Blockade of CD49d (α_4 Integrin) on Intrapulmonary but Not Circulating Leukocytes Inhibits Airway Inflammation and Hyperresponsiveness in a Mouse Model of Asthma

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Abstract

Immunized mice after inhalation of specific antigen have the following characteristic features of human asthma: airway eosinophilia, mucus and Th2 cytokine release, and hyperresponsiveness to methacholine. A model of late-phase allergic pulmonary inflammation in ovalbumin-sensitized mice was used to address the role of the α_4 integrin (CD49d) in mediating the airway inflammation and hyperresponsiveness. Local, intrapulmonary blockade of CD49d by intranasal administration of CD49d mAb inhibited all signs of lung inflammation, IL-4 and IL-5 release, and hyperresponsiveness to methacholine. In contrast, CD49d blockade on circulating leukocytes by intraperitoneal CD49d mAb treatment only prevented the airway eosinophilia. In this asthma model, a CD49d-positive intrapulmonary leukocyte distinct from the eosinophil is the key effector cell of allergen-induced pulmonary inflammation and hyperresponsiveness. (*J. Clin. Invest.* 1997. 100:3083–3092.) Key words: eosinophil • interleukin-4 • interleukin-5 • mucus • very late antigen-4

Introduction

Asthma in humans is characterized by increased reactivity of the airways to a wide variety of physical and chemical stimuli. This bronchial hyperresponsiveness has been attributed to chronic airway inflammation, as the severity of asthma and increased airway reactivity correlate with the infiltration of the airways by eosinophils and lymphocytes. Consequently, there has been great interest in the mechanisms involved in the recruitment to and activation of inflammatory cells in the airways. Leukocyte migration into the lung requires the interaction between surface-adhesion receptors on leukocyte and counter-structures on pulmonary endothelium and ligands in extravascular tissue. The α_4 integrin subunit CD49d associates with either the β_1 (CD29) or β_7 subunit to form the integrin heterodimers very late antigen (VLA)[1]-4 ($\alpha_4\beta_1$; CD49d/CD29) and $\alpha_4\beta_7$ (1, 2). VLA-4 is expressed at significant levels on all circulating leukocytes except mature neutrophils, and binds to vascular cell adhesion molecule (VCAM)-1 (CD106), a member of the Ig gene superfamily that is expressed on cytokine-activated endothelial cells, and to the matrix protein fibronectin. $\alpha_4\beta_7$ is expressed on a subset of T and B cells, natural killer cells, and eosinophils. It binds to the mucosal vascular addressin (MAdCAM-1), a member of the Ig and mucin-like families of adhesion molecules, as well as to VCAM-1 and fibronectin (2, 3).

Studies in vitro have demonstrated that VLA-4 interaction with VCAM-1 is involved in mononuclear leukocyte and eosinophil adherence to endothelium and transendothelial migration (1, 4). $\alpha_4\beta_7$ is thought to be involved primarily in leukocyte recruitment to gut-associated lymphoid tissue (3). Studies in vivo using blocking mAbs have established a role for CD49d in leukocyte recruitment in a variety of inflammatory and immune disorders (4). CD49d appears to play a particularly important role in inflammatory cell recruitment in allergic disorders. CD49d mAbs have been reported to inhibit the emigration of eosinophils to inflammatory lesions in guinea pig skin (5) and the accumulation of eosinophils and/or lymphocytes after antigen challenge in rat (6), sheep (7), mouse (8), and guinea pig (9, 10) lung. CD49d blockade was also found to attenuate bronchial hyperreactivity after allergen challenge in the guinea pig (9), rat (11, 12), and sheep (7). However, it remains controversial whether there is a causal link between the effect of CD49d blockade on bronchial activity and eosinophil recruitment.

Pretolani et al. (9) and Fryer et al. (10) examined the effect of a CD49d mAb on antigen-induced airway hyperreactivity and cellular infiltration in the guinea pig airways. They noted a decrease in both airway hyperreactivity and cellular infiltration of eosinophils in bronchoalveolar lavage (BAL) fluid and bronchial tissue. In contrast, Laberge et al. (11) and Rabb et al. (12) reported that a CD49d mAb prevented the increase in airway responsiveness in allergen-challenged rats, although the CD49d mAb treatment did not alter eosinophil accumulation in the lung. Abraham et al. (7) reported that a CD49d mAb modified the antigen-induced late bronchial response and airway hyperresponsiveness, and reduced eosinophil accumulation after acute antigen challenge in a sheep model. However, the administration of the CD49d mAb by aerosol also attenuated bronchial hyperresponsiveness without substantially reducing eosinophil numbers. The latter three studies suggest that

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1. *Abbreviations used in this paper:* alum, aluminum potassium sulfate; AP, alkaline phosphatase; BAL, bronchoalveolar lavage; C_{dyn}, dynamic compliance; G_L, conductance; HPRT, hypoxanthine-guanine phosphoribosyl transferase; ICC, immunocytochemistry; OVA, ovalbumin; *R*, resistance; RT, reverse transcriptase; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

CD49d blockade may modulate allergen-induced airway reactivity by a mechanism other than inflammatory cell recruitment. The observations that a leukotriene synthesis inhibitor (13) or IL-5 mAb (14, 15) inhibits eosinophil accumulation without affecting airway hyperreactivity also suggest that eosinophils do not mediate allergen-induced airway hyperresponsiveness. Taken together, these studies suggest that treatment with a CD49d mAb may reduce airway hyperreactivity after allergen challenge by targeting a cell other than the eosinophil.

To define further the role of CD49d in allergic airway inflammation and hyperreactivity, we examined the effect of cell CD49d blockade on eosinophil recruitment, bronchial hyperresponsiveness to methacholine, mucus hypersecretion, and Th2 cytokine synthesis in a mouse model of asthma (13, 16). CD49d mAb was targeted to intrapulmonary leukocytes by intranasal (i.n.) administration or to circulating leukocytes by intraperitoneal (i.p.) administration. We demonstrate that local intrapulmonary blockade of CD49d abrogated allergen-induced bronchial hyperresponsiveness, release of Th2 cytokines IL-4 and IL-5, and all manifestations of lung inflammation, whereas blockade of CD49d on circulating leukocytes prevented eosinophil accumulation in BAL fluid but did not affect airway hyperreactivity, mucus hypersecretion, or Th2 cytokine synthesis. We conclude that a CD49d-positive intrapulmonary leukocyte other than an eosinophil is a critical effector of allergen-induced airway hyperreactivity and lung inflammation in this model.

Methods

Special reagents. 500 μg/ml crystalline ovalbumin (OVA; Pierce Chemical Co., Rockford, IL) in saline was mixed with equal volumes of 10% (wt/vol) aluminum potassium sulfate (alum; Sigma Chemical Co., St. Louis, MO) in distilled water and incubated for 60 min at room temperature after adjustment to pH 6.5 using 10 N NaOH. After centrifugation at 750 *g* for 5 min, the OVA/alum pellet was resuspended to the original volume in distilled water. Rat IgG_{2b} mAb anti–mouse CD49d α_4 chain (PS/2 batch CS071995, 1.6 mg/ml) was purified by protein A and gel filtration chromatography and generously provided by Dr. Roy R. Lobb (Biogen, Inc., Cambridge, MA). Purified rat IgG_{2b} (1 mg/ml, clone R35–38) mAb was purchased from PharMingen (San Diego, CA). The endotoxin level of each purified antibody was ≤ 0.01 ng/μg of protein. Hamster IgG mAb (>1 μg/ml) secreted from hybridoma cell line N418 (HB 224; American Type Culture Collection, Rockville, MD), which reacts with CD11c from mouse dendritic cells, was kindly provided by Dr. Andrew G. Farr (University of Washington, Seattle, WA).

Allergen sensitization/challenge protocol. Female BALB/c mice (6–8 wk of age) received an i.p. injection of 100 μg OVA (0.2 ml of 500 μg/ml in normal saline) complexed with alum on days 0 and 14. On days 14, 25, 26, and 27, the mice underwent anesthesia with 0.2 ml i.p. of ketamine (0.44 mg/ml)/xylazine (6.3 mg/ml) in normal saline before receiving a 0.05-ml i.n. dose of either 100 μg OVA in saline (day 14) or 50 μg OVA in saline (days 25, 26, and 27). Control animals received i.p. saline with alum on days 0 and 14 and i.n. saline without alum on days 14, 25, 26, and 27. All animal study protocols were approved by the University of Washington Animal Care Committee.

Antibody treatment. To assess the role of adhesion molecules in airway inflammation and hyperresponsiveness, antiadhesion molecule and control antibodies were given by either i.p. or i.n. administration based in part on dosing regimens reported by Nakajima et al. (8), Burkly et al. (17), and Tsuyuki et al. (18). For i.p. treatment, mice received 384 μg of CD49d mAb i.p. on day 24, and 96 μg of CD49d mAb i.p. on days 25, 26, and 27. For i.n. treatment, mice received 11–56 μg of rat IgG_{2b} or CD49d mAbs i.n. on days 25, 26, and 27. The i.p. and i.n. administrations of each mAb were given 30 min before the i.n. OVA challenge on days 25, 26, and 27.

Pulmonary function testing. 24 h after i.n. challenge with either OVA or saline, airway reactivity was measured on day 28. Dose–response curves to intravenous infusions of methacholine in mice were determined by plethysmography as modified (13) from previously described procedures of Amdur and Mead (19) and Martin et al. (20). The mice were anesthetized with pentobarbital (70–90 mg/kg, i.p.) before undergoing thoracotomy. An angled 18-gauge blunt needle and 8-cm silastic tubing were used for tracheal intubation and jugular vein cannulation, respectively. The mice were connected to a ventilator (Harvard Apparatus, Inc., South Natick, MA) and placed in a supine position in one compartment (0.25 ml dead space) of a two-chamber whole body plethysmograph system. Normal arterial blood gases were maintained with the following minute ventilation: 0.2 ml/20 g tidal vol, 120 breaths/min frequency, and 2.5–3 cm H_2O positive end–expiratory pressure (21). The change in box pressure (P_{box} = lung pleural pressure) of the first chamber as determined by a sensitive transducer ±0.7 cm H_2O represents the change in lung volume (Δvol = P_{box}) of the mouse. Copper mesh in the plethysmograph served as a heat sink to reduce rapid pressure fluctuations due to heat produced by gas compression during each breath. Increases in temperature in the first chamber resulting from the animal's body heat were corrected for by a microvalve (time constant ∼ 4.5 s) placed between the two compartments; a second microvalve (time constant ∼ 11 s) was open to ambient air to maintain mean P_{box} near zero. A transducer measuring pressure movements at the opening of the tracheal tube (P_{aw}) was referenced to P_{box} for calculation of transpulmonary pressure ($P_{tp} = P_{aw} - P_{box}$). The lungs were inflated initially to a P_{aw} of 30–35 cm H_2O and also 1–2 min before each measurement to reexpand any areas of collapsed lung. P_{tp} and P_{box} were measured at 5-ms intervals by an analog-to-digital data acquisition system (Strawberry Tree, Inc., Sunnyvale, CA). To bring P_{tp} into phase with the P_{box} data, the digital P_{tp} data were offset by 15-ms. To dampen the background noise in P_{box}, a smoothing function was applied as described previously (13). Basal pulmonary function was calculated during the first 10 min from data (collected three times) from seven consecutive breaths. Increasing concentrations of methacholine (3, 12, 48, 120, and 480 mg/kg) were then injected into the jugular vein over 10 s with 1.5–3-min intervals between injections for pulmonary function to return to basal levels. Dynamic compliance (C_{dyn}), resistance (R), and lung conductance ($G_L = 1/R$) were calculated for the basal period and the peak response to each dose of methacholine injected. C_{dyn} was calculated as the change in tidal volume (Vt) divided by the difference between P_{tp} when flow is zero at the end of inspiration and expiration ($C_{dyn} = \Delta Vt/\Delta P_{tp}$). R was calculated as the difference in P_{tp} and air flow at midtidal volume on inflation and deflation; tracheal tube resistance (0.63 cm $H_2O \cdot ml^{-1} \cdot s$) was subtracted from each measurement of airway resistance. C_{dyn} and G_L were expressed as the percentage of basal value at each methacholine dose. The concentration of methacholine reducing C_{dyn} and G_L to 75 and 50% of the control values (ED_{75} and ED_{50}, respectively, expressed as geometric mean + log SD) was calculated from the log dose–peak response curves.

BAL. After pulmonary function testing, the mice underwent exsanguination by cardiac puncture and then BAL (0.4 ml of saline three times) of the right lung after tying off the left lung at the mainstem bronchus. Total BAL fluid cells were counted from a 0.05-ml aliquot, and the remaining fluid was centrifuged at 200 *g* for 10 min at 4°C. The supernatants were stored at −70°C until assay of mucus glycoproteins and cytokine protein levels. The cell pellets were resuspended in saline containing 10% BSA, and smears were made on glass slides. Eosinophils were stained for 5–8 min with Discombe's diluting fluid containing 0.05% aqueous eosin and 5% (vol/vol) acetone in distilled water, rinsed with distilled water, and counterstained with 0.07% methylene blue as described previously (13).

Lung histology. After BAL, the trachea and upper and lower

lobes of the left lung were removed and fixed for 15 h at 4°C in Carnoy's solution. The tissues were embedded in paraffin and cut into 5-μm sections before staining with Discombe's solution and counterstaining with methylene blue. Airway mucus was identified by the following histochemical procedures: mucin by mucicarmine staining; acidic sulfated mucosubstances, hyaluronic acid, and sialomucins by alcian blue, pH 2.5 staining; neutral and acidic mucosubstances by alcian blue, pH 2.5 and periodic acid-Schiff (PAS) reaction; and sialic acid–rich, nonsulfated mucosubstances by toluidine blue, pH 4.5 staining as described previously (13).

Immunocytochemistry (ICC). Paraffin sections of lung tissue of OVA-treated mice given either i.p. or i.n. administration of rat IgG CD49d mAb were deparaffinized, hydrated, and washed in PBS. To localize CD49d mAb–positive cells, the sections were incubated at room temperature for 2 h with biotinylated rabbit anti–rat IgG (DAKO Corp., Carpinteria, CA) diluted 1:100 in 5% nonfat dry milk with 1% rabbit serum (Vector Laboratories, Inc., Burlingame, CA). Control slides were treated with 5% nonfat dry milk with 1% rabbit serum only. After rinsing in PBS, the sections were incubated for 1 h with alkaline phosphatase (AP) solution of Vectastain® ABC-AP Standard kit (Vector Laboratories, Inc.) in PBS. The sections were rinsed in PBS and incubated at room temperature for 30 min with the AP solution of Vector® Red AP-Substrate kit (Vector Laboratories, Inc.) in Tris-HCl buffer, pH 8.2, containing 1% levamisol (Vector Laboratories, Inc.); AP reaction products stain pink. The sections were rinsed with distilled water and counterstained with 0.4% methylene blue in 70% ethanol and 0.01% NaOH (22). The sections were dehydrated in a series of ethanol concentrations up to 100%, cleared in xylene, and mounted with Permount (Fisher Scientific Co., Pittsburgh, PA). The number of intravascular and extravascular CD49d mAb–positive cells per unit airway area (2,200 μm^2) was determined by morphometry as described previously (13, 23).

The phenotype of resident lung cells binding CD49d mAb was examined by double-staining ICC. The lung sections from OVA-treated mice given i.n. CD49d mAb which had been single-stained for CD49d mAb localization as described above, before counterstaining were incubated with 0.3% H_2O_2 for 30 min to block endogenous peroxidase. The sections were incubated with hamster IgG mAb against mouse CD11c. The sections were rinsed with PBS and incubated with hamster IgG anti–mouse CD11c for 60 min followed by incubation with biotinylated goat anti–hamster IgG (10 μg/ml in 5% nonfat dry milk with 1% goat serum; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). To detect the biotinylated antibody, the sections were incubated for 45 min with the horseradish peroxidase solution of the ABC Elite kit (Vector Laboratories, Inc.). The sections were developed with HRP-Substrate VIP kit (Vector Laboratories, Inc.) for 25 min at room temperature; the double-labeled AP and peroxidase reaction products stain brownish red. Sections were counterstained with 2% aqueous methyl green, dehydrated in ethanol, and processed as described above.

Determination of CD49d mAb binding on circulating blood cells. To determine the level of CD49d mAb on circulating blood cells by FACS®, whole blood was obtained on day 28 (24 h after i.n. challenge with OVA) from OVA-treated mice that had received no other treatment, i.n. control rat IgG_{2b} administration, or CD49d mAb either by i.p. or i.n. administration. The blood was put immediately on ice, and 100 μl of blood was aliquoted into 6-ml polystyrene tubes. In some tubes, 1 μg/ml CD49d mAb or 15–50 μg/ml rat IgG_{2b} was added 20 min before the lysing step. Red blood cells were lysed by adding 1.3 ml of 1× lysing solution (prepared on the day of the experiment by mixing nine parts sterile water to one part 10× lysing solution; 1.68 M NH_4Cl, 0.1 M $KHCO_3$, 1 mM EDTA, pH 7.3) to 100 μl of blood for 7 min on ice. The tubes were centrifuged at 260 *g* for 7 min at 4°C, the supernatant was discarded, and the cell pellet was incubated in 100 μl HBSS buffer containing 15 μg/ml FITC-labeled goat anti–rat secondary antibody (Caltag Labs., Inc., Burlingame, CA) for 30 min at 4°C. The tubes were centrifuged, and the cell pellet was reconstituted in 500 μl of HBSS buffer, kept on ice, and analyzed immediately using a flow cytometer (EPICS XL; Coulter Corp., Hialeah, FL). Granulocyte, monocyte, and lymphocyte populations were delineated using forward- and side-scattering properties of the three cell populations. Fluorescence intensities were measured within the gated leukocyte populations. Mean fluorescence values were determined from histograms of the gate populations, and are expressed as relative fluorescence intensity.

Assay of BAL fluid mucus glycoproteins. BAL fluid (0.05 ml) was blotted onto prewetted nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell, Keene, NH) by water suction vacuum in a 72-well slot blot apparatus (Minifold II; Schleicher & Schuell), and mucus glycoproteins were visualized by PAS reaction and quantitated by reflectance densitometry as described previously (13, 24, 25). PAS images were digitized using a scanner (ScanJet IIcx; Hewlett-Packard Co., Palo Alto, CA) and assayed on a 256 gray level scale using a Dimension XPS P90 computer with UltraScan 17ES monitor (Dell Corp., Austin, TX) with Image-Pro® Plus software (Media Cybernetics Inc., Silver Spring, MD). The BAL fluid mucus content was quantitated by comparison with a standard curve of human respiratory mucin glycoprotein (26) (generously provided by Dr. James H. Shelhamer, National Institutes of Health, Bethesda, MD) that underwent the same slot blotting and PAS-staining procedures as the BAL fluid samples.

Cytokine protein assays. Biotrak® mouse IL-4 and IL-5 ELISA systems (Amersham Corp., Arlington Heights, IL) were used for quantitation of IL-4 and IL-5 levels in the BAL fluids. These assays are based on a solid-phase ELISA using antibodies (raised against recombinant IL-4 and IL-5) bound to microtiter plate wells and conjugated to horseradish peroxidase. IL-4 was measured in the range 15–375 pg/ml, and IL-5 in the range 20–320 pg/ml.

Reverse transcriptase (RT)-PCR for cytokine gene expression. After dissection, bronchial lymph nodes were frozen on dry ice slabs and homogenized in TRI REAGENT® solution (1 ml/50–100 mg tissue; Molecular Research Center, Inc., Cincinnati, OH) using a 5-ml grinder with Teflon pestle. Total RNA was isolated according to the manufacturer's instructions and reverse-transcribed as described previously (16). 3 μg of total RNA in 10 μl diethyl pyrocarbonate/water was reverse-transcribed in a final 20 μl reaction mixture containing 0.5 mM dNTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 25 ng/μl random hexamer primers (Pharmacia Biotech, Piscataway, NJ), 10 U/μl Moloney murine leukemia virus transcriptase (GIBCO BRL, Gaithersburg, MD), 1 U/μl placental ribonuclease (RNasin) inhibitor (Promega Corp., Madison, WI), 5 mM DTT, 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris-HCl, pH 8.3 (GIBCO BRL). The mixture was incubated first at 37°C for 60 min for RT, and then at 95°C for 5 min to inactivate the transcriptase. The RT-cDNA template (1–5 μl) underwent PCR in a 50-ml reaction mixture containing 200 μM dNTP, 0.03 U/μl *Taq* polymerase (Amersham Corp.), 1 μM sense-strand primer, 1 μM antisense-strand primer, 1.5 mM $MgCl_2$, 20 mM ammonium sulfate, and 50 mM Tris-HCl, pH 9.0 (Amersham Corp.). The samples underwent denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min. To verify that equal amounts of cDNA were added in each PCR reaction within an experiment and a uniform amplification process, the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was also reverse-transcribed and amplified in each assay. The coding and noncoding strand primers for PCR of IL-4 and HPRT transcripts were 5′-CATCGGCATTTTGAACGAGGTCA and 3′-CTTATCGATGAATCCAGGCATCG (IL-4), and 5′-GTTGGATACAGGCCAGACTTTGTTG and 3′-GAGGGTAGGCTGGCCTATAGGCT (HPRT) (27). Under these conditions, 34 and 25 PCR cycles were used for each respective IL-4 and HPRT primer pair to produce a linear relationship between the final PCR products and the beginning amount of mouse bronchial lymph node RNA. PCR products were separated by electrophoresis in 1.8% agarose gels (Life Technologies, Inc., Gaithersburg, MD) containing 0.5 μg/ml ethidium bromide in Tris-borate buffer. Type 55 film (Polaroid Corp., Cambridge, MA) was used to photograph the gels for estimation of relative levels of IL-4 to

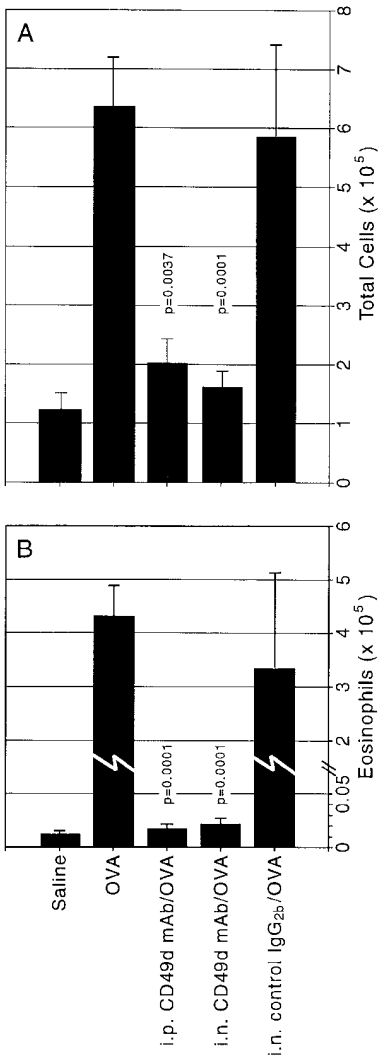

Figure 1. CD49d mAb by either i.n. or i.p. administration blocks eosinophil infiltration of BAL fluid in OVA-treated mice. BAL fluid was obtained from sham-sensitized and saline-challenged mice (*Saline*; $n = 13$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 14$) or presence of i.p. CD49d mAb (*i.p. CD49d mAb/OVA*; $n = 8$), i.n. CD49d mAb (*i.n. CD49d mAb/OVA*; $n = 10$), or i.n. control rat IgG_{2b} (*i.n. control IgG_{2b}/OVA*, $n = 3$). The number (mean±SEM) of total cells (*A*) and eosinophils (*B*) per total lavage fluid obtained is shown. *P* values, i.p. CD49d mAb/OVA or i.n. CD49d mAb/OVA vs. OVA.

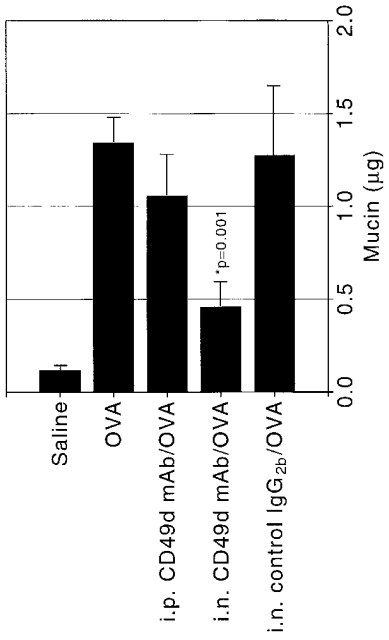

Figure 2. CD49d mAb by i.n. but not by i.p. administration blocks airway mucus release in OVA-treated mice. BAL fluid was obtained from sham-sensitized and saline-challenged mice (*Saline*; $n = 10$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 8$) or presence of i.p. CD49d mAb (*i.p. CD49d mAb/OVA*; $n = 6$), i.n. CD49d mAb (*i.n. CD49d mAb/OVA*; $n = 5$), or i.n. control rat IgG_{2b} (*i.n. control IgG_{2b}/OVA*; $n = 2$) treatment. BAL fluid (0.5 ml) was blotted onto nitrocellulose with mucus glycoproteins visualized by PAS reaction and quantitated by reflectance densitometry as described in Methods. Human respiratory mucin glycoprotein (0.05–0.75 ml, 2 µg/ml) was assayed in the same manner to establish a standard curve for quantitation. *P* value, i.n. CD49d mAb/OVA vs. OVA.

HPRT gene expression. The photographic negatives were scanned on a flatbed scanner (Arcus II, Agfa Division, Bayer Corp., Ridgefield Park, NJ), and the digital data of the band intensities were analyzed with Image-Pro® Plus software (Media Cybernetics Inc.) (16). The sum of pixel values of each single band for IL-4 and HPRT was calculated with gel background absorbence subtracted from these integrated band intensities to determine the ratio of IL-4– to HPRT-amplified products as described previously by Gavett et al. (28).

Statistical analyses. The data are reported as the mean±SEM of the combined experiments. Student's two-tailed *t* test for independent means was used to determine significant differences ($P < 0.05$). ANOVA using the protected least significant difference method (Statview II; Abacus Concepts, Inc., Berkeley, CA) was used to evaluate the pulmonary function data. By this method, a multiple *t* statistic determines all possible pairwise comparisons for both equal and unequal pair sample sizes.

Results

Inhibition of allergen-induced airway eosinophil infiltration by CD49d mAb. As we have reported previously (13), i.n. OVA challenge on days 25, 26, and 27 in mice sensitized previously by i.p. OVA on days 0 and 14 and by i.n. OVA on day 14 causes a marked influx of eosinophils into the bronchial interstitium and BAL fluid 24 h after the last i.n. challenge with antigen (Fig. 1). In OVA-sensitized/challenged mice, total BAL fluid eosinophils increased 360-fold ($P = 0.0001$) compared with saline (Fig. 1 *B*) and comprised 67.8% of the total BAL fluid cells compared with < 1% of total cells in control mice. CD49d mAb by i.p. or i.n. administration reduced this influx of eosinophils into the lungs by > 99% (Fig. 1 *B*; $P = 0.0001$, i.p. CD49d mAb/OVA vs. OVA; $P = 0.0001$, i.n. CD49d mAb/OVA vs. OVA). Control rat IgG_{2b} when given by i.n. administration 30 min before OVA challenge did not affect significantly the influx of eosinophils into the lungs observed in sensitized mice (Fig. 1 *B*). The parenchymal and submucosal eosinophil infiltration observed in the OVA-treated mice was reduced after administration of either i.p. or i.n. CD49d mAb.

Lymphocyte accumulation in the BAL fluid was inhibited significantly by either i.p. or i.n. CD49d mAb but not by i.n. control rat IgG_{2b}: $1.3\pm0.2 \times 10^5$ lymphocytes, OVA versus (*a*) $0.3\pm0.1 \times 10^5$ lymphocytes, saline, $P = 0.0001$; (*b*) $0.5\pm0.1 \times 10^5$ lymphocytes, i.p. CD49d mAb/OVA, $P = 0.0031$; (*c*) $0.3\pm0.1 \times 10^5$ lymphocytes, i.n. CD49d mAb/OVA, $P = 0.0001$; and (*d*) $1.2\pm0.3 \times 10^5$ lymphocytes, i.n. IgG_{2b}, $P = 0.7762$.

Inhibition of allergen-induced airway mucus release by i.n. but not by i.p. administration of CD49d mAb. The amount of mucus glycoprotein recovered in BAL fluid was 13.5-fold greater in OVA-treated mice than in control mice (Fig. 2; $P = 0.0001$, OVA vs. saline). Whereas the i.p. administration of CD49d mAb failed to block airway mucin release (Fig. 2 and Fig. 3 *A*), i.n. treatment with CD49d mAb (Fig. 2 and Fig. 3 *B*) reduced significantly the amount of mucus glycoprotein recovered in the BAL fluid of OVA-sensitized/challenged mice ($P = 0.001$, i.n. CD49d mAb/OVA vs. OVA).

To assess the cellular localization of CD49d mAb after i.p. and i.n. administration, ICC of lung tissue and FACS® analysis of peripheral blood were performed in OVA-sensitized/chal-

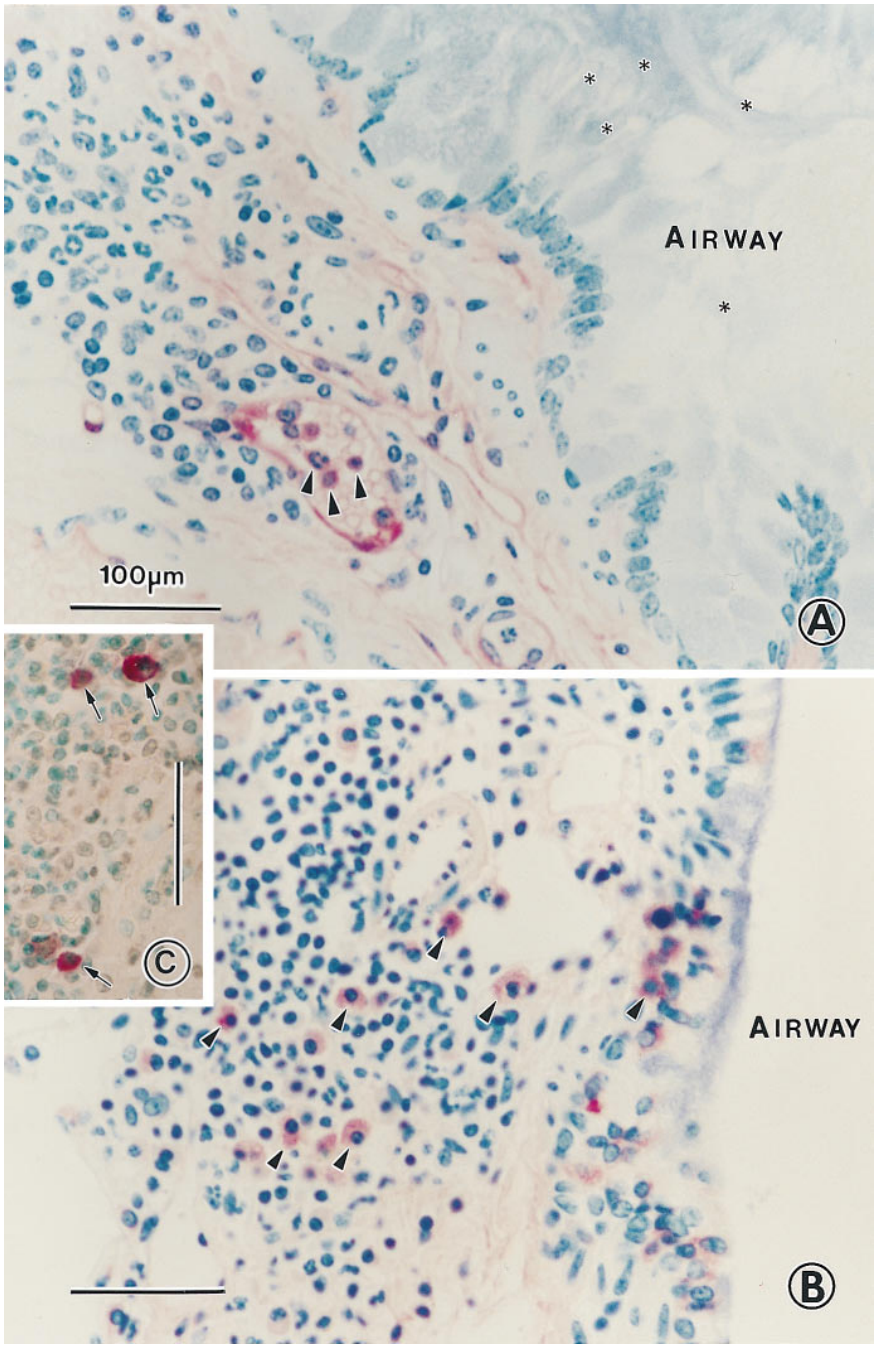

Figure 3. Immunocytochemical detection of CD49d mAb in lungs after administration of CD49d mAb in OVA-treated mice. Lung tissue was obtained from OVA-immunized/challenged mice in the presence of either i.p. (*A*) or i.n. (*B* and *C*) administration of CD49d mAb. Lung sections underwent single-staining ICC for localization of CD49d mAb binding (*A* and *B*) or double-staining ICC for colocalization of CD49d mAb and CD11c (*C*) as described in Methods. (*A*) The airway lumen contains large amounts of released mucus (*). Immunocytochemical localization of the i.p.-administered CD49d mAb, as indicated by the pink AP reaction products, is confined to mononuclear cells (*arrowheads*) within the pulmonary vessels. (*B*) Airway mucus secretion is not observed when i.n. CD49d mAb is administered before i.n. OVA. Mononuclear leukocytes in the airway epithelium and interstitial space react strongly (*arrowheads*) for the i.n.-administered CD49d mAb by ICC. Staining of intravascular leukocytes (*C*) for CD49d mAb is not seen. Eosinophil infiltration of the interstitial space is reduced markedly after either i.p. (*A*) or i.n. (*B*) administration of CD49d mAb. AP reaction products were not present in the lung sections from OVA-treated mice receiving either i.p. or i.n. CD49d mAb when the rabbit anti–rat IgG antibody was omitted from the staining protocol (not shown). (*C*) By double-staining ICC, mononuclear cells binding CD49d mAb were all positive for CD11c, as indicated by the brownish red reaction products (*arrows*). *Bar*, 100 μm.

lenged mice. After i.p. administration, CD49d mAb was localized by ICC to mononuclear leukocytes within the lung vessels, with little staining of these cells in the interstitial space outside of the blood vessels (Fig. 3 *A*). In contrast, after i.n. administration, mononuclear cells with morphologic features of monocyte/macrophages or dendritic cells in the airway epithelium and interstitium but not within pulmonary vessels were strongly reactive for CD49d mAb by ICC (Fig. 3 *B*). By morphometric analysis, ~ 19-fold more intravascular CD49d mAb–positive pulmonary mononuclear cells were present after i.p. versus i.n. administration of CD49d mAb (i.n. CD49d mAb/OVA vs. i.p. CD49d mAb/OVA, $P = 0.0015$; Table I). Significantly fewer extravascular CD49d mAb–positive pulmonary mononuclear cells were found after i.p. versus i.n. administra-

Table I. Intravascular and Extavascular Pulmonary Cells: Effect of i.p. versus i.n. CD49d mAb

	Mononuclear cells	
Group	Intravascular	Extravascular
i.p. CD49d mAb/OVA	5.8±0.7	0.5±0.1
i.n. CD49d mAb/OVA	0.3±0.1*	5.4±0.7*

Data represent mean±SEM. The number of CD49d mAb–positive intravascular and extravascular pulmonary mononuclear cells per unit area (2,200 μm^2) of lung tissue after i.p. ($n = 3$) versus i.n. ($n = 3$) administration of CD49d mAb in OVA-treated mice was quantitated by ICC as described in Methods. *$P < 0.005$ vs. i.p. CD49d mAb/OVA.

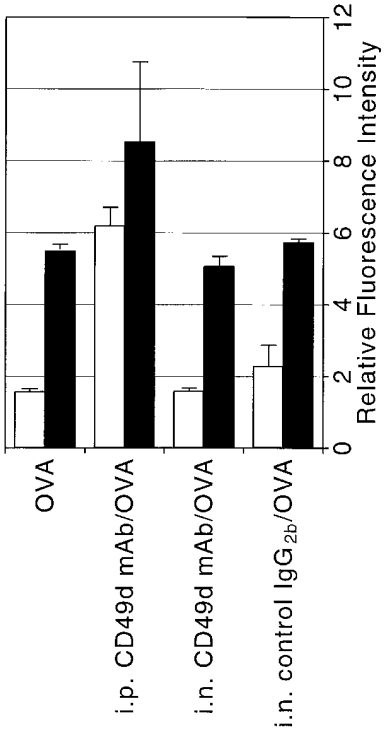

Figure 4. CD49d mAb reacts with circulating mononuclear cells after i.p. but not i.n. administration. Blood was obtained from OVA-sensitized/challenged mice in the absence (*OVA*; n = 2) or presence of i.n. control rat IgG_{2b} (*i.n. control* IgG_{2b}*/OVA*; n = 2), i.n. CD49d mAb (*i.n. CD49d mAb/OVA*; n = 6), or i.p. CD49d mAb (*i.p. CD49d mAb/OVA*; n = 2) treatment. The mononuclear leukocyte cell population was analyzed for binding of CD49d mAb by flow cytometry as described in Methods. *White bars*, Mean channel fluorescence after addition of secondary FITC-conjugated goat anti–rat antibody ex vivo. *Black bars*, Samples treated first ex vivo with CD49d mAb at 1 μg/ml, then with the secondary FITC-conjugated goat anti–rat antibody.

tion of CD49d mAb/OVA (P = 0.0025; Table I). The phenotype of the resident lung mononuclear cells binding CD49d mAb given by i.n. administration was examined. By double-staining ICC (Fig. 3 *C*), the CD49d mAb–binding cells all co-stained for CD11c, which is expressed on mouse dendritic cells and activated macrophages (29, 30).

Mice treated with i.n. CD49d mAb showed no binding by FACS® of mAb to blood mononuclear leukocytes compared with untreated mice or those treated with i.n. isotype-matched irrelevant rat mAb (Fig. 4). In contrast, mice treated with i.p. CD49d mAb showed marked binding by FACS® of mAb to blood mononuclear leukocytes (Fig. 4). Similar qualitative results were found for the lymphocyte population, with lower absolute values of antibody staining for the spiked samples and the i.p. CD49d mAb sample. No changes in staining levels were found with the granulocyte population composed predominantly of neutrophils, which lack significant CD49d expression. Dosing of mouse blood ex vivo with 15–50 μg/ml of nonspecific rat IgG_{2b} (a dose likely to far exceed plasma levels achieved with the i.p. dosing schedule) did not produce specific binding to peripheral blood leukocytes in whole blood on flow cytometric analysis (relative fluorescence: second antibody alone = 1.0, rat IgG_{2b} + second antibody = 1.5±0.3 [n = 3], and CD49d mAb + second antibody = 5.4±0.2 [n = 3]).

Inhibition of allergen-induced airway hyperreactivity to methacholine by i.n. but not by i.p. administration of CD49d mAb. Administration of i.p. and i.n. OVA in mice increased airway reactivity to methacholine compared with mice receiving only saline. Significantly lower concentrations of methacholine were needed to decrease G_L (Fig. 5 *A*) and C_{dyn} (Fig. 5 *B*) in OVA-sensitized/challenged mice compared with controls treated with saline. CD49d mAb i.n. administration on days 25, 26, and 27 blocked the increased airway response to methacholine (Fig. 5 and Table II). In contrast, i.p. CD49d mAb failed to alter the methacholine-induced airway hyperresponsiveness in OVA-treated mice (Fig. 5 and Table II).

Inhibition of allergen-induced Th2 cytokines. BAL fluid protein levels of the Th2 cytokines IL-4 and IL-5 were increased 2.8- and 3.0-fold, respectively, in OVA-treated mice compared with saline-treated controls (Fig. 6). These Th2 cytokines were inhibited significantly in OVA-treated mice by i.n. but not by i.p. CD49d mAb treatment (Fig. 6; P = 0.0419 [IL-4] and P = 0.0323 [IL-5], i.n. CD49d mAb/OVA vs. OVA).

To confirm suppression of Th2 cytokine synthesis in immunized/challenged mice by i.n. CD49d mAb, IL-4 gene expression was examined in the bronchial lymph nodes of saline- and OVA-treated mice by RT-PCR. IL-4 transcripts relative to HPRT products were increased 2.1-fold in OVA-sensitized/challenged mice compared with sham-sensitized/saline-challenged mice (Fig. 7). Whereas neither i.p. CD49d mAb nor i.n. control rat IgG_{2b} affected IL-4 gene expression in OVA-treated mice, i.n. CD49d mAb inhibited significantly IL-4 relative to HPRT transcripts (Fig. 7; P = 0.0487, i.n. CD49d mAb/OVA vs. OVA).

Discussion

The major findings of this study are (*a*) blockade of CD49d on circulating leukocytes by mAb given by i.p. administration before allergen challenge prevents eosinophil recruitment into BAL fluid, but does not inhibit allergen-induced airway hyperreactivity, mucus hypersecretion, or IL-4 and IL-5 synthesis; and (*b*) blockade of CD49d on intrapulmonary leukocytes by i.n. administration of mAb immediately before allergen challenge abrogates airway hyperreactivity, IL-4 and IL-5 synthesis, and mucus hypersecretion, as well as eosinophil and lymphocyte accumulation in BAL fluid.

The inhibition of eosinophil emigration into BAL fluid after antigen challenge by systemically administered CD49d mAb has been reported previously in rats (6), sheep (7), mice (8), and guinea pigs (9, 10). It is assumed that this effect is due in large part to blockade of CD49d on circulating leukocytes, preventing leukocyte binding to VCAM-1 on pulmonary endothelium. This conclusion is supported by observations that antigen-induced eosinophil emigration was abolished in sensitized mice treated with anti–VCAM-1 mAb (8) or in animals with VCAM-1 deficiency due to a targeted, hypomorphic mutation (31). In several of these studies, the effect of CD49d mAb treatment on antigen-induced airway hyperreactivity was also examined. In the guinea pig (9, 10) model, the CD49d mAb was noted to reduce bronchial hyperresponsiveness as well as eosinophil accumulation. However, Laberge et al. (11) and Rabb et al. (12) found that intravenously administered CD49d mAb inhibited airway reactivity in allergen-challenged rats without affecting eosinophil accumulation in BAL fluid, leading them to suggest that eosinophils were not causally related to bronchial hyperresponsiveness. Similarly, Abraham et al. (7) found that the inhibition of airway hyperresponsiveness by CD49d mAb in the sheep model did not correlate with the reduction in eosinophil accumulation, and suggested that a major effect of the CD49d mAb was on cell activation rather than leukocyte recruitment. This discordance was also ob-

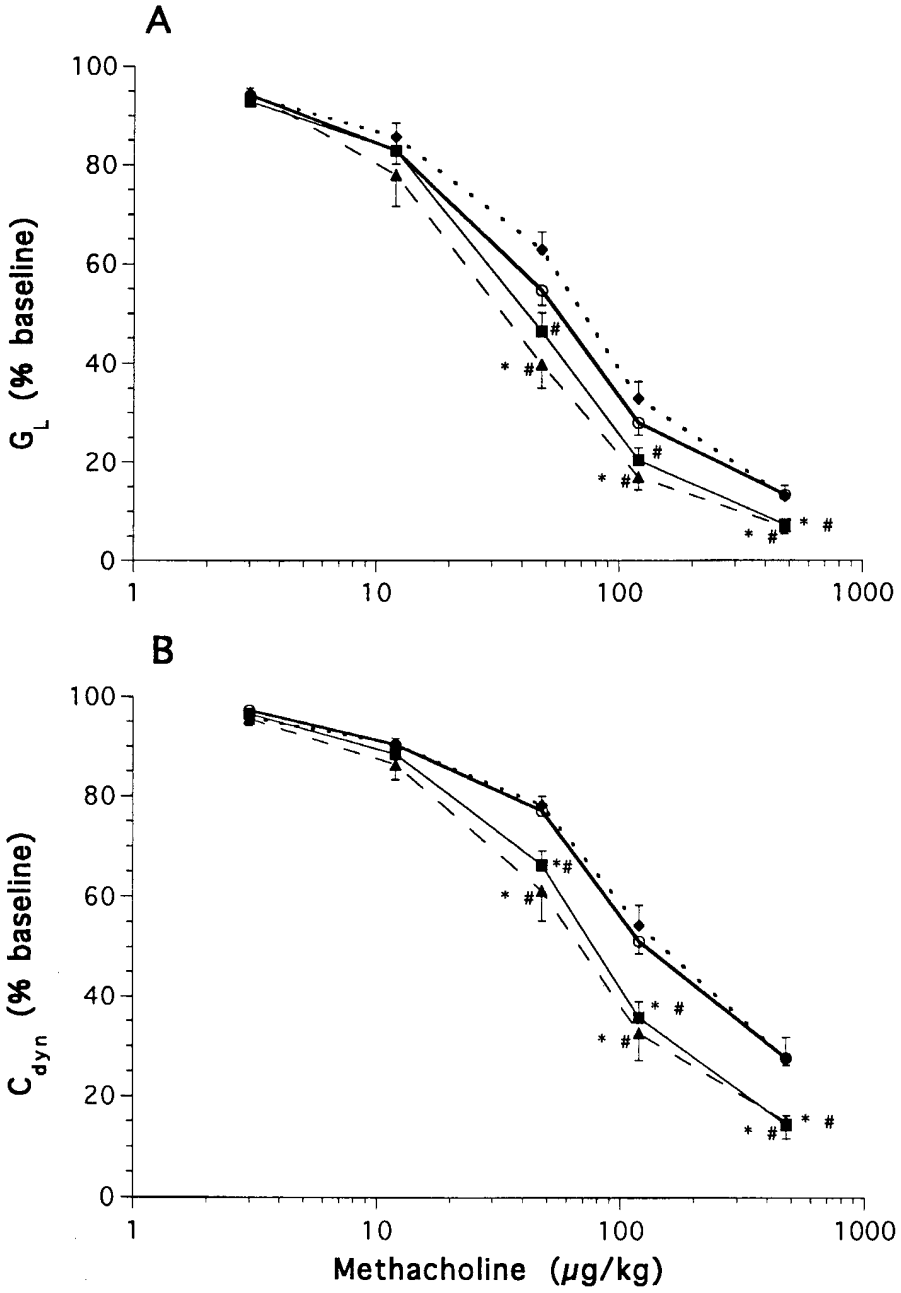

Figure 5. i.n. but not i.p. administration of CD49d mAb blocks methacholine-induced airway responsiveness in OVA-treated mice. Airway responsiveness to increasing concentrations of methacholine was calculated in sham-sensitized and saline-challenged mice (*circles*; $n = 11$) and OVA-sensitized/challenged mice in the absence (*squares*; $n = 10$) or presence of i.p. CD49d mAb (*triangles*; $n = 6$) or i.n. CD49d mAb (*diamonds*; $n = 10$) treatment. (*A*) Airway conductance (G_L) and (*B*) dynamic lung compliance (C_{dyn}) were determined as described in Methods. The data are mean±SEM. $*P < 0.05$ by ANOVA, sham-sensitized and saline-challenged vs. the OVA-sensitized/challenged, i.p. CD49d mAb/OVA, and i.n. CD49d mAb/OVA groups.

served in guinea pig models. Ishida et al. (32) found that a platelet-activating factor antagonist inhibited antigen-induced airway hyperresponsiveness in a guinea pig model without affecting airway eosinophilia. Lilly et al. (33) demonstrated that the intratracheal administration of IL-5 in guinea pigs produced pulmonary eosinophilia but not airway hyperresponsiveness. Also, transgenic mice expressing IL-4 constitutively in the lung developed an inflammatory cell infiltrate including eosinophilia without resulting in an alteration in airway reactivity to inhaled methacholine (34).

Our studies show that CD49d mAb given by i.p. administration at doses that saturate circulating leukocytes blocked antigen-induced eosinophil accumulation in BAL fluid but did not prevent airway hyperreactivity, mucus hypersecretion, or IL-4 and IL-5 release. This finding of airway hyperreactivity without eosinophilia is in keeping with our previous studies in this mouse model, in which inhibitors of leukotriene synthesis prevented allergen-induced eosinophil recruitment without affecting bronchial hyperreactivity, and also with those of Corry et al. in mice (14) and Mauser et al. in guinea pigs (15), in which an anti–IL-5 mAb prevented eosinophil recruitment after allergen challenge without affecting airway hyperreactivity. Similarly, Elwood et al. in rats (35) found that cyclosporin A failed to attenuate allergen-induced airway hyperresponsiveness while suppressing eosinophilia in BAL fluid. Taken together, these studies indicate that allergen-induced airway hyperreactivity can be reduced without affecting airway eosinophilia (7, 11, 12, 32), airway eosinophilia alone does not provoke airway hyperreactivity (33, 34), and allergen-induced airway hyperresponsiveness develops in the absence of eosino-

Table II. Pulmonary Mechanics to Methacholine in OVA-treated Mice: Effect of i.p. versus i.n. CD49d mAb

	Conductance (G_L)			Dynamic compliance (C_{dyn})		
		ED_{50} methacholine			ED_{50} methacholine	
Group	Basal	Geometric mean	log SD	Basal	Geometric mean	log SD
	ml/s × cm H_2O	*μg/kg*		*ml/s × cm H_2O*	*μg/kg*	
Saline	1.52±0.08	54	0.17	0.033±0.001	133	0.07
OVA	1.45±0.04	42*	0.17	0.031±0.001*	80*‡	0.14
i.p. CD49d mAb/OVA	1.40±0.05	32*‡	0.21	0.030±0.001*‡	68*‡	0.22
i.n. CD49d mAb/OVA	1.56±0.07	70	0.05	0.034±0.001	160	0.08

Data represent the mean±SD. G_L and C_{dyn} were determined in sham-sensitized and saline-challenged mice (*Saline*; $n = 11$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 10$) or presence of i.p. CD49d mAb ($n = 6$) or i.n. CD49d mAb ($n = 10$) treatment as described in Methods. $P < 0.05$ by ANOVA, ‡vs. saline, *vs. i.n. CD49d mAb/OVA.

phil accumulation in the lungs, as shown in this and other studies (13–15, 35). Thus, it appears that eosinophil accumulation in the lung is neither necessary nor sufficient alone for the development of airway hyperreactivity. However, a conflicting conclusion was reached by Foster et al. (36) and Hogan et al. (37). In IL-5–deficient (36, 37) mouse models, the eosinophilia, lung damage, and airway hyperreactivity resulting from allergen challenge were abolished, leading them to conclude that eosinophils are central mediators in the pathogenesis of allergic lung disease. The reason for the discrepancy in this mouse model regarding the role of eosinophils in airway hyperreactivity is not clear, and the causal role of the eosinophils in human asthma likewise remains uncertain.

The remarkable inhibitory activity of the CD49d mAb by i.n. but not by i.p. administration suggests strongly that the primary target is an intrapulmonary rather than circulating leukocyte. This proposal is supported by the finding that the i.n. CD49d mAb did not bind to circulating leukocytes as assessed by flow cytometry. Since total levels of eosinophils and lymphocytes were comparably reduced by i.n. or i.p. CD49d mAb treatment, these data suggest that these recruited cells were not required for the late effector phase of the model. Also, ICC analysis indicated that the CD49d mAb after i.n. administration penetrated the lung interstitium to a greater extent than after i.p. administration. Although our data indicate that the i.p. dosing of CD49d mAb saturated CD49d on circulating eosinophils, the doses we used might not have allowed sufficient levels of circulating unbound antibody to enter airway inflammatory sites and act on resident lung effector cells.

There are several candidate intrapulmonary target cells for the i.n.-administered CD49d mAb, including mast cells, eosinophils, T-lymphocytes, and dendritic cells or interstitial macrophages. Mast cells and eosinophils are unlikely to be the intrapulmonary targets of the CD49d mAb, as sensitized mast cell–deficient mice still develop eosinophilia (38, 39) and air-

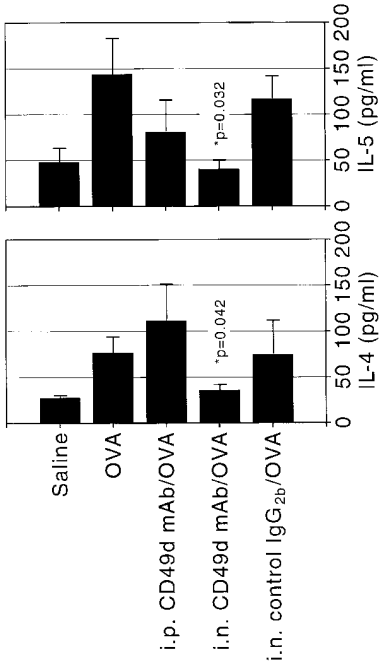

Figure 6. CD49d mAb by i.n. but not by i.p. administration inhibits Th2 cytokine protein levels in OVA-treated mice. ELISA was performed for IL-4 and IL-5 protein levels in BAL fluid from sham-sensitized and saline-challenged mice (*Saline*; $n = 10$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 11$) or presence of i.p. CD49d mAb (*i.p. CD49d mAb/OVA*; $n = 8$), i.n. CD49d mAb (*i.n. CD49d mAb/OVA*; $n = 10$), or i.n. IgG_{2b} (*i.n. control IgG_{2b}/OVA*; $n = 2$) treatment. The data are the mean±SEM. *P value, i.n. CD49d mAb/OVA vs. OVA.

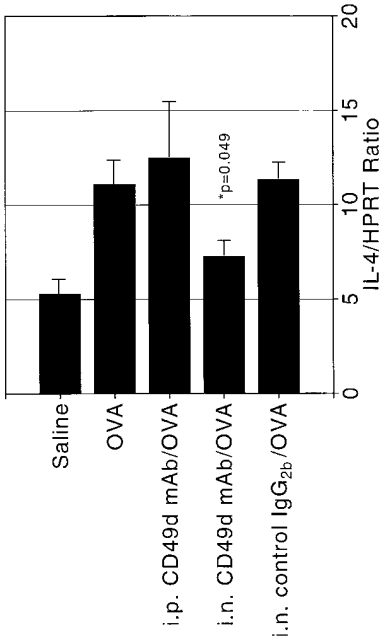

Figure 7. i.n. but not i.p. administration of CD49d mAb blocks Th2 cytokine gene expression in OVA-treated mice. RT-PCR was performed for detection of IL-4 mRNA in bronchial lymph nodes from sham-sensitized and saline-challenged mice (*Saline*; $n = 4$) and OVA-sensitized/challenged mice in the absence (*OVA*; $n = 5$) or presence of i.p. CD49d mAb (*i.p. CD49d mAb/OVA*; $n = 3$), i.n. CD49d mAb (*i.n. CD49d mAb/OVA*; $n = 4$), or i.n. IgG_{2b} (*i.n. control IgG_{2b}/OVA*, $n = 3$) treatment. The ratio of IL-4 transcripts relative to HPRT transcripts was calculated for each condition as described in Methods. The data are the mean±SEM. *P value, i.n. CD49d mAb/OVA vs. OVA.

way hyperreactivity (Henderson, W.R., Jr., R.K. Albert, W.J.E. Lamm, and E.Y. Chi, manuscript in preparation) after allergen challenge, and antigen-induced bronchial hyperresponsiveness develops in the absence of eosinophil infiltration in our (13) and other (14, 15, 35) models of asthma. T-lymphocytes and dendritic cells or interstitial macrophages are more likely candidate target cells. The CD4-positive T-lymphocyte has been proposed to orchestrate the chronic inflammation of asthma (40). Like eosinophils, CD4-positive lymphocytes infiltrate airways of asthmatic subjects (41, 42), and antigen challenge recruits additional CD4-positive cells into the airways (43). The CD4 cells in these patients exhibit a Th2 phenotype with secretion of IL-4 and IL-5 (44). Depletion of CD4 cells by mAb pretreatment before allergen challenge (but after allergen sensitization) prevents airway reactivity (45) and eosinophil recruitment (45, 46). However, the ICC studies show prominent staining of bound CD49d mAb by an interstitial mononuclear cell after the administration of i.n. CD49d mAb. The ICC data suggest that these are either activated macrophages or dendritic cells since they express CD11c, a marker of murine dendritic cells or activated macrophages (29, 30). The mature dendritic cell is a potent antigen-presenting cell (47). Dendritic cells are prominent in the mucosal lining of the conducting airways and in alveolar septa (48), and their number is increased in the airways of asthmatics (49). Tissue dendritic cells are specialized for acquisition and processing of antigen, but are relatively ineffective in presentation of antigen; this property is fully acquired after migration to the regional lymph node (47). Moreover, dendritic cells have now been identified in germinal centers within the lung parenchyma after allergen challenge (50), and dendritic cells capable of stimulating T cells have been identified in germinal centers (51). Finally, the ICC studies are also consistent with the interstitial macrophage as the CD49d mAb–binding cell. The role of activated interstitial macrophages in the lung response to inhaled allergen is unexplored.

The mechanisms by which i.n. CD49d mAb modulates the effector functions of a critical intrapulmonary cell such as a CD4-positive lymphocyte, macrophage, or dendritic cell are unknown. Integrin receptors clearly transduce signals upon engagement of ligand (52). VLA-4–mediated T-lymphocyte binding to fibronectin (53, 54) or VCAM-1 (55) provides a potent costimulatory signal for T cell activation after CD3 cross-linking, which is thought to mimic antigen engagement. T cells have been shown to bind to VCAM-1 expressed on airway smooth muscle cells (56). Additional studies are required to determine whether fibronectin, VCAM-1, or the mucosal vascular addressin MAdCAM-1 is the critical intrapulmonary ligand(s) for CD49d. Blockade of this costimulatory signal by i.n. CD49d mAb could prevent full activation of antigen-specific T cells in response to allergen challenge. The inhibition of Th2 gene expression in bronchial lymph nodes and of protein levels in BAL fluid in animals receiving i.n. CD49d mAb is consistent with this effect. The i.n. CD49d mAb could also provoke apoptosis of critical resident effector cells in the lung. Integrin signaling has been reported to transduce survival signals, preventing apoptosis in a variety of cell types, including leukocytes (57–59). Abrogation of this survival signal by inhibition of CD49d-dependent adherence to fibronectin could trigger apoptosis of a critical intrapulmonary target cell. Finally, CD49d blockade could inhibit VLA-4–dependent trafficking of antigen-presenting dendritic cells from the airway to T-lymphocytes in germinal centers in the lung parenchyma or to peribronchial lymph nodes.

Further studies to define the precise target cell and mechanism of action of the CD49d mAb in the lung are in progress. It remains to be determined whether similar effects are observed with blocking mAbs directed to other leukocyte integrin receptors.

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