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Blockade of Airway Inflammation and Hyperresponsiveness by HIV-TAT-Dominant Negative Ras

Shigeharu Myou, Xiangdong Zhu, Saori Myo, Evan Boetticher, Angelo Y. Meliton, Jie Liu, Nilda M. Munoz, and Alan R. Leff

We have reported previously that HIV-TAT-dominant negative (dn) Ras inhibits eosinophil adhesion to ICAM-1 after activation by IL-5 and eotaxin. In this study, we evaluated the role of Ras in Ag-induced airway inflammation and hyperresponsiveness by i.p. administration into mice of dnRas, which was fused to an HIV-TAT protein transduction domain (TAT-dnRas). Uptake of TAT-dnRas (t1/2 = 12 h) was demonstrated in leukocytes after i.p. administration. OVA-sensitization significantly increased eosinophil and lymphocyte numbers in bronchoalveolar lavage fluid 24 h after final challenge. Treatment of animals with 3–10 mg/kg TAT-dnRas blocked the migration of eosinophils from 464 ± 91 × 10³/ml to 288 ± 79 × 10³/ml with 3 mg/kg TAT-dnRas (p < 0.05), and further decreased to 116 ± 63 × 10³/ml after 10 mg/kg TAT-dnRas (p < 0.01). Histological examination demonstrated that inflammatory cell infiltration (largely eosinophils and mononuclear cells) and mucin production around the airways caused by OVA were blocked by TAT-dnRas. OVA challenge also caused airway hyperresponsiveness to methacholine, which was dose dependently blocked by treatment with TAT-dnRas. TAT-dnRas also blocked Ag-induced IL-4 and IL-5, but not IFN-γ, production in lung tissue. Intranasal administration of IL-5 caused eosinophil migration into the airway lumen, which was attenuated by pretreatment with TAT-dnRas. By contrast, TAT-green fluorescent protein or dnRas lacking the TAT protein transduction domain did not block airway inflammation, cytokine production, or airway hyperresponsiveness. We conclude that Ras mediates Th2 cytokine production, airway inflammation, and airway hyperresponsiveness in immune-sensitized mice.

transduced TAT-dnRas into mice in vivo. We found that TAT-dnRas suppressed OVA-induced infiltration of eosinophils and lymphocytes, reduced Th2 cytokine secretion in the lung, and diminished airway hyperresponsiveness to methacholine.

Materials and Methods

Generation of TAT-dnRas and its in vivo administration

Generation of TAT dN-Ras protein was accomplished by a method we have described previously (23). Briefly, a cDNA fragment encoding dn H-Ras was amplified by PCR from the H-Ras cDNA (17N mutant) in pUSEamp (Upstate Biotechnology, Lake Placid, NY). The amplified PCR products were inserted into a pcDNA3.1(C-terminal-green fluorescent protein (GFP)-TOPO vector (Invitrogen, Carlsbad, CA). Site-directed mutagenesis was conducted to create an Age I cutting site in pcDNA3.1-dnRas, and the mutated plasmids were digested with Age I and ligated into an Age I-digested pTAT vector (kindly provided by Dr. S. Dowdy, University of California San Diego, La Jolla, CA) using T4 ligase. TAT-dnRas was purified by sonication of high expressions BL21(DE3)pLyS E cells in 10 ml of buffer Z (8 M urea, 20 mM HEPES (pH 8.0), 100 mM NaCl). Cellular lysates were resolved by centrifugation, loaded onto 5-ml Ni-NTA columns in buffer Z, washed, and eluted with imidazole in buffer Z sequentially in 100, 250, and 500 mM concentrations. Urea and imidazole were removed from the resultant protein solution using a low-volume, 10,000 M.W. cut-off Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). Each fusion protein was flash-frozen at −80°C.

Mice

Male C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free biohazard level 2 facility maintained by the University of Chicago Animal Resources Center (Chicago, IL). The studies reported in this paper conform to the principles outlined by the Animal Welfare and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

In protocols detailed below, animals receive i.p. TAT-dnRas or control injection. The animals receiving injection of TAT-dnRas or control proteins (His-dnRas or TAT-GFP) were assigned randomly to the experimental groups consisting of six animals each. In additional experiments, TAT fusion proteins were administered intranasally to mice to assess their effect on airway inflammation after Ag challenge. Two hours before OVA challenge, mice received intranasal administration of 50 μl of 0.3 or 1.0 mg/ml TAT-dnRas through a 24-g catheter.

Flow cytometric analysis of the TAT fusion protein transduction into blood leukocytes

Either TAT-dnRas or control protein lacking the TAT PTD was labeled with FITC (Sigma-Aldrich, St. Louis, MO) according to manufacturer’s instructions. Mice were sacrificed, and blood was aspirated by cardiac puncture into a heparinized syringe for isolation of whole blood leukocytes at specified times after i.p. injection of TAT-dnRas or control dnRas protein. Whole blood leukocytes were isolated by hypotonic lysis of RBC. Isolated leukocytes were resuspended in calcium- and magnesium-free HBSS (pH 6.8) containing 1% BSA. The pH of the sheath fluid of the flow cytometer was adjusted to 6.8 to quench FITC fluorescence on the outside of the cell (24), and at least 10,000 cells were analyzed on a FACScan (BD Biosciences, Mansfield, MA).

Immunization and airway challenge with OVA

Mice were sensitized and challenged as described previously (29). Briefly, mice were immunized with 10 μg of OVA and 1.125 mg of aluminum hydroxide (Imject Alum; Pierce) in 0.2 ml of sterile saline i.p. on days 0, 7, and 14. On days 21–24, mice were challenged intranasally with 100 μg of OVA in 1% saline for 40 min. To examine the effect of TAT-dnRas on Ag-induced airway reactions, the animals received i.p. injection of TAT-dnRas or control dnRas protein lacking TAT PTD (Fig. 1B) every 12 h during OVA inhalations. For measurement of airway responsiveness, animals also received TAT vehicle lacking dnRas (Fig. 1C).

Measurement of airway responsiveness to methacholine

Methacholine challenge was performed 24 h after the final dose of OVA. The assessment of respiratory mechanics was done with a computer-controlled small-animal ventilator (SAV) (Flexivent; Scireq, Montreal, Canada) as previously described (30). Briefly, mice were anesthetized with 30 mg/kg xylazine and 80 mg/kg ketamine i.p., and the trachea was cannulated with an 18-gauge metal needle connected to the SAV. Regular mechanical ventilation was applied, and animals were ventilated quasiisosomoidaly at a frequency of 120 breaths/min at a tidal volume of 6 ml/kg. The expiratory valve of the SAV allowed the animal to empty passively through a water trap adjusted to maintain a positive end-expiratory pressure of 2.0 cm H2O. In preliminary experiments, this positive end-expiratory pressure was shown to be optimal for the determination of methacholine-induced changes in respiratory system resistance (30). Increasing doses of methacholine (31.3–4,000 μg/kg) were infused through a jugular vein catheter at 5-min intervals.

Collection and analysis of bronchoalveolar fluid cells

Airway inflammation was assessed 24 h after the final Ag challenge with OVA. Bronchoalveolar lavage (BAL) was performed by delivering 0.8 ml cold PBS into the airway through a tracheal cannula and gently aspirating the fluid. The lavage was repeated three times to recover a total volume of 2–3 ml. The cells were stained with trypan blue to determine viability, and with Turko solution to obtain total nucleated cell counts using a hemocytometer. Cytospin (Cytospin 2; Thermo Shandon, Pittsburgh, PA) slides were prepared from the BAL, and were then fixed and stained using Diff-Quick (Dade Diagnostics, Aguada, PR). Differential cell counts were determined by counting a minimum of 300 cells/slide using standard morphological criteria in a single-blind method.

Measurement of cytokine concentrations in the lung

Whole lungs were excised from mice 24 h after the last OVA challenge. Frozen lung tissue was chopped after thawing, and was resuspended in 1 ml of 50 mM HEPES buffer containing 1 mM EDTA and 50 mM PMSF. Samples were homogenized for 3 s at 15,000 rpm in a Ultra-Turrax homogenizer and centrifuged at 16,000 × g for 10 min at 4°C. The concentrations of IL-4, IL-5, and IFN-γ were measured using a Mouse Th1/Th2 Cytokine BCA kit according to the manufacturer’s protocol (BD Biosciences). The detection limits were 5 pg/ml for IL-4, 5 pg/ml for IL-5, and 2.5 pg/ml for IFN-γ.

Lung histology

Lungs removed from the chest cavity were fixed by injection of 4% buffered parafformaldehyde into the tracheal cannula at a pressure of 20 cm H2O, and immersed in parafformaldehyde for 24 h. Lobes were sectioned sagittally, embedded in paraffin, cut into 5-μm sections, and stained with H&E for histological analysis. Additional sections were stained with Alcian blue/periodic acid-Schiff (PAS) to identify mucus-containing cells. The severity of peribronchial inflammation was graded semiquantitatively for the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells two to four cells thick; 3, a ring of inflammatory cells two to four cells thick; 4, a ring of inflammatory cells of more than four cells deep. The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, <0.5% PAs-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75% (31).

IL-5-induced airway eosinophil accumulation

TAT-dnRas (3 or 10 mg/kg) or a control dnRas protein (excluding TAT PTD) was i.p. administered 2 h before challenge with IL-5. Animals were anesthetized with i.p. 4.4 mg/kg xylazine and 65 mg/kg ketamine (32), and then challenged intranasally with 100 μl of 100 ng/ml IL-5 dissolved in saline for 40 min. To examine the effect of TAT-dnRas on Ag-induced airway reactions, the animals received i.p. injection of TAT-dnRas or control dnRas protein lacking TAT PTD (Fig. 1B) every 12 h during OVA inhalations. For measurement of airway responsiveness, animals also received TAT vehicle lacking dnRas (Fig. 1C).

FIGURE 1. Purified proteins used in this study. A, Structure of TAT-dnRas fusion protein. Six His residues and the 11-aa TAT peptide precede the N terminus of the dn N17 H-Ras protein. The 11 aa of TAT are the PTD. B, Control dnRas protein lacking the PTD. C, TAT-GFP, a control protein lacking dnRas.
PBS through a 24-g catheter. BAL was performed 12 h after IL-5 instillation as described above.

Statistical analysis

Variation between three or more groups was analyzed using ANOVA followed by Fisher’s least protected difference test. Statistical significance was claimed whenever \( p < 0.05 \).

Results

*Kinetics of TAT-dnRas transduction*

Mice were injected i.p. with 10 mg/kg FITC-labeled TAT-dnRas or control dnRas protein lacking TAT PTD. Flow cytometry of whole blood leukocytes isolated 2–24 h after i.p. injection demonstrated the quantitative presence of TAT-dnRas. TAT-dnRas was maximally expressed at 2 h and returned to baseline over 24 h. The \( t_{1/2} \) for TAT-dnRas was 12 h. By contrast, dnRas protein lacking the TAT transduction domain was undetectable in blood leukocytes at any time (Fig. 2). Accordingly, TAT-dnRas was administered every 12 h in the subsequent experiments involving Ag challenge.

*Ag-induced airway inflammation and mucus secretion*

OVA inhalation significantly increased eosinophil \( (p < 0.01) \) and lymphocyte numbers \( (p < 0.01) \) in BAL fluid 24 h after challenge (Fig. 3). By trypan blue exclusion, cells in the BAL were \( \geq 98\% \) viable. Eosinophils were absent in BAL fluid collected from the saline-challenged group. After OVA challenge, the number of eosinophils in the BAL fluid increased to \( 464 \pm 91 \times 10^3 \) cells \( (p < 0.01) \). Treatment of animals with 3–10 mg/kg TAT-dnRas blocked the migration of eosinophils 24 h after OVA challenge in concentration-dependent manner. The eosinophil number decreased from \( 464 \pm 91 \times 10^3 \) to 288 \( \pm 79 \times 10^3/\text{ml} \) with 3 mg/kg of TAT-dnRas \( (p < 0.05) \), and further decreased to 116 \( \pm 63 \times 10^3/\text{ml} \) after 10 mg/kg TAT-dnRas \( (p < 0.01) \). By contrast, neither dnRas protein lacking the TAT protein-transduction domain (Fig. 3A) nor TAT-GFP lacking dnRas (Fig. 3C) blocked OVA-induced eosinophilia in BAL fluid.

Lymphocytes in BAL increased from \( 0.19 \pm 0.11 \times 10^3/\text{ml} \) for saline-challenged control to \( 47.6 \pm 9.9 \times 10^3/\text{ml} \) 24 h after OVA challenge \( (p < 0.01) \). TAT-dnRas also blocked the increase in lymphocytes in the airway lumen after OVA challenge in concentration-dependent manner. The number of lymphocytes decreased from \( 18.3 \pm 6.5 \times 10^3 \) at 3 mg/kg \( (p < 0.01) \) and \( 10.3 \pm 5.1 \times 10^3/\text{ml} \) at 10 mg/kg TAT-dnRas \( (p < 0.01 \text{ vs saline-challenged control, above}) \). Neither dnRas protein lacking the TAT PTD (Fig. 3B) nor TAT-GFP lacking dnRas (Fig. 3C) block the OVA-induced increase in lymphocyte number in BAL fluid.

OVA challenge also induced inflammatory cell infiltration (largely eosinophils and mononuclear cells) around the airways and pulmonary blood vessels and in the interstitium (Fig. 4, A and B). Pretreatment with 3–10 mg/kg TAT-dnRas blocked cell infiltration progressively at 24 h (Fig. 4, A3 and B, left panel). Although many mucus-containing epithelial cells were apparent in OVA-challenged mice (Fig. 4A5), very few of these cells were detected in mice treated with 10 mg/kg TAT-dnRas (Fig. 4, A6 and B, right panel).

*Ag-induced cytokine production*

TAT-dnRas also blocked production of Th2 cytokines in OVA-challenged mice. The OVA-challenge alone caused a significant

FIGURE 2. Kinetics of TAT-dnRas transduction into whole blood leukocytes in mice measured by flow cytometry. Whole blood leukocytes were isolated at 0, 2, 6, 12, and 24 h after i.p. injection of FITC-labeled TAT-dnRas (A) or control dnRas protein (B), which lacks the HIV-TAT domain, to determine time course and specificity of transmembrane uptake of TAT-dnRas. Nonspecific cell surface fluorescence was quenched by resuspension in pH 6.8 HBSS.

FIGURE 3. Effect of TAT-dnRas on airway inflammation after OVA sensitization and challenge. Mice were given i.p. TAT-dnRas or control injection every 12 h during Ag challenge. A. Analysis of inflammatory cells from BAL fluid 24 h after last OVA challenge. Positive (●) and negative (□) controls are OVA- and saline-challenged animals, respectively. These animals received no other treatment. B. Increase in lymphocytes is recorded on a different scale (see ordinate). C. Effect of TAT-GFP on airway inflammation after OVA challenge. Each bar represents the mean ± SEM of four to six mice. *, \( p < 0.05 \) and **, \( p < 0.01 \) compared with positive control group. N.D., not detectable. Ma, macrophage; Ly, lymphocyte; Eo, eosinophil; Ne, neutrophil.
increase in the concentration of IL-4 and IL-5, which was concentration dependently inhibited by TAT-dnRas (Fig. 5). There was no inhibition of production of these Th2 cell cytokines in control tissues receiving dnRas lacking the TAT domain. OVA challenge also increased concentration of INF-γ, a Th-1 cytokine, in lung tissue. TAT-dnRas at 10 mg/kg had no inhibitory effect on INF-γ production.

**Ag-induced airway hyperresponsiveness**

TAT-dnRas attenuated Ag-induced airway hyperresponsiveness to methacholine (Fig. 6). At 24 h, OVA challenge caused a substantial increase of the response to methacholine in Ag-sensitized mice. This response was blocked in dose-dependent manner by TAT-dnRas, but not by dnRas lacking the TAT-domain. We also established that TAT-GFP lacking dnRas had no effect on the airway response to methacholine. In both nonsensitized and OVA-sensitized mice, methacholine challenge curves were comparable for animals receiving either i.p. TAT-GFP or buffer (PBS).

**IL-5-induced airway inflammation**

Further studies were performed to determine the role of Ras in IL-5-induced eosinophil migration into airways. Eosinophils were not detected in BAL fluid in nonsensitized animals; 12 h after intranasal instillation of IL-5, the eosinophil count in BAL fluid was 4.8 ± 0.6 × 10³/ml (Fig. 7, p < 0.01). Pretreatment of animals with TAT-dnRas concentration dependently blocked BAL eosinophilia to 3.0 ± 0.7 × 10³/ml at 3 mg/kg TAT dnRas (p < 0.05) and 1.9 ± 0.6 × 10³/ml at 10 mg/kg TAT-dnRas (p < 0.01).

**Effect of intranasal delivery of TAT-dnRas on airway inflammation**

In further studies, we examined the effect of intranasal delivery of TAT-dnRas on airway inflammation. TAT-dnRas blocked OVA-induced eosinophil and lymphocyte accumulation in the airway (Fig. 8). Pretreatment of animals with TAT-dnRas blocked BAL eosinophilia caused by OVA challenge from 503.0 ± 34.9 to 229.9 ± 47.0 × 10³/ml at 50 µg TAT-dnRas (p < 0.01). The number of lymphocytes in BAL decreased after pretreatment from 55.7 ± 16.8 to 25.2 ± 4.4 × 10³ with 15 µg (p < 0.05) and to 13.4 ± 3.8 × 10³/ml with 50 µg/kg TAT-dnRas (p < 0.01 vs positive control). By contrast, 50 µg/kg TAT-GFP had no effect on the increase of eosinophils and lymphocytes in BAL fluids (Fig. 8).
mice.

neutrophil.

not detectable. Ma, macrophage; Ly, lymphocyte; Eo, eosinophil; Ne, neutrophil.

FIGURE 7. Effects of TAT-dnRas on eosinophil accumulation in BAL fluid 12 h after intranasal administration of IL-5. Cell counts were obtained from animals receiving IL-5 plus either control dnRas (lacking TAT domain) or TAT-dnRas. Each bar represents the mean \pm SEM of four to six mice. *, p < 0.05 and **, p < 0.01 compared with positive control group. N.D., not detectable.

Discussion

Our results show that TAT-dnRas dose dependently attenuated Ag-induced airway infiltration of eosinophils and lymphocytes, mucus secretion, Th2 cytokine production, and airway hyperresponsiveness to methacholine. These findings suggest that blockade of Ras-mediated events, which causes ERK phosphorylation and up-regulates integrin adhesion (23), also attenuates Ag-induced airway inflammation and hyperresponsiveness in mice. TAT-dnRas administration blocked OVA-induced airway infiltration of eosinophils and lymphocytes (Figs. 3 and 4). The mechanism of these effects is unknown. Decreased eosinophil infiltration may result from one or more of the following: 1) decreased eosinophil adhesion to vascular endothelial cells (23), 2) decreased Th2 cytokine production, or 3) decreased eosinophil survival (24). We have recently demonstrated that the cytosolic group IV-PLA2 and its upstream activators, ERK and Ras, regulate \( \beta_2 \)-integrin-dependent adhesion of eosinophils in vitro (22, 23). Ras activation of ERK signaling causes up-regulation of Mac-1 (CD11b/CD18) by a known precursor for ERK1/2 phosphorylation and subsequent cytosolic transduction of protein directly into the inflammatory cells of living animals. This report is the first to use this approach in the examination of potential signaling pathways in the ontogeny of airway remodeling and hyperresponsiveness. In this study, TAT-dnRas had a profound inhibitory effect on Ag-induced airway hyperresponsiveness that could not be attributed to either the TAT-protein alone or dnRas, which did not cross the outer membrane of inflammatory leukocytes. Likewise, leukocytes remained fully viable after TAT-protein transduction, and there was a clear dose-response relationship in the reduction of airway hyperresponsiveness, cellular infiltration after Ag challenge, and cytokine production. Whereas prior investigations have demonstrated that pharmacological inhibitors of ERK phosphorylation (33) or phosphoinositide 3-kinase (34) attenuate Ag-induced airway reactions, the introduction of a highly specific dn protein inhibitor, i.e., dnRas, establishes specifically the role of this protein in the induction of airway inflammation and hyperresponsiveness. Ras is a known precursor for ERK1/2 phosphorylation and subsequent cytosolic group IV-PLA2, phosphorylation in human eosinophils in vitro. Pharmacological inhibition of group IV-PLA2 in guinea pigs have been shown to prevent airway hyperresponsiveness and eosinophil infiltration (27). Our data comport with prior pharmacological suggestions that a Ras-mediated pathway may be critical in development of immune hyperresponsiveness in mice. They also may suggest potential targets for future therapeutic approaches for treatment of human asthma.

It is important to recognize specific limitations of our findings. Our data were obtained in mice with experimentally induced Ag-mediated airway hyperresponsiveness. Although the histological changes in these airways resemble those of human asthma, these data cannot be extrapolated to the human state. Nonetheless, these data suggest both a possible pathogenetic mechanism and potential for therapeutic intervention in human asthmatic states. For example, we have shown previously that TAT-dnRas blocks integrin-mediated adhesion of human eosinophils to ICAM-1 by a mechanism that prevents the focal clustering and active conformation of CD11b/CD18. We conclude that Ras-initiated signaling mediates affinity and avidity caused by IL-5, fMLP, or eotaxin (23). Ras also has been shown to be involved in Th2 cell differentiation and Th2 cytokine secretion (19, 21). Yamashita et al. (19) demonstrated that Ras-induced mitogen-activated protein kinase activation is required for Th2 cell differentiation, and controls IL-4R-mediated signaling.

In this study, lung content of Th2 cytokines such as IL-4 and IL-5 increased after OVA challenge. This increase in Th2 cytokine was attenuated by TAT-dnRas. By contrast, TAT-dnRas had no effect on the Th1 cytokine, IFN- \( \gamma \), in OVA-challenged mice (Fig. 5). These findings suggest that Ras may be involved in Ag-induced activation of Th2 lymphocytes, but not Th1 cells. However, IL-5-induced eosinophil migration was also blocked by TAT-dnRas (Fig. 7), suggesting that Ras not only is involved in Th2 cytokine production, but also in eosinophil migration induced by Th2 cytokines. Furthermore, Ras-induced ERK activation also is involved in IL-5-induced eosinophil survival (24). The relative contribution of each to the blockade of airway eosinophilic inflammation cannot be determined from this study.

Our data demonstrate that blockade of Ras protein by i.p. administration of TAT-dnRas also blocks nonspecific airway hyperresponsiveness to methacholine, which was not blocked by TAT-protein vehicle lacking dnRas or dnRas lacking TAT PTD. These data suggest that Ras regulates both the inflammatory component and subsequent airway hyperresponsiveness caused by Ag sensitization.

The use of TAT-dnRas affords a unique opportunity for intracellular transduction of protein directly into the inflammatory cells of living animals. This report is the first to use this approach in the examination of potential signaling pathways in the ontogeny of airway remodeling and hyperresponsiveness. In this study, TAT-dnRas had a profound inhibitory effect on Ag-induced airway hyperresponsiveness that could not be attributed to either the TAT-protein alone or dnRas, which did not cross the outer membrane of inflammatory leukocytes. Likewise, leukocytes remained fully viable after TAT-protein transduction, and there was a clear dose-response relationship in the reduction of airway hyperresponsiveness, cellular infiltration after Ag challenge, and cytokine production. Whereas prior investigations have demonstrated that pharmacological inhibitors of ERK phosphorylation (33) or phosphoinositide 3-kinase (34) attenuate Ag-induced airway reactions, the introduction of a highly specific dn protein inhibitor, i.e., dnRas, establishes specifically the role of this protein in the induction of airway inflammation and hyperresponsiveness. Ras is a known precursor for ERK1/2 phosphorylation and subsequent cytosolic group IV-PLA2, phosphorylation in human eosinophils in vitro. Pharmacological inhibition of group IV-PLA2 in guinea pigs have been shown to prevent airway hyperresponsiveness and eosinophil infiltration (27). Our data comport with prior pharmacological suggestions that a Ras-mediated pathway may be critical in development of immune hyperresponsiveness in mice. They also may suggest potential targets for future therapeutic approaches for treatment of human asthma.
Th2 cytokine production, airway inflammation, and airway hyper-responsiveness in sensitized, Ag-challenged mice. The specific effect on chemotaxis may relate to impaired integrin adhesion, but the precise pathways and mediators involved in this process remain to be defined.

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References