β but not SDF-1 α protein (Fig. 1E–F, Supplementary Fig. 3D). Notably, the recruitment of CD11b+ myeloid cells to tumors paralleled the overall expression of IL-1 β and SDF-1 α in the tumor (Fig. 1G, Supplementary Fig. 3E), suggesting functional relationships between expression of these factors and myeloid cell recruitment.

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

derived cells and stimulate angiogenesis. As tumor cells express SDF-1 α , and myeloid cells express IL-1 β , these studies suggest that both tumor and myeloid cells promote tumor inflammation.

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

To determine whether SDF-1 α and IL-1 β recruit myeloid cells during LLC tumor progression, mice bearing LLC tumors were treated for 10 days with antagonists of SDF-1 α or IL-1 β ; tumor inflammation and growth was analyzed. Anti-IL1 β significantly suppressed tumor growth (Fig. 2A) as well as myeloid cell recruitment and angiogenesis (Fig. 2B). These results suggest that inhibition of myeloid cell recruitment by IL-1 β antagonists decreased tumor blood vessel formation. Similarly, blockade of SDF-1 α 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-1 α , IL-1 β , IL-6, TNF α and VEGF-A in tumors (Fig. 2E), of IL-1 β , IL-6 and VEGF-A in tumor-derived macrophages (Fig. 2F) and of IL-1 β in tumor-derived CD11b+Gr1hi granulocytes (Supplementary Fig. 4). Taken together, these findings indicate that IL-1 β and SDF-1 α 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 suppressed tumor growth.

Inflammatory factors induce integrin $\alpha 4 \beta 1$ 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. Fluorescently labeled murine myeloid cells exhibited increased adhesion to vascular endothelium after a brief (30 minute) stimulation by purified SDF-1 α , IL-1 β , or LLC tumor cell-conditioned medium (TCM, which contains largely SDF-1 α , TNF- α and VEGF-A), (Fig. 3A). This adhesion event was mediated by integrin $\alpha 4$, as antagonists of integrin $\alpha 4$ and siRNA-mediated knockdown of integrin $\alpha 4$ expression blocked myeloid cell adhesion to endothelium, while antagonists of other myeloid cell integrins, including αv , $\alpha 5$, and αM , had no effects (Fig. 3A, Supplementary Fig. 5A–B).

We therefore investigated the role of receptor-mediated signaling in integrin $\alpha 4$ -mediated cell adhesion. Myeloid cells from MyD88 $^{-/-}$ mice, which are defective in TLR/IL-1 β receptor signaling, failed to adhere to the endothelium in the presence of IL-1 β but adhered normally in the presence of SDF-1 α (Fig. 3B), indicating that IL-1R-mediated signal transduction is required for IL-1 β -induced adhesion. Similarly, pertussis toxin and AMD3100 inhibited SDF-1 α -induced, but not IL-1 β induced adhesion, indicating that CXCR4-mediated signal transduction is necessary for SDF-1 α and TCM-induced cell adhesion to the endothelium (Fig. 3B), indicating that SDF-1 α is a key active component in TCM (Fig. 3B). Together, these data indicate that diverse tumor-derived chemoattractants promote integrin $\alpha 4 \beta 1$ -mediated myeloid cell adhesion using non-overlapping 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 $\beta 1$ 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-1 α , IL-1 β , or Mn $^{2+}$ (a positive control) and performed flow cytometry to detect binding of HUTS21 and P4C10, an antibody that recognizes $\beta 1$ integrins regardless of conformation. SDF-1 α and IL-1 β

increased HUTS21 binding but had no effect on P4C10 binding, indicating that SDF-1 α and IL-1 β can activate myeloid cell β 1 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-1 α and IL-1 β on integrin α 4 β 1 clustering. Both SDF-1 α and IL-1 β rapidly (within 5 minutes) stimulated integrin α 4 clustering in human and murine myeloid cells (Fig. 3D, Supplementary Fig. 6). Receptor-mediated signaling is required for IL-1 β and SDF-1 α -induced integrin α 4 clustering, as IL-1 β -stimulated MyD88 $^{-/-}$ cells and SDF-1 α -stimulated wildtype cells treated with PTX or AMD3100 exhibited no integrin α 4 clustering (Fig. 3E). These studies support the hypothesis that tumor-derived factors stimulate integrin α 4 β 1-mediated myeloid cell adhesion by increasing both the affinity and the avidity of integrin α 4 β 1.

Inflammatory factors regulate integrin α 4 activation by increasing integrin α 4 interactions with paxillin and talin

Activation of integrin α 4 β 1 depends on association of talin with an NPXY domain in β 1 cytoplasmic tails, and its adhesion under flow depends on association of paxillin with tyrosine991 within the integrin α 4 cytoplasmic tail (20, 23, 27–29). We therefore evaluated integrin α 4 β 1 co-clustering with paxillin and talin in myeloid cells from WT mice and from mice expressing an α 4 Y991A mutation (α 4Y991A), which disrupts the α 4 integrin-paxillin binding site and partially disrupts α 4 β 1 interactions with talin (23). IL-1 β and SDF-1 α rapidly (within 5 minutes) stimulated integrin α 4 clustering in WT but not α 4Y991A cells (Fig. 4A). Inflammatory mediators induced co-clustering of integrin α 4 β 1 with paxillin and talin in WT but not α 4Y991A myeloid cells (Fig. 4B–C), even though integrin α 4 surface expression levels are identical in WT and α 4Y991A cells (Supplementary Fig. 7). In addition, paxillin and talin co-immunoprecipitated with integrin α 4 β 1 in myeloid cells from WT but not α 4Y991A mice upon stimulation with TCM (Fig. 4D). Importantly, α 4Y991A myeloid cells failed to adhere in response to SDF-1 α or IL-1 β (Fig. 4E) and exhibited defective cell migration on the α 4 integrin ligand VCAM-1 but not on vitronectin, an α v integrin ligand (Fig. 4F).

Combination of integrin α 4 antagonists with inflammatory inhibitors effectively block tumor inflammation and growth

As SDF-1 α and IL-1 β can stimulate integrin α 4 β 1-mediated adhesion to endothelium in vitro, we asked whether these factors stimulate integrin α 4 β 1 mediated trafficking of myeloid cells into tumors in vivo. Fluorescently labeled myeloid cells injected intravenously into mice rapidly accumulated in established tumors (Figure 5A). In contrast, myeloid cells that were co-injected with antagonists of SDF-1 α , IL-1 β or integrin α 4 β 1 failed to accumulate in tumors (Fig. 5A). Notably, combined blockade of SDF-1 α and IL-1 β reduced the recruitment of myeloid cells to tumors to the same extent as integrin α 4 β 1 blockade, supporting the idea that activation of integrin α 4 β 1 by both SDF-1 α and IL-1 β plays a critical role in tumor inflammation. Importantly, trafficking of myeloid cells to other organs was not affected (Fig. 5A). Our results demonstrate that tumor-derived SDF-1 α and macrophage-derived IL-1 β activate integrin α 4 and together promote myeloid cell recruitment to the tumor microenvironment.

To determine whether antagonists of integrin α 4, IL-1 β and SDF-1 α in combination could have beneficial anti-tumor effects, we evaluated the effects of treating LLC tumors with the integrin α 4 function-blocking antibody PS2, the SDF-1 α inhibitor AMD3100 and anti-IL-1 β antibodies alone or in combination. Treatment with AMD3100, anti-IL-1 β , or anti- α 4 alone inhibited tumor growth, reducing tumor burdens to approximately 50% that of untreated

tumors (Fig. 5B). Combined administration of anti-IL-1 β and AMD3100 further suppressed tumor growth, while combined blockade of integrin α 4 and SDF-1 α or IL1 β 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 α 4 and inflammatory factors such as IL-1 β or SDF-1 α may provide therapeutic benefit in the treatment of solid tumors.

Targeting integrin α 4 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 α 4, ELN476063, on myeloid cell adhesion *in vitro* and on tumor inflammation *in vivo*. ELN476063 efficiently blocked IL-1 β , SDF-1 α and TCM stimulated myeloid cell adhesion to the integrin α 4 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 α 4 antagonists on spontaneous breast tumor growth by treating PyMT+ FVB mice with the ELN476063 integrin α 4 antagonist (from postnatal 6w–9w). 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/ α 4Y991A breast tumors exhibited reduced tumor inflammation, growth and progression (24). Altogether, our data indicate that blockade of integrin α 4 inhibits the recruitment of pro-angiogenic myeloid cells to the tumor microenvironment, therefore impairing tumor progression.

Blockade of integrin α 4 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 (VDAs) 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 α 4 antagonist. OXi4503 inhibited short-term tumor growth (Fig. 7A), but stimulated inflammation (Fig. 7B–C) and new blood vessel formation in tumors (Fig. 7C). However, when OXi4503 was combined with integrin α 4 β 1 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 α 4 inhibitors could synergize to reduce tumor growth and metastasis, we treated Lewis lung carcinomas and orthotopic murine Panc02 pancreatic carcinomas with gemcitabine, anti-integrin α 4 or both together for three weeks. Combination of gemcitabine and anti- α 4 β 1 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 α 4 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+Gr1Ly6G[−] monocytic cells, CD11b+Gr1Ly6G⁺ granulocytic cells, to the tumor microenvironment by regulating the activation state of integrin $\alpha 4\beta 1$ on these cells. IL-1 β and SDF-1 α are commonly found in the tumor microenvironment (25–26). In the tumors studied here, only tumor-associated myeloid cells expressed IL-1 β , while only tumor cells expressed SDF-1 α . These results suggest that SDF-1 α (33) initially recruits the first myeloid cells to tumors and that these newly recruited myeloid cells secrete IL-1 β to recruit additional waves of myeloid cells, thereby establishing a chronic cascade of inflammation in the tumor microenvironment. Some tumor cells express IL-1 β , 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 chemoattractants, yet targeting the downstream effector integrin $\alpha 4\beta 1$ is an effective strategy to inhibit tumor inflammation and progression.

Here we show that tumor-derived factors, such as SDF-1 α and IL-1 β , activate integrin $\alpha 4\beta 1$ on myeloid cells, thereby increasing adhesion and recruitment to the tumor microenvironment. The chemokine SDF-1 α and the pro-inflammatory cytokine IL-1 β both increase integrin $\alpha 4\beta 1$ activity, clustering and adhesion of primary myeloid cells to the endothelium. As blockade of receptor signaling abolished inside-out activation of integrin $\alpha 4\beta 1$, our findings indicate that myeloid cell trafficking is tightly controlled by receptor mediated signal transduction.

Integrin $\alpha 4\beta 1$ activation is regulated by binding of the $\alpha 4$ integrin cytoplasmic tail to the adaptor protein paxillin, which stabilizes integrin $\alpha 4\beta 1$ -ligand binding (27–28). Here, we observed that paxillin-integrin $\alpha 4$ interactions are required for integrin clustering and for myeloid cell adhesion to the endothelium. Similarly, the adaptor molecule talin also interacts with integrin $\alpha 4\beta 1$ upon stimulation. Binding of talin to the integrin $\beta 1$ chain promotes conformational changes of integrin $\alpha 4\beta 1$ to its active state. Our data provide evidence that blockade of $\alpha 4\beta 1$ with these adapter proteins affects the biological function of primary myeloid cells.

In conclusion, our studies indicate that integrin $\alpha 4\beta 1$ activation by inflammatory mediators plays a critical role in tumor inflammation. Tumor cells likely initiate recruitment of pro-angiogenic myeloid cells by expressing factors such as SDF-1 α and that myeloid cells recruit further myeloid cells by expressing IL-1 β . Our results indicate that blockade of integrin $\alpha 4$ in combination with cytokine inhibitors or chemotherapeutic agents could provide substantial benefit in cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002; 420:860–867. [PubMed: 12490959]
2. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010; 140:883–899. [PubMed: 20303878]
3. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. 2004; 6:409–421. [PubMed: 15488763]
4. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*. 2006; 66:11238–11246. [PubMed: 17114237]
5. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res*. 2005; 65:5278–5283. [PubMed: 15958574]
6. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast Cancer Res Treat*. 2010 Sep 15. [Epub ahead of print].
7. Oosterling SJ, van der Bij GJ, Meijer GA, Tuk CW, van Garderen E, van Rooijen N, et al. Macrophages direct tumour histology and clinical outcome in a colon cancer model. *J Pathol*. 2005; 207:147–155. [PubMed: 16104052]
8. Du R, Petritsch C, Liu P, Ganss R, Passequé E, Song H, et al. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell*. 2008; 13:206–220. [PubMed: 18328425]
9. Ahn GO, Brown JM. Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell*. 2008; 13:193–205. [PubMed: 18328424]
10. Hildenbrand R, Dilger I, Horlin A, Stutte HJ. Urokinase and macrophages in tumour angiogenesis. *Br J Cancer*. 1995; 72:818–823. [PubMed: 7547226]
11. Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, et al. Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. *J Natl Cancer Inst*. 2002; 94:1134–1142. [PubMed: 12165638]
12. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol*. 2009; 182:4499–4506. [PubMed: 19342621]
13. Nagaraj S, Gabrilovich DI. Tumor escape mechanism governed by myeloid-derived suppressor cells. *Cancer Res*. 2008; 68:2561–2563. [PubMed: 18413722]
14. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med*. 2003; 198:1741–1752. [PubMed: 14657224]
15. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol*. 2008; 180:2011–2017. [PubMed: 18250403]
16. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res*. 2000; 6:3282–3289. [PubMed: 10955814]
17. Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science*. 2006; 313:1785–1787. [PubMed: 16990548]
18. Jin H, Aiye A, Su J, Borgstrom P, Stupack D, Friedlander M, et al. A homing mechanism for bone marrow-derived progenitor cell recruitment to the neovasculature. *J Clin Invest*. 2006; 116:652–662. [PubMed: 16498499]
19. Jin H, Su J, Garmy-Susini B, Kleeman J, Varner J. Integrin alpha4beta1 promotes monocyte trafficking and angiogenesis in tumors. *Cancer Res*. 2006; 66:2146–2152. [PubMed: 16489015]

20. Arnaout MA, Mahalingam B, Xiong JP. Integrin structure, allostery, and bidirectional signaling. *Annu. Rev. Cell Dev. Biol.* 2005; 21:381–410. [PubMed: 16212500]
21. Luque A, Gómez M, Puzon W, Takada Y, Sánchez-Madrid F, Cabañas C. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355–425) of the common beta 1 chain. *J Biol Chem.* 1996; 271:11067–11075. [PubMed: 8626649]
22. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol.* 2003; 163:2113–2126. [PubMed: 14578209]
23. Feral CC, Rose DM, Han J, Fox N, Silverman GJ, Kaushansky K, et al. Blocking the alpha 4 integrin-paxillin interaction selectively impairs mononuclear leukocyte recruitment to an inflammatory site. *J Clin Invest.* 2006; 116:715–723. [PubMed: 16470243]
24. Schmid MC, Avraamides CJ, Dippold HC, Franco I, Foubert P, Ellies LG, et al. Receptor Tyrosine Kinases and TLR/IL1Rs Unexpectedly Activate Myeloid Cell PI3K γ , A Single Convergent Point Promoting Tumor Inflammation and Progression. *Cancer Cell.* 2011; 19:715–727. [PubMed: 21665146]
25. Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R. Chemokines direct endothelial progenitors into tumor neovessels. *Proc Natl Acad Sci U S A.* 2005; 102:18111–18116. [PubMed: 16326806]
26. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res.* 2007; 67:10019–10026. [PubMed: 17942936]
27. Rose DM, Han J, Ginsberg MH. Alpha4 integrins and the immune response. *Immunol Rev.* 2002; 186:118–124. [PubMed: 12234367]
28. Grabovsky V, Feigelson S, Chen C, Bleijs DA, Peled A, Cinamon G, et al. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med.* 2000; 192:495–506. [PubMed: 10952719]
29. Alon R, Feigelson SW, Manevich E, Rose DM, Schmitz J, Overby DR, et al. Alpha4beta1-dependent adhesion strengthening under mechanical strain is regulated by paxillin association with the alpha4-cytoplasmic domain. *J Cell Biol.* 2005; 171:1073–1084. [PubMed: 16365170]
30. Ferrara N. Role of myeloid cells in vascular endothelial growth factor-independent tumor angiogenesis. *Curr Opin Hematol.* 2010; 17:219–214. [PubMed: 20308892]
31. Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol.* 2007; 25:911–920. [PubMed: 17664940]
32. Shaked Y, Henke E, Roodhart JM, Mancuso P, Langenberg MH, Colleoni M, et al. Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. *Cancer Cell.* 2008; 14:263–273. [PubMed: 18772115]
33. Furusato B, Mohamed A, Uhlen M, Rhim JS. CXCR4 and cancer. *Pathol Int.* 2010; 60:497–505. [PubMed: 20594270]
34. Apte RN, Dotan S, Elkabets M, White MR, Reich E, Carmi Y, Song X, et al. The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. *Cancer Metastasis Rev.* 2006; 25:387–408. [PubMed: 17043764]
35. Naldini A, Filippi I, Miglietta D, Moschetta M, Giavazzi R, Carraro F. Interleukin-1 β regulates the migratory potential of MDAMB231 breast cancer cells through the hypoxia-inducible factor-1 α . *Eur J Cancer.* 2010; 46:3400–3408. [PubMed: 20801015]
36. Goldberg JE, Schwertfeger KL. Proinflammatory cytokines in breast cancer: mechanisms of action and potential targets for therapeutics. *Curr Drug Targets.* 2010; 11:1133–1146. [PubMed: 20545607]
37. Voronov E, Reich E, Dotan S, Dransh P, Cohen I, Huszar M, et al. Effects of IL-1 molecules on growth patterns of 3-MCA-induced cell lines: an interplay between immunogenicity and invasive potential. *J Immunotoxicol.* 2010; 7:27–38. [PubMed: 20001788]

38. Apte RN, Voronov E. Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? *Immunol Rev.* 2008; 222:222–241. [PubMed: 18364005]

Figure 1. SDF-1α and IL-1β in the tumor microenvironment recruit pro-angiogenic myeloid cells

(A) Percent of CD11b+Gr1^{lo/neg} and CD11b+Gr1^{hi} myeloid cells from LLC tumors that express Ly6C, Ly6G, F4/80, or CD14.

(B) Chemoattractant gene expression in cultured LLC cells and LLC tumors (n=3).

(C) Chemoattractant gene expression in CD11b+ myeloid cells and tumor cells freshly isolated from d14 subcutaneous LLC tumors (n=3).

(D) Chemoattractant gene expression in CD11b+Gr1^{hi} granulocytic cells and CD11bGr1^{lo/neg}F4/80+ 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 *Il1b* and *Sdf-1a* mRNA expression in tumors over time (n=3),

*P<0.01.

(H) Mice were reconstituted with GFP+ bone marrow and stimulated with growth factor depleted Matrigel saturated with saline, IL-1β, or SDF-1α for three weeks (n=6). *Images*: 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 vs saline.

Scale bars indicate 40 μm . Error bars represent SEM. Statistical significance was determined by two tailed Student's t-test.

Figure 2. Blockade of IL-1 β and SDF-1 α inhibits tumor inflammation and growth

(A–D) Mice were treated for 10d after subcutaneous implantation of 1×10^6 LLC cells ($n=8-14$) with (A–B) isotype-matched control (cIgG) or anti-IL1 β antibodies or (C–D) AMD3100 or saline. (A,C) Tumor volumes and weights per treatment group. (B,D) *Images*: Representative micrographs of F4/80+ macrophages (red) and CD31+ blood vessels (green) in tumor sections. *Graph*: Quantification of F4/80+ or CD31+ pixels/field ($n=10$). (E–F) Chemoattractant gene expression in LLC tumors or tumor-derived CD11b+ Gr1^{lo}F4/80+ macrophages isolated from mice from A–D ($n=3$). Scale bars indicate 40 μ m. Error bars represent SEM. **P* < 0.01 vs control group.

Figure 3. Tumor-derived factors promote integrin $\alpha 4 \beta 1$ 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- $\alpha 4$, anti- αM , anti- $\alpha 5$ and anti- αv integrin antibodies ($n=3$) or after transfection with $\alpha 4$ integrin and control siRNAs, $*P<0.01$ vs IgG. (B) Adhesion to endothelium of chemoattractant-treated WT and MyD88 $^{-/-}$ myeloid cells and WT myeloid cells in the absence or presence of pertussis toxin (PTX) or AMD3100 ($n=3$). $*P<0.01$ vs WT. (C) Histogram of human myeloid cells stained to detect active (HUTS21, upper panel) and total (P4C10, lower panel) human integrin $\beta 1$ in the absence (unstimulated, gray filled) or presence (stimulated, black line) of SDF-1 α , IL-1 β , or Mn2 $^{+}$, a positive control activator. IgG negative control appears as thin line ($n=5$). (D) *Images*: clustering of integrin $\alpha 4 \beta 1$ (green, arrowheads) in human myeloid cells incubated with SDF-1 α , IL-1 β , or human serum albumin (HSA)-coated microspheres and counterstained for DNA (blue). *Graph*: Percentage of integrin $\alpha 4 \beta 1$ clustering per myeloid cell ($n>15$). $*P<0.001$ vs HSA.

(E) Percentage of integrin $\alpha 4 \beta 1$ clustering of WT, MyD88 $^{-/-}$, pertussis toxin (PTX) and AMD3100-treated WT myeloid cells after incubation with SDF-1 α or IL-1 β -coated microspheres ($n>15$). $*P<0.001$ vs WT.

Scale bars indicate 5 μm . Error bars represent SEM.

Figure 4. Integrin $\alpha 4\beta 1$ colocalization with paxillin and talin promotes myeloid cell adhesion and migration

(A) Percentage of integrin $\alpha 4\beta 1$ clustering in WT (black bars) and $\alpha 4Y991A$ (white bars) myeloid cells after incubation with BSA, SDF-1 α , IL-1 β , or TCM-coated microspheres ($n > 15$). * $P < 0.01$.

(B–C) *Images*: clustering of (B) paxillin (red, arrowheads) or (C) talin (red, arrowheads) with integrin $\alpha 4$ (green, arrowheads) in CD11b $^{+}$ myeloid cells from WT and $\alpha 4Y991A$ mice incubated with SDF-1 α -coated microspheres and counterstained for DNA (blue). *Graph*: Percentage of (B) integrin $\alpha 4$ -paxillin and (C) integrin $\alpha 4$ -talin co-localization in myeloid

cells from WT (black bars) and $\alpha 4 Y 991 A$ mice (white bars) incubated with TCM, IL-1 β , and SDF-1 α -coated microspheres (n>100). * $P < 0.01$. Scale bars indicate 5 μm .

(D) Integrin $\alpha 4$ immunoprecipitates of WT and $\alpha 4 Y 991 A$ myeloid cells incubated in the presence (+) or absence (–) of LLC tumor conditioned medium (TCM) and immunoblotted for integrin $\alpha 4$, talin, paxillin and IgG. Histograms: normalized ratios of talin and paxillin to integrin $\alpha 4$.

(E) Adhesion of WT (black bars) and $\alpha 4 Y 991 A$ (white bars) myeloid cells to endothelial cell monolayers after treatment with basal medium, SDF-1 α , IL-1 β and TCM (n=3).

* $P < 0.01$.

(F) Migration of WT (black bars) and $\alpha 4 Y 991 A$ (white bars) myeloid cells on VCAM-1 or vitronectin-coated transwells (n=4). * $P < 0.01$.

Error bars represent SEM.

Figure 5. Synergistic suppression of tumor growth by integrin $\alpha 4$, IL-1 β and SDF-1 α inhibitors

(A) Percent of fluorescent myeloid cells trafficking to LLC tumors or spleen in the presence or absence of AMD3100, anti-IL-1 β or anti- $\alpha 4\beta 1$, or combinations of these inhibitors.

(B) LLC tumor weights after treatment for 14d with AMD3100, anti-IL-1 β , anti- $\alpha 4\beta 1$, and combinations of these inhibitors (n=12–14).

(C) Graph and corresponding images of CD11b+ myeloid cell density in LLC tumors from B.

Error bars indicate SEM.

Figure 6. Integrin α 4 small molecule inhibitor blocks tumor inflammation and growth

(A) Percent myeloid cell adhesion to VCAM-1 induced by SDF-1 α , IL-1 β or TCM in the presence of ELN 476063, a small molecule inhibitor of integrin α 4.

(B) Volumes and weights of tumors from mice with subcutaneous LLC tumors that were treated for 21d with ELN476063 or saline control (n=10).

(C) Images of cryosections from tumors in (B) immunostained to detect CD11b+ myeloid cells or CD31+ blood vessels and counterstained with Dapi. Graphs: quantification of CD11b+ pixels/field and CD31+ pixels/field. Scale bars indicate 40 μ m.

(D) Spontaneous PyMT+ breast tumors were treated with ELN476063 for 3 weeks starting at the age of 6 weeks. (Left) Tumor weights, (Middle) F4/80+ pixels/field, and (Right) CD31+ pixels/field.

Error bars represent SEM. $*P=0.03$, $**P<0.01$.

Figure 7. Integrin $\alpha 4 \beta 1$ antagonists synergize with chemotherapeutic agents

(A–D) LLC tumors were treated for 3 days with saline, OXi-4503, anti- $\alpha 4$ (PS2) or PS2 + OXi-4503. (A) Tumor volumes. Dashed line indicates tumor volumes prior to treatment. ** $P < 0.001$ vs control group. (B) CD11b+ cells/ 10^4 tumor cells, ** $P < 0.001$ vs control group. (C) CD31+ pixels/field in tumor cryosections, * $P < 0.01$, ** $P < 0.001$ vs control group. Images: Tumor cryosections immunostained to detect CD31 (red) and CD11b (green). Scale bar, 40 μ m. (D) Pimonidazole hydrochloride staining of necrosis in LLC tumors ($n > 20$ fields/group), * $P < 0.05$, ** $P < 0.01$ vs. control.

(E–F) Mice bearing LLC tumors were treated from d7–d21 with saline, function-blocking $\alpha 4$ antibodies, gemcitabine, or gemcitabine plus anti- $\alpha 4$ antibodies. (E) Tumor volumes and weights. (F) Percent tumor-associated CD11b+Gr1+ cells.

(G–I) Mice bearing two week old orthotopic Panc02 pancreatic carcinomas (n=8–10) were treated from d14–d45 with saline, anti- $\alpha 4$ antibodies, gemcitabine, or gemcitabine plus anti- $\alpha 4$ antibodies. (G) Tumor weights. (H) CD11b+Gr1+ myeloid cells in tumors (percent of total cell number). (I) Incidence of metastases in diaphragm and liver.

Error bars indicate SEM.