$\alpha 5 \beta 1$ Integrin Blockade Inhibits Lymphangiogenesis in Airway Inflammation

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The integrin $\alpha 5 \beta 1$ has been previously implicated in tumor angiogenesis, but its role in the remodeling of both blood vessels and lymphatics during inflammation is at an early stage of understanding. We examined this issue using a selective, small-molecule inhibitor of $\alpha 5 \beta 1$ integrin, 2-arylamino-3-[4-[(pyridin-2-ylaminomethyl)heterocyclyl]phenyl]propionic acid (JSM8757), in a model of sustained airway inflammation in mice with *Mycoplasma pulmonis* infection, which is known to be accompanied by robust blood vessel remodeling and lymphangiogenesis. The inhibitor significantly decreased the proliferation of lymphatic endothelial cells in culture and the number of lymphatic sprouts and new lymphatics in airways of mice infected for 2 weeks but did not reduce remodeling of blood vessels in the same airways. In inflamed airways, $\alpha 5$ integrin immunoreactivity was present on lymphatic sprouts, but not on collecting lymphatics or blood vessels, and was not found on any lymphatics of normal airways. Macrophages, potential targets of the inhibitor, did not have $\alpha 5$ integrin immunoreactivity in inflamed airways. In addition, macrophage recruitment, assessed in infected airways by quantitative reverse transcription-polymerase chain reaction measurements of expression of the marker protein ionized calcium-binding adapter molecule 1 (Iba1), was not reduced by JSM8757. We conclude that inhibition of the $\alpha 5 \beta 1$ integrin reduces lymphangiogenesis in inflamed airways after *M. pulmonis* infection because expression of the integrin is selectively increased on lymphatic sprouts and plays an essential role in lymphatic growth.

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Integrins, a multifunctional family of heterodimeric transmembrane receptors,^{1,2} play an important role in embryogenesis, hemostasis, immune responses, and tumor progression^{3–5} by binding to the extracellular matrix and other cellular adhesion receptors.^{3,6–8} Integrin engagement leads to activation of signaling processes that are essential for regulating cell survival, proliferation, and migration. Some integrins act in concert with soluble growth factors.^{9,10}

Multiple integrins, including $\alpha v \beta 3$, $\alpha v \beta 5$, and $\alpha 5 \beta 1$, have been implicated in angiogenesis, and $\alpha 5 \beta 1$, $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, $\alpha 4 \beta 1$, and $\alpha 9 \beta 1$ are thought to participate in lymphangiogenesis.^{1,11–15} The $\alpha 5 \beta 1$ integrin, also known as very late antigen-5, is the only integrin heterodimer that contains the $\alpha 5$ integrin subunit.^{3,16,17} $\alpha 5 \beta 1$ integrin functions as a receptor for fibronectin and certain other extracellular matrix proteins.¹⁶

$\alpha 5 \beta 1$ integrin has little or no expression in most quiescent endothelial cells but is up-regulated in blood vessels of tumors.^{1,5,18} Exceptions are normal hepatic sinusoids and high endothelial venules of lymph nodes, which have strong $\alpha 5$ integrin immunoreactivity.¹⁹

The $\alpha 5 \beta 1$ integrin has an essential role in blood vessel development. Loss of the gene encoding $\alpha 5$ integrin subunit leads to embryonic lethality in mice.^{7,20} Inhibition of $\alpha 5 \beta 1$ integrin reduces growth factor-induced angiogenesis, tumor angiogenesis in chick chorioallantoic

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membrane assays,⁸ tumor growth rate,²¹ and choroidal neovascularization in animal models.^{22,23}

Lymphatic vessels form a system of tubes with unidirectional flow to return extravasated protein-rich fluids in tissues back into the bloodstream.^{24,25} Lymph fluid and cells enter initial lymphatics, which are blind-ended vessels with valve-like openings between endothelial cells joined by unique button-like intercellular junctions.²⁵ Collecting lymphatics, which have tight, zipper-like endothelial cell junctions and have smooth-muscle cells to propel lymph and valves to prevent backflow, deliver lymph fluid via lymph nodes into the venous circulation.^{24,26} In addition to their role in maintenance of tissue homeostasis, lymphatics are important in immune surveillance and inflammation^{27,28} and serve as routes for metastatic spread of cancer cells.^{26,29,30}

Growth of new lymphatics from existing lymphatic vessels (lymphangiogenesis) is an integral part of many pathological conditions. The action of vascular endothelial growth factor (VEGF)-C and VEGF-D on VEGF receptor-3 (VEGFR-3) plays a central role in lymphangiogenesis.^{31–33} Lymphatic vessel growth under many conditions can be blocked by inhibition of VEGFR-3 signaling.^{24,34}

Several lines of evidence are consistent with a role of $\alpha 5 \beta 1$ integrin in lymphangiogenesis mediated through VEGFR-3 signaling. Human lymphatic endothelial cells in culture express $\alpha 5$ integrin,^{15,35} and $\alpha 5$ integrin immunoreactivity has been found on some lymphatics in mouse eyes.¹¹ Integrin $\alpha 5 \beta 1$ is important for activation of VEGFR-3 signaling *in vitro*, and integrin subunits $\alpha 5$ or $\beta 1$ are associated with activated VEGFR-3.³⁵ Selective inhibition of $\alpha 5 \beta 1$ integrin reduces lymphangiogenesis in a mouse model of suture-induced corneal inflammation.¹¹ Furthermore, endostatin, which can inhibit endothelial cell migration by binding to $\alpha 5 \beta 1$ integrin,³⁶ reduces lymphangiogenesis in skin tumors.³⁷

The mouse tracheal mucosa has a distinctive segmented vascular architecture, in which repetitive arrangements of blood vessels and lymphatics are aligned with the framework of cartilage rings.³⁸ Blood capillaries span the rings in a ladder-like pattern, and arterioles, venules, and lymphatics are restricted to the regions between the rings.³¹ This stereotype architecture makes it straightforward to detect and quantify the growth and remodeling of blood vessels and lymphatics in inflammatory conditions.³¹

Respiratory tract infection caused by *Mycoplasma pulmonis* results in conspicuous growth and remodeling of blood vessels and lymphatics in the airways.^{31,39–41} We used the vascular changes accompanying *M. pulmonis* infection to determine whether $\alpha 5 \beta 1$ integrin plays an essential role in blood vessel remodeling and lymphangiogenesis in airway inflammation. The role of $\alpha 5 \beta 1$ integrin was tested by using 2-arylamino-3-[4-[(pyridin-2-ylaminomethyl)heterocyclyl]phenyl]propionic acid (JSM8757), a novel selective small molecule inhibitor of $\alpha 5 \beta 1$ integrin function. JSM8757 is an orally available analogue of JSM6427, which was previously shown to inhibit choroidal neovascularization and inflammatory lymphangiogenesis in the cornea.^{11,22,42}

Our approach was first to determine the distribution of $\alpha 5 \beta 1$ integrin in the trachea by using $\alpha 5$ integrin subunit immunoreactivity as a readout. Next, we examined the efficacy of JSM8757 in inhibiting proliferation of cultured lymphatic endothelial cells and then on vascular remodeling and lymphangiogenesis after *M. pulmonis* infection. We found that the $\alpha 5 \beta 1$ integrin blockade reduced lymphatic sprouting and growth in airway inflammation but did not reduce blood vessel remodeling or macrophage recruitment.

Materials and Methods

Mice

Specific pathogen-free 8-week-old female C57BL/6 mice (Charles River, Hollister, CA) were housed under barrier conditions. Mice were anesthetized by intramuscular injection of ketamine (83 mg/kg) and xylazine (13 mg/kg). All experimental procedures were approved by the Institutional Animal Care and Use Committees of the University of California at San Francisco. All reagents were purchased from Sigma unless indicated otherwise.

Integrin Inhibitor JSM8757

JSM8757, which was synthesized at Jerini AG (Berlin, Germany) with a purity of >99%, is a small-molecule antagonist of integrin $\alpha 5 \beta 1$. JSM8757 mimics the RGD (Arg-Gly-Asp) recognition motif in fibronectin, where it interacts with $\alpha 5$ and $\beta 1$ integrin subunits with subnanomolar IC₅₀ values. The agent inhibits $\alpha 5 \beta 1$ /ligand binding with an IC₅₀ value of 0.9 nmol/L assessed by enzyme-linked immunosorbent assay and an IC₅₀ value of 30 nmol/L in blocking the adhesion of $\alpha 5 \beta 1$ -positive HEK293 cells to fibronectin. JSM8757 has much higher affinity for $\alpha 5 \beta 1$ integrin than for other RGD-binding integrins, with IC₅₀ values >670-fold greater for $\alpha v \beta 3$ integrin, >13,300-fold greater for $\alpha v \beta 5$ integrin, and >34,000-fold greater for $\alpha 11 \beta 3$ integrin. The enzyme-linked immunosorbent assay and IC₅₀ determination was performed as described previously.⁴²

Mouse Model of Inflammation

Mice at 8 weeks of age were anesthetized and inoculated intranasally on day 0 with 50 μ l of broth containing 10⁶ colony-forming units of *M. pulmonis* organisms (strain CT7) as described previously.³¹ *M. pulmonis* organisms activate an immune response with a time course similar to other airway infections.⁴³ Mice were concurrently injected intraperitoneally with vehicle or JSM8757 (100 mg/kg) twice a day for 14 days. Previous pharmacokinetic studies show that this dose of JSM8757 results in plasma concentrations (C_{max}) of approximately 40 μ mol/L. At 14 days after *M. pulmonis* infection, mice were anesthetized again and tissues were harvested for further studies.

Immunohistochemistry

Mice were perfused for 2 minutes with fixative (1% paraformaldehyde in phosphate-buffered saline; PBS, pH 7.4) from a cannula inserted through the left ventricle into the aorta.³¹ Tracheas were removed and immersed in fixative for 1 hour at 4°C. Tissues were washed and stained immunohistochemically by incubating whole mounts or 60- μ m cryostat sections with one or more primary antibodies diluted in PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin, 5% normal goat serum, and 0.1% sodium azide, as described previously.³¹ The following antibodies were used at the indicated concentrations: lymphatics: lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), 1:500 (rabbit polyclonal 07-538; Abcam, Del Mar, CA); endothelial cells: CD31, 1:500 (hamster anti-mouse PECAM-1, clone 2H8; Thermo, Waltham, MA); monocyte/macrophages: CD11b, 1:500 (rat anti-mouse CD11b, clone M1/70; eBioscience, San Diego, CA), ionized calcium-binding adapter molecule 1 (Iba1), 1:500 (rabbit polyclonal 019-19741; Wako Pure Chemical Industries, Osaka, Japan); α 5 integrin/CD49e: 1:500 (rat anti-mouse CD49e, clone 5H10-27; BD Biosciences Pharmingen, San Diego, CA). Because α 5 integrin subunits (CD49e) pair solely with β 1 subunits, anti- α 5 integrin antibody was regarded as binding exclusively to α 5 β 1 integrin.^{6,16,17} Secondary antibodies were labeled with fluorescein isothiocyanate, Cyanine3 (Cy3), or Cy5, 1:500 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Specimens were viewed with an Axiophot fluorescence microscope (Carl Zeiss) with a 3-CCD low light RGB video camera (CoolCam; SciMeasure Analytical Systems, Atlanta, GA) or a LSM510 confocal microscope (Carl Zeiss) using AIM confocal software (version 3.2.2).

Morphometric Measurements

Measurements were performed using digitizing software interfaced to the Axiophot microscope and CoolCam camera. The number of lymphatic sprouts was measured in projected real-time fluorescent images of tracheal whole mounts. Lymphatic sprouts were counted and defined as tapered LYVE-1-positive projections visible at a screen magnification of 180 $\times$, in five regions per trachea, each 1.5 mm² in area.³¹ Area densities (percentage of total tissue area) of LYVE-1-positive lymphatics and CD31-positive blood vessels viewed in real-time fluorescent images of tracheal whole mounts were measured by stereological point counting of 10 regions per trachea, each 1.7 mm² in area.³¹

Fluorescence Intensity Measurements

The fluorescence intensity was measured as an estimate of the expression of α 5 integrin in the mucosa overlying cartilage rings. The fluorescence intensity was measured on digital images stained for α 5 integrin immunoreactivity using ImageJ software.^{44,45} A fluorescence intensity of 15 (range, 0–255) was established as the threshold for distinguishing pixels of α 5 integrin immunoreactive tissues from those of the background. The fluorescence intensity represented the average brightness of all α 5

integrin immunoreactive tissues in the mucosa overlying cartilage rings. This value was calculated from all pixels with fluorescence intensities ≥ 15 as described previously.⁴⁶ The mean value was calculated from 10 images of the regions of interest in each trachea. The distribution of intensity values in the regions of interest was visualized using the surface plot function of ImageJ software.⁴⁴

Lymphatic Endothelial Cell Proliferation Assay

Human lymphatic microvascular endothelial cells (HMVEC-dLy-Ad-der; Cambrex Bio Science, Walkersville, MD) were cultured in EGM2-MV medium (Cambrex Bio Science) according to instructions of the manufacturer. Ninety-six-well plates were precoated with 10 μ g/ml fibronectin (F1904, Chemicon Europe, Hampshire, UK) and blocked with 1% bovine serum albumin. HMVECs were seeded at a density of 4×10^3 cells per well in the presence of indicated concentrations of the integrin α 5 small-molecule antagonist (JSM8757). Vehicle dimethyl sulfoxide concentrations were adjusted and did not exceed 0.1%. Cells were grown for 3 days in growth medium containing serum and supplements. Plates were washed once with PBS and adherent cells were fixed with 5% glutaraldehyde for 30 minutes, rinsed three times with PBS, and stained with 0.1% crystal violet for 1 hour. After rinsing with PBS, 10% acetic acid was added and plates were analyzed at 570 nm with a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA). To determine net cell proliferation, some cells were fixed 3 hours after seeding on day 0. The difference of absorbance on day 0 and on day 3 is the net proliferation. Experiments were performed in triplicate.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Mice were anesthetized and briefly perfused via the aorta with sterile PBS. Tracheas were removed and stored in RNeasy lysis reagent, homogenized, and total RNA was extracted using RNeasy fibrous tissue kit (Qiagen, Hilden, Germany). cDNA was generated using random primers (Invitrogen, Carlsbad, CA) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Samples of 1 ng of cDNA were subjected to reverse transcription-polymerase chain reaction using SYBR Green protocols and ER qPCR SuperMix Universal (Invitrogen). Primers were designed using data from PrimerBank (<http://pga.mgh.harvard.edu/primerbank>, last accessed April 23, 2008) or published sequences from the literature. The forward and reverse primers were: β -actin, 5'-GAAGCTGTGCTATGTTGCTCTA-3' and 5'-GGAGGAAGAGGATGCGGCA-3'; and Iba1, 5'-ATCAACAAGCAATTCCTCGATGA-3' and 5'-CAGC-ATTCGCTCAAGGACATA-3'. Reverse transcription-polymerase chain reaction analysis was done using a MyiQ reverse transcription-polymerase chain reaction machine (Bio-Rad, Hercules, CA). Samples were tested in duplicate and relative gene expression data were normalized to β -actin. Results are presented as fold increases of mRNA relative to β -actin.

Figure 1. Distribution of $\alpha 5$ integrin immunoreactivity in normal tracheal blood vessels. Confocal microscopic images of tracheal whole mounts stained for CD31 (green, blood vessels) and $\alpha 5$ integrin (red) immunoreactivities of a small arteriole (**A** and **B**, arrows), venule (**C** and **D**), and unusual capillary (**E** and **F**) from a pathogen-free mouse. **G** and **H**: Tracheal capillary, like most tracheal capillaries, without detectable $\alpha 5$ integrin immunoreactivity. Arrowheads in **A** and **B** mark a small venule. Scale bar = 10 μ m.

Statistical Analysis

Values are presented as means $\pm$ SEM with four or five mice per group unless otherwise indicated. The significance of differences between means was assessed by analysis of variance followed by the Dunn-Bonferroni test for multiple comparisons with P values <0.05 considered significant.

Results

Distribution of $\alpha 5$ Integrin Immunoreactivity in Normal Tracheal Microvasculature

In tracheal whole mounts of pathogen-free mice, CD31-immunoreactive arterioles had mostly diffuse $\alpha 5$ integrin

immunoreactivity (Figure 1, **A** and **B**). Venules also had $\alpha 5$ integrin immunoreactivity (Figure 1, **C** and **D**). Although some capillaries had $\alpha 5$ integrin immunoreactivity (Figure 1, **E** and **F**), most had none (Figure 1, **G** and **H**). Fibronectin immunoreactivity was widely distributed around the tracheal vasculature (data not shown).

Inhibition of Human Lymphatic Endothelial Cell Proliferation by JSM8757

To determine whether $\alpha 5 \beta 1$ integrin was essential for proliferation of lymphatic endothelial cells, we examined the effect of JSM8757 on cultured human lymphatic endothelial cells. We found that JSM8757 significantly reduced the proliferation of lymphatic endothelial cells and

Figure 2. Inhibition of lymphatic endothelial cell proliferation by JSM8757. Human lymphatic endothelial cells were cultured on fibronectin for 3 days, fixed with 5% glutaraldehyde, stained with crystal violet, and measured for absorbance at 570 nm. The difference between absorbance at day 0 (onset value for cells fixed on day 0) and absorbance of control cells (grown for 3 days in control culture medium containing 0.1% dimethyl sulfoxide) reflected the amount of endothelial cell proliferation. Values were significantly lower when grown in the presence of JSM8757 at concentrations of 25 or 50 $\mu\text{mol/L}$ ($*P < 0.01$ versus control). Data are from one of three independent experiments. Values are means $\pm$ SD.

at concentrations of 25 and 50 $\mu\text{mol/L}$ fully inhibited the net expansion of the cells (Figure 2).

Effect of JSM8757 on Lymphangiogenesis and Blood Vessel Remodeling in Inflamed Tracheas

Having confirmed the effect of JSM8757 on lymphatic endothelial cell growth *in vitro*, we next examined effect of JSM8757 on blood vessel remodeling and lymphangiogenesis in mouse airways after *M. pulmonis* infection. Blood vessels (strong CD31 immunoreactivity) and lymphatics (strong LYVE-1 immunoreactivity) in tracheas of pathogen-free mice were segmented and aligned with the framework of the cartilage rings. Most arterioles and venules were between the rings, and most capillaries were in the mucosa over the rings (Figure 3A).

After *M. pulmonis* infection, all mice lost about 10% of their body weight over the first 2 days and then JSM8757-treated mice returned to their original weight and continued to grow. By comparison, after infection, vehicle-treated mice stayed below their original weights (Figure 3B). At 14 days after infection, lymphatics not only were located between cartilage rings but also were abundant over the rings, where none were normally present (Figure 3C). However, mice treated with JSM8757 during the infection had few lymphatics in regions of the tracheal mucosa over the rings (Figure 3D). The area density of lymphatics over rings was significantly greater in *M. pulmonis*-infected mice than in pathogen-free mice, but was 57% less in JSM8757-treated mice than in vehicle-treated mice (Figure 3E).

Consistent with the vascular remodeling that occurs after *M. pulmonis* infection,^{39,40} blood vessels were enlarged. The amount of enlargement was similar in both groups of mice (Figure 3, F and G), as reflected by the increase in area density of CD31-positive blood vessels

(Figure 3H). JSM8757 had no significant effect on the amount of enlargement (Figure 3H). Similarly, the mean diameter of tracheal blood vessels was not significantly different in the two groups ($12.6 \pm 0.6 \mu\text{m}$ after vehicle compared with $11.9 \pm 0.7 \mu\text{m}$ after JSM8757).

The surface of lymphatics in pathogen-free airways was smooth and lacked sprouts (Figure 3I). After infection, many lymphatics had sprouts (Figure 3J), but sprouts were much more abundant in vehicle-treated mice than in JSM8757-treated mice (Figure 3K). The number of lymphatic sprouts was significantly greater in infected mice than in pathogen-free mice, but JSM8757-treated mice had 66% fewer lymphatic sprouts than vehicle-treated mice (Figure 3L).

$\alpha 5$ Integrin Immunoreactivity of Initial Lymphatics in Inflamed Airways

Little or no $\alpha 5$ integrin immunoreactivity was detected on initial lymphatics or collecting lymphatics in tracheas of pathogen-free mice (Figure 4, A–C). However, after infection for 14 days, newly formed lymphatics identified by LYVE-1 immunoreactive vessels over cartilage rings had patchy but clear $\alpha 5$ integrin immunoreactivity, both on sprouts and on initial lymphatics (Figure 4, D–F). The stronger $\alpha 5$ integrin immunoreactivity after infection and the patchy nature of the staining were evident in surface plots of fluorescence intensity (Figure 4G). High-intensity spots were more numerous in infected mice (Figure 4G). The mean intensity of $\alpha 5$ integrin immunofluorescence was 34% greater in infected mice (Figure 4H).

Effect of JSM8757 on Lymphatic Sprouts but Not on Macrophages in Infected Airways

Lymphatic sprouts had patchy but clear $\alpha 5$ integrin immunoreactivity (Figure 5, A–C). No $\alpha 5$ integrin immunoreactivity was found in collecting lymphatics of infected mice (Figure 5, D and E). Tissues around lymphatics also had patchy $\alpha 5$ integrin immunoreactivity (Figure 5, A–F).

To assess the possible contribution of macrophages as a target of JSM8757 in blocking lymphangiogenesis after *M. pulmonis* infection, we compared the number of CD11b immunoreactive cells in whole mounts of infected tracheas after vehicle or JSM8757 treatment for 14 days and found no treatment-related difference between the two groups (Figure 5, F and G). We also measured mRNA expression of the macrophage marker protein, ionized calcium-binding adapter molecule 1 (Iba1).^{46,47} Iba1 mRNA was greater in *M. pulmonis*-infected mice than in pathogen-free mice (Figure 5H), but the increase was about the same in vehicle-treated mice and JSM8757-treated mice (Figure 5H). Iba1-immunoreactive macrophages in inflamed airways did not have $\alpha 5$ integrin immunoreactivity (Figure 5, I and J).

Discussion

These experiments provided multiple lines of evidence that favor the involvement of $\alpha 5\beta 1$ integrin in lymphangiogen-

Figure 3. Contrasting effects of JSM8757 on lymphatic growth and blood vessel remodeling. Confocal microscopic images of tracheal whole mounts stained for CD31 (green, blood vessels) and LYVE-1 (red, lymphatics) immunoreactivities in pathogen-free mouse (A) or after *M. pulmonis* infection for 14 days with concurrent treatment with vehicle or JSM8757 (C, D, F, G, I–K). **A:** Overview of arrangement of blood vessels and lymphatics in trachea of a pathogen-free mouse. **B:** Body weights of infected mice treated with vehicle or JSM8757 from day 0 to day 14. Initial body weight was considered 100%. * $P < 0.05$ compared with corresponding vehicle-treated mice. **C and D:** Comparison of lymphatics in the trachea of infected mice treated with vehicle (C) or JSM8757 (D). **Arrows** indicate newly grown lymphatics in the mucosa overlying cartilage rings. **E:** Area density of lymphatics in tracheal mucosa over cartilage rings in pathogen-free mice or infected mice treated with vehicle or JSM8757. **F and G:** Similarity of blood vessel caliber in infected mice treated with vehicle (F) or JSM8757 (G). **H:** Blood vessel area density in the mucosa over cartilage rings in pathogen-free mice or in infected mice treated with vehicle or JSM8757. **I:** No sprouts are present on lymphatics in pathogen-free mouse (I), but lymphatics in infected mouse treated with vehicle have numerous sprouts (J, **arrows**). Fewer sprouts are present on lymphatics of infected mouse treated with JSM8757 (K, **arrow**). **L:** Number of lymphatic sprouts in tracheas of pathogen-free or infected mice treated with vehicle or JSM8757. $P < 0.05$ compared with pathogen-free mice (*) or to vehicle-treated infected mice (†). Values are means $\pm$ SEM, five mice per group. Scale bar in K applies to all panels: 80 μ m in A, C, D, F, and G; 30 μ m in I–K.

Figure 4. $\alpha 5$ integrin immunoreactivity of lymphatics in infected tracheas. Confocal microscopic images of tracheal whole mounts, stained for LYVE-1 (green) and $\alpha 5$ integrin (red) immunoreactivities, from pathogen-free mice (**A–C**) or mice infected with *M. pulmonis* for 14 days (**D–F**). **A–C:** Absence of $\alpha 5$ integrin immunoreactivity (red) on a collecting lymphatic in a pathogen-free mouse shown by lack of colocalization with LYVE-1 (green). **B:** Gray scale image of **A** to highlight staining of the lymphatic. **D–F:** An initial lymphatic in an infected mouse showing patchy $\alpha 5$ integrin immunoreactivity. **E:** Gray scale image of **D**. **G:** Surface plot of the intensity of $\alpha 5$ integrin immunofluorescence in tracheal mucosa over a cartilage ring. **H:** Mean fluorescence intensity of $\alpha 5$ integrin in mucosa over cartilage rings in infected and pathogen-free mice. Values are means $\pm$ SEM. * $P < 0.05$ compared with pathogen-free mice. Five mice per group. Scale bar = 10 μ m.

esis after *M. pulmonis* infection. Sprouting and growth of lymphatics in the airways after infection were significantly reduced by JSM8757, a selective small-molecule inhibitor of $\alpha 5\beta 1$ integrin. Similarly, proliferation of lymphatic endothelial cells in culture was reduced by JSM8757. Unlike lymphatics in pathogen-free airways, lymphatic sprouts and initial lymphatics that formed after infection had $\alpha 5$ integrin immunoreactivity. The effect of JSM8757 on lymphangiogenesis was not a reflection of general toxicity, as the

treated mice increased in body weight after infection while their vehicle-treated counterparts lost weight. Indeed, JSM8757 seemed to have had a protective effect after infection. Because JSM8757 did not affect macrophage influx after infection, the antilymphangiogenic action was apparently through a direct effect on tracheal lymphatics.

The presence of $\alpha 5$ integrin immunoreactivity in lymphatic sprouts in inflamed airways, but not in pathogen-

Figure 5. $\alpha 5$ integrin immunoreactivity of lymphatic sprouts but not macrophages in infected airways. Confocal microscopic images of tracheas stained for LYVE-1 (green) and $\alpha 5$ integrin (red) immunoreactivities from mice infected with *M. pulmonis* for 14 days and concurrently treated with vehicle or JSM8757. **A–C:** Lymphatic sprouts (arrows) in infected trachea from vehicle-treated mouse. **D and E:** Collecting lymphatic of infected mouse trachea showing lack of colocalization of $\alpha 5$ integrin (red) and LYVE-1 (green) immunoreactivities. **F and G:** Lymphatics stained for LYVE-1 (red) and a monocyte/macrophage marker CD11b (green) after vehicle or JSM8757 treatment. The number of CD11b immunoreactive cells was similar between infected tracheas treated with vehicle (**F**) or JSM8757 (**G**). **H:** Quantitative reverse transcription-polymerase chain reaction measurements of Iba1 mRNA expression in macrophages in tracheas of pathogen-free mice and infected mice treated with vehicle or JSM8757. Values are means $\pm$ SEM, five mice per group. * $P < 0.05$ compared with pathogen-free values. **I and J:** Trachea of infected mouse showing lack of colocalization of $\alpha 5$ integrin (red) and Iba-1 (green) immunoreactivities. Scale bar in **J** applies to all panels: 10 μ m in **A–C**; 30 μ m in **D and E**; 120 μ m in **F and G**; 15 μ m in **I and J**.

free airways, suggests that the integrin is up-regulated in growing lymphatics. To our knowledge, this is the first report of the selective induction of integrin expression in inflamed lymphatics. Although fibroblast-like connective tissue cells in the airway mucosa also had $\alpha 5$ integrin immunoreactivity, colocalization with LYVE-1 and surface plots of $\alpha 5$ integrin immunofluorescence confirmed the presence of the integrin on lymphatics.

In our study, $\alpha 5$ integrin immunoreactivity had a patchy distribution on lymphatics, suggestive of focal adhesion sites. Sprouts on lymphatic endothelial cells were particularly common sites of staining. The immunoreactivity also had a patchy distribution in some regions of the surrounding tissue. Signaling complexes involving integrin $\alpha 5 \beta 1$ have also been reported to have a patchy distribution on cultured cells.⁸

The reduction in sprouting and proliferation of lymphatics that occur after *M. pulmonis* infection in the presence of JSM8757 is consistent with inhibition of lymphangiogenesis through inhibition of binding of $\alpha 5 \beta 1$ integrin to fibronectin, which reduces endothelial cell proliferation and sprouting. $\alpha 5 \beta 1$ integrin-mediated signaling has been linked to endothelial cell survival and proliferation. Adhesion via $\alpha 5$ integrin has been shown to support cell proliferation by activation of mitogen-activated protein kinases and transcriptional regulation of cell cycle proteins.⁴⁸ Antagonists of $\alpha 5 \beta 1$ integrin can cause apoptosis of endothelial cells by induction of protein kinase A and activation of caspase-8.⁴⁹

In addition to blocking binding to fibronectin, JSM8757 may also reduce binding to fibrillin,⁵⁰ which is another integrin ligand that is a main component of lymphatic anchoring filaments.⁵¹ Anchoring filaments are a characteristic feature of initial lymphatics and connect lymphatic endothelial cells to elastic fibers in the perivascular extracellular matrix.⁵¹ Integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$ are reported to be receptors for fibrillin in some cultured cells.⁵⁰ If this applies to airway lymphatics, $\alpha 5 \beta 1$ integrin blockade could inhibit lymphatic growth by blocking the binding of anchoring filaments to lymphatic endothelial cells. Part of the antilymphangiogenic action of JSM8757 could also result from the association of $\alpha 5 \beta 1$ integrin with activation of VEGFR-3.³⁵

Macrophages are believed to be among the immune cells that drive lymphangiogenesis.^{31,52} As macrophages express $\alpha 5 \beta 1$ integrin in some inflammatory conditions,⁵³ a potential mechanism for inhibition of lymphangiogenesis by JSM8757 is prevention of macrophage recruitment. In this context, JSM6427, an $\alpha 5 \beta 1$ integrin inhibitor similar to JSM8757, had no effect on macrophage recruitment in a model of corneal inflammation.¹¹ Similarly, we found that macrophages were recruited to infected airways, but the amount of recruitment, assessed by mRNA of the macrophage marker *Iba1*,^{46,47} was not significantly reduced by JSM8757. Moreover, we did not detect $\alpha 5$ integrin immunoreactivity on recruited macrophages. These results suggest that in airway inflammation produced by *M. pulmonis* infection, JSM8757 inhibits lymphangiogenesis by mechanisms other than blocking of macrophage recruitment and favor effects on lymphatic endothelial cells.

In conclusion, in mouse airways, $\alpha 5 \beta 1$ integrin was distributed on most blood vessels larger than capillaries, but selective blockade of the integrin did not affect blood vessel remodeling after *M. pulmonis* infection. In contrast, $\alpha 5 \beta 1$ integrin was not found on lymphatics in normal airways but was strongly up-regulated on lymphatic sprouts and new lymphatics, where it had a patchy distribution suggestive of focal adhesion sites. Blockade of $\alpha 5 \beta 1$ integrin by JSM8757 significantly reduced lymphangiogenesis after *M. pulmonis*. Based on these findings, we propose a mechanism by which $\alpha 5 \beta 1$ integrin is involved in lymphangiogenesis in inflammation by promoting sprouting and proliferation of lymphatic endothelial cells. Our results suggest a novel approach for inhibiting lymphangiogenesis by exploiting the selective expression of $\alpha 5 \beta 1$ integrin on activated lymphatics. Inhibition of lymphangiogenesis could be beneficial in

chronic inflammatory airway disease, psoriasis, tumor cell spread via lymphatics, kidney transplant rejection, or other clinical conditions where lymphatic proliferation or abnormalities play a role.^{24,54}

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