Glycobiology and Extracellular Matrices:

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Inter-α-trypsin Inhibitor Promotes Bronchial Epithelial Repair after Injury through Vitronectin Binding*

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Pulmonary epithelial injury is central to the pathogenesis of many lung diseases, such as asthma, pulmonary fibrosis, and the acute respiratory distress syndrome. Regulated epithelial repair is crucial for lung homeostasis and prevents scar formation and inflammation that accompany dysregulated healing. The extracellular matrix (ECM) plays an important role in epithelial repair after injury. Vitronectin is a major ECM component that promotes epithelial repair. However, the factors that modify cell-vitronectin interactions after injury and help promote epithelial repair are not well studied. Inter-α-trypsin inhibitor (IaI) is an abundant serum protein. IaI heavy chains contain von Willebrand A domains that can bind the arginine-glycine-aspartate domain of vitronectin. We therefore hypothesized that IaI can bind vitronectin and promote vitronectin-induced epithelial repair after injury. We show that IaI binds vitronectin at the arginine-glycine-aspartate site, thereby promoting epithelial adhesion and migration *in vitro*. Furthermore, we show that IaI-deficient mice have a dysregulated response to epithelial injury *in vivo*, consisting of decreased proliferation and epithelial metaplasia. We conclude that IaI interacts not only with hyaluronan, as previously reported, but also other ECM components like vitronectin and is an important regulator of cellular repair after injury.

Epithelial injury is a crucial component in the pathogenesis of many lung diseases. Bronchial epithelial injury occurs chronically in asthmatic patients (1, 2). Furthermore, alveolar and bronchiolar epithelial injury are early triggers in idiopathic pulmonary fibrosis (3) and in lung transplant rejection (4), respectively. In acute respiratory distress syndrome, diffuse alveolar epithelial injury initiates the inflammatory and fibrotic response that leads to lung dysfunction (5). It is now believed that a dysregulated response to epithelial injury ultimately causes fibroproliferation, scar formation, and respiratory failure in acute as well as chronic lung injury (3). It is therefore important to understand the epithelial repair process after pulmonary epithelial injury, if we are to develop causal treatments for these diseases.

The mechanisms governing epithelial repair are incompletely understood. Epithelial repair encompasses cell proliferation, migration, and differentiation. All of these processes require cell interactions with the extracellular matrix (ECM).[2] ECM components like tenascin C, fibronectin, and vitronectin promote epithelial regeneration through integrin binding. Vitronectin (Vn) is a pluripotent 75-kDa plasma and ECM glycoprotein that regulates a number of biological processes such as coagulation, complement activation, and wound healing. Vn promotes cell adhesion and migration via binding primarily to integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$. After cell binding, Vn can protect bronchial epithelial cells from apoptosis (6) by inducing Akt phosphorylation and preventing caspase and Fas-associated with death domain (FADD) activation (7). Vn also binds to non-integrin cell receptors such as urokinase-type plasminogen activator receptor to promote changes in cell morphology, migration, and signal transduction (8). Consequently, Vn deficiency impairs bronchial (6) and alveolar (9) epithelial repair.

Cell-Vn interactions are modulated by other extracellular factors. For example, plasminogen activator inhibitor 1 (PAI-1) is bound to circulating Vn and forms multimers with Vn upon extravasation to the extracellular space (10), thus possibly activating Vn into an adhesive form (11). Furthermore, Vn possesses several domains that can function as possible ligands, such as a somatomedin B domain, an arginine-glycine-aspartate (RGD) domain, and a heparin-binding domain. However, the extent to which other serum or ECM factors may interact with Vn and influence cell-Vn interactions is unclear.

In this report, we investigated possible interactions between Vn and the serum and ECM protein inter-α-trypsin inhibitor. Inter-α-trypsin inhibitor (IaI) is a complex protein found in relatively high concentrations in mammalian plasma. It is made up of a light chain (called bikunin for its two Kunitz domains), which confers the protease inhibitory activity, as well as two heavy chains (12). The precise functions of the heavy chains are unknown. Heavy chains contain a von Willebrand Type A (vWA) domain, and they have been shown to bind to hyaluronan and thereby stabilize the extracellular matrix. However, vWA domains are fairly promiscuous and can bind to a large array of proteins, including RGD domains. Furthermore, IaI is expressed by epithelial cells under stress conditions and is incorporated into *de novo* ECM structures produced by stressed epithelia (13). We therefore hypothesized that IaI may

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[2] The abbreviations used are: ECM, extracellular matrix; IaI, inter-α-trypsin inhibitor; vWA, von Willebrand A domain; Vn, vitronectin; RGD, arginine-glycine-aspartate; PAI-1, plasminogen activator inhibitor 1; BrdUrd, bromodeoxyuridine; UTI, urinary trypsin inhibitor; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay.

interact with Vn and thus promote epithelial survival after injury.

EXPERIMENTAL PROCEDURES

Materials—IaI proteins were derived from human plasma samples and contained both major forms of IaI proteins (IaI and pre-α inhibitors) at >95% purity (ProThera Biologics, East Providence, RI). IaI was first isolated from cryoprecipitated clotting factor VIII/von Willebrand factor concentrate (Octapharma Pharmaceuticals, Vienna, Austria). IaI proteins were then purified by ion exchange high performance liquid chromatography followed by size exclusion high performance liquid chromatography (Biosec; Merck). Monoclonal function blocking integrin antibodies (anti-α_v clone P3G8, anti-α_2 clone P1E6, anti-β_1 clone 6S6, anti-β_3 clone B3A, anti-$\alpha_v\beta_5$ clone P1F6, and anti-$\alpha_v\beta_6$ clone 10D5) and monoclonal actin antibody (clone C4) were from Chemicon (Billerica, MA). Monoclonal Vn antibody (clone VN58-1) and polyclonal sheep Vn antibody were from Abcam (Cambridge, UK). Rabbit anti-human IaI immunoglobulin was from Dako (Glostrup, Denmark), and horseradish peroxidase goat anti-rabbit immunoglobulin was from Jackson Immunoresearch Laboratories (West Grove, PA). Secondary antibodies Alexa 488 anti-sheep and Alexa 594 anti-rabbit were from Molecular Probes (Carlsbad, CA). Human recombinant Vn was from Chemicon and American Diagnostica (Stamford, CT). Naphthalene, corn oil, BrdUrd, RGDS, GRGDS, and SDGRG, sulfo-sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate, heparin sodium, and polylysine were from Sigma. BEAS-2B cells and A549 cells were purchased from ATCC and grown in bronchial epithelial basal medium/bronchial epithelial growth medium from Lonza/Clonetics (Walkersville, MD) or RPMI, respectively. Urinary trypsin inhibitor (UTI) was purchased, and RGDSC and RGESC peptides were custom made by GenScript (Scotch Plains, NJ). PAI-1 and PAI-1 Q123 (Vn-) were from Molecular Innovations (Detroit, MI). Rat anti-BrdUrd was from Accurate Chemical (Westbury, NY).

Isothermal Titration Calorimetry—Isothermal titration calorimetry measurements were carried out in phosphate-buffered saline-calcium-magnesium free without KCl buffer using an ITC_{200} MicroCalorimeter (Micro Cal, Inc.) at 20 °C. A 50 μM vitronectin substrate solution was injected into a reaction cell containing 5 μM of IaI. Fifteen injections of 0.4 μl at 180-s intervals were performed. Data acquisition and analysis were performed by the MicroCal Origin software package. Data analysis was performed by generating a binding isotherm and best fit using the following fitting parameters: N (number of sites), ΔH (cal/mol), ΔS (cal/mol/deg), and K (binding constant in M^{-1}) and the standard Levenberg-Marquardt methods (14). Following data analysis, K (M^{-1}) was then converted to K_d (μM).

ELISA-IaI-vitronectin Interaction—Maxisorb plates (Nunc, Rochester, NY) were coated with purified human Vn at indicated concentrations. Purified human IaI was added to the wells at indicated concentrations. Detection was performed with rabbit anti-human IaI immunoglobulin (1:2000) and 50 μl of horseradish peroxidase goat anti-rabbit immunoglobulin (1:3000). Color development was performed with TMB solution, and the absorbance at 450 nm was measured on a VersaMAX spectrophotometer (Molecular Devices, Sunnyvale, CA).

RGD Binding and IaI Antagonism—In the first series of experiments, the plates were coated with human recombinant Vn, blocked, and then incubated with UTI at 250 μg/ml or with IaI at 20 μg/ml. Some wells received additional RGDS or GRGDS or SDGRG (1 mg/ml) or PAI-1 (5 μg/ml) or heparin (3000 u/ml). Detection was as above, and the absorbance at 450 nm was measured with a PowerWave XS Spectrophotometer. In another experiment, the plates were coated with human recombinant Vn, blocked, and then incubated with IaI at 20 μg/ml and increasing concentrations of bovine serum albumin, GRGDS, or SDGRG. Detection and absorbance measurements were as above.

A549 in Vitro Wound Model—A549 cells were plated in a 96-well plate (10^5/well) in Dulbecco's modified Eagle's medium, 10% fetal bovine serum supplemented with antioxidants, L-glutamine, and sodium pyruvate. The cells were grown to confluence and then serum-starved for 24 h. The cell layer was then scraped with the tip of a 1-ml pipette, the wells were washed to remove debris, and experimental medium was added. Control wells received Dulbecco's modified Eagle's medium, 0.1% fetal bovine serum, and experimental wells received control medium and IaI at 50 μg/ml, or control medium, IaI and PAI-1 (1 μg/ml), or control medium, IaI and PAI-1 Q123 at 1 μg/ml, or control medium and PAI-1. Photos were taken immediately after injury and at 24 and 48 h, the denuded area was measured using ImagePro software (Media Cybernetics, Bethesda, MD), and the amount of wound closure was expressed as percentage of the initial area. In subsequent experiments the cells were grown after injury in control medium, control medium + IaI (25 μg/ml), control medium + UTI (5 μg/ml), or control medium + IaI (25 μg/ml) with anti-integrin (β_1, or β_3, α_v, or isotype control) at 20 μg/ml concentration.

Cell Adhesion Assay—BEAS-2B cells were cultured as previously described (15). Briefly, the cells were grown in bronchial epithelial basal medium with bronchial epithelial growth medium SingleQuots® at 37 °C. Tissue culture dishes (96-well plates) were coated with human recombinant Vn at 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml, and 1 μg/ml Vn, and control wells were coated with 32 μg/ml polylysine. Nonspecific binding sites were blocked with 2% bovine serum albumin solution. BEAS-2B cells were suspended in medium with IaI (50 or 200 μg/ml) or phosphate-buffered saline vehicle at 3.0×10^5 cells/ml and immediately transferred into precoated wells. the plates were incubated at 37 °C for 45 min. Following incubation, nonadherent cells were removed by aspiration and washing, adherent cells were fixed in 6% glutaraldehyde and stained with 0.5% crystal violet in 20% methanol, excess dye was removed, and intracellular stain was solubilized by the addition of 1% SDS. Absorbances at 595 nm were determined using a SpectraMax M2 microplate reader. The results were standardized as a percentage of adhesion to polylysine after background adhesion to uncoated wells was subtracted.

Naphthalene Model of Bronchial Epithelial Injury—IaI-deficient mice were generated by knocking out the light chain bikunin, which is needed for the assembly of IaI, and have been

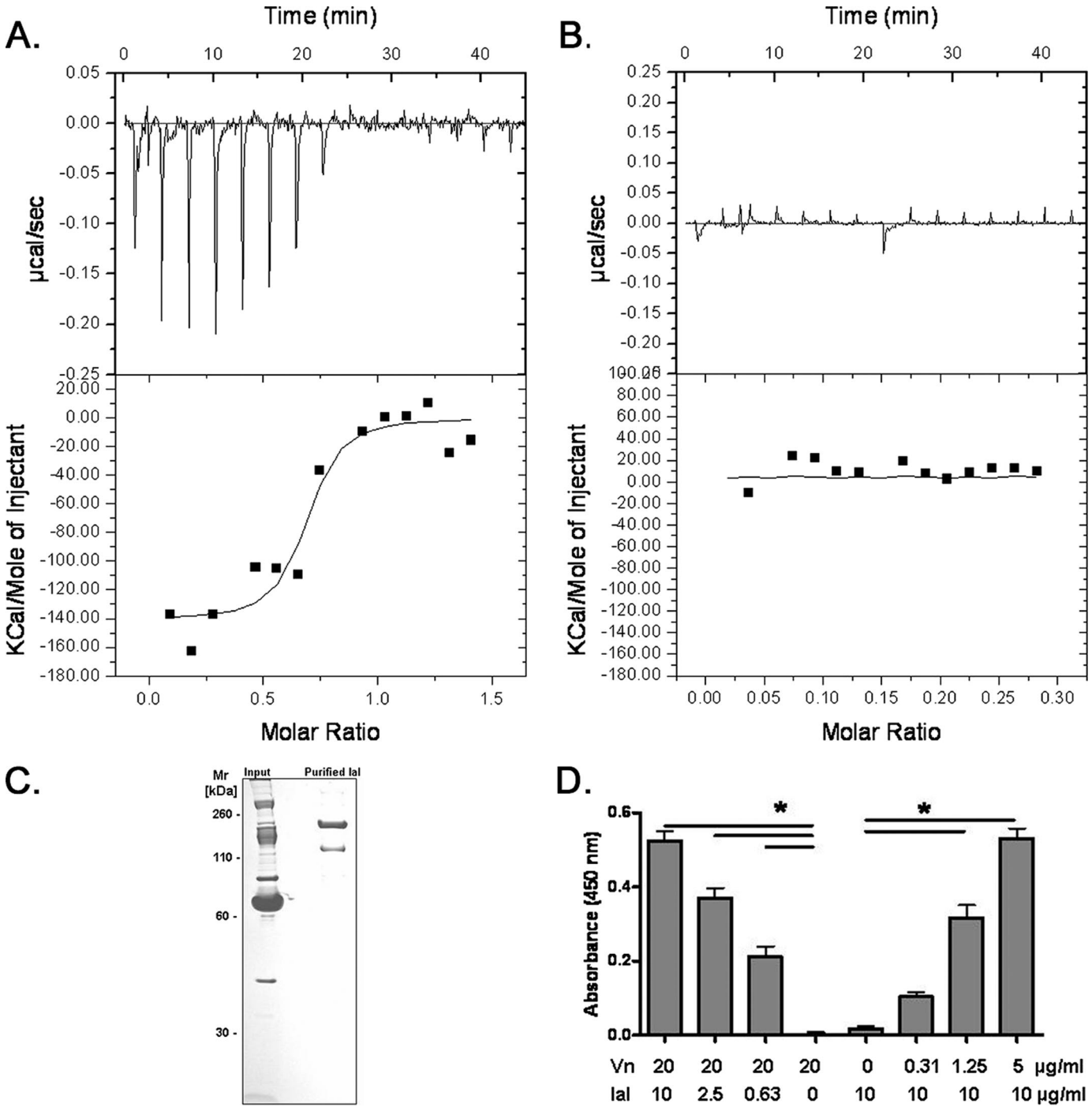

FIGURE 1. *A*, calorimetric profile of Vn binding to IaI. The reaction cell contained a solution of IaI (50 μM) in phosphate-buffered saline. The syringe contained 5 μM Vn in the same buffer. *Top panel*, raw calorimetric data obtained from the injection of 3 μl aliquots of Vn at 3-min intervals. The *lower panel* shows the integrated binding isotherm with the experimental points (■) and best fit. *B*, calorimetric profile of Vn binding to buffer (negative control). *C*, gel showing the purity of IaI used in ITC experiments and enzyme assays. The *first lane* contains the serum input, and the *second lane* contains purified IaI. There are visible bands at ~220 kDa (IaI) and 120 kDa (pre-α inhibitor). 4–12% SDS gel with Coomassie staining. *D*, binding ELISA of various concentrations of IaI to various concentrations of immobilized Vn. *, $p < 0.001$ (ANOVA with Bonferroni post-hoc test).

backcrossed onto C57BL/6 for >10 generations (16). Fully wild-type and heterozygote littermates (which have normal serum IaI levels at base line) were used as controls. The mice received a one-time dose of naphthalene intraperitoneally (275 mg/kg of body weight, dissolved in sterile corn oil) or control vehicle only and were sacrificed 3 and 7 days later, at which point there is normally epithelial recovery from injury. Mice received 50 mg/kg BrdUrd intraperitoneally 2 h before the 3-day sacrifice to evaluate cell proliferation histologically. Naïve mice also received BrdUrd and were sacrificed 2 h later for cell proliferation analysis. The right lungs were snap frozen and stored at −80 °C until used for mRNA analysis, and the left lungs were inflated with 10% formalin, paraffin-fixed, and used for histological staining.

Histology—Formalin-fixed mouse lungs were sectioned at 5 μm and stained with hematoxylin and eosin or with rat anti-BrdUrd at 1:800 after antigen retrieval with 0.01% trypsin. For quantification of Pathology Index, we counted abnormal-looking cells (vacuolated, mitotic, apoptotic, and metaplastic) and normalized for length of underlying basal membrane (arbitrary units). For quantification of BrdUrd staining, we counted BrdUrd-positive cells and normalized for total bronchial nuclei count. The measurements were undertaken for at least 20 airways/mouse and at least four mice/strain. For confocal immunohistochemistry, the lungs were stained with rabbit anti-IaI and sheep anti-Vn antibodies and appropriate secondary antibodies and visualized with a Zeiss LSM 510 NLO microscope (Carl Zeiss, Thornwood, NY).

Real Time Reverse Transcription PCR—mRNA was isolated from frozen lungs and livers with TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real time reverse transcription-PCR was performed for the IaI heavy chain 1 (forward, GGTCTTTGGCTCTAAAGTGCAATC; and reverse, GGTGGCTTCCTTGAGCTTTGT) and heavy chain 2 (forward, GCCATCCACATCTTCAATGAGAG; and reverse, CGCTTGAGAAAGCTGTAGAGCTG) using the SYBR-Green assay and the 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The housekeeping protein 18s transcript was used as the reference mRNA.

Statistical Methods and Analysis—ANOVA with Bonferroni and Tukey's honestly significant difference post-tests, Student's *t* test, and the Hill transformation for adhesion curves were performed using SPSS (Chicago, IL) and GraphPad (San Diego, CA) software. A p value <0.05 was considered statistically significant.

RESULTS

IaI Binds Vitronectin at the RGD Site—We first investigated whether IaI binds Vn *in vitro* by isothermal titration calorimetry. The calorimetric profile showed that Vn bound to IaI at a molecular ratio of 1:1 (0.854 ± 0.033) with a K_d of 85.47 ± 9.43 picomolar, $\Delta H = -113.2 \pm 6.283$ kcal/mol, $\Delta S = -0.340$ kcal/mol/deg, and $\Delta G = -13.58 \pm 6.283$ kcal/mol. The measured K_d is comparable with published K_d values of Vn interactions with other proteins (17, 18). Control calorimetry using vitronectin and phosphate-buffered saline was negative (Fig. 1, *A* and *B*). We then performed a binding ELISA using immobilized Vn, detected IaI and found a concentration-dependent increase in absorbance at 450 nm (Fig. 1*D*), suggesting that IaI can bind immobilized Vn. Vn has several domains to which IaI could potentially attach: the vWA domain of IaI can bind the RGD site at Vn (19), the chondroitin sulfate chain could bind one of several heparin-binding domains (20), and finally Vn has at least two PAI-1-binding sites with unclear attributes. We therefore performed competitive ELISAs using RGD peptides, heparin, or PAI-1 as antagonists (Fig. 2*A*). We found that only the RGD peptides, but not a scrambled control peptide, competed with IaI for binding. To further confirm our findings, we performed a competition ELISA using GRGDS or the control SDGRG (Fig. 2*B*). Again we found that the RGD peptide, but not the scrambled peptide, inhibited IaI-Vn binding in a dose-dependent manner. Collectively these results suggest that IaI binds Vn at its RGD site.

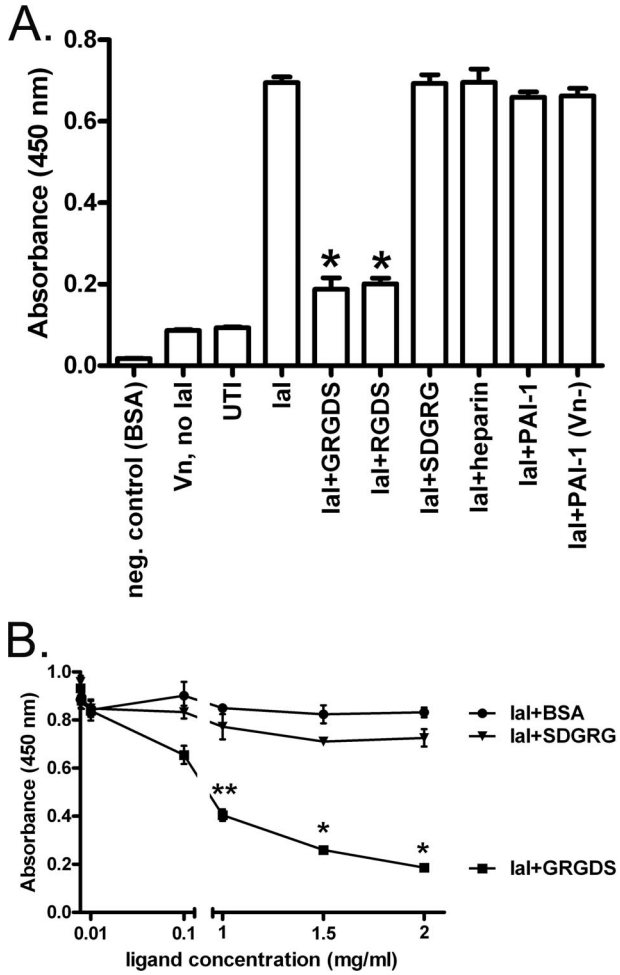

FIGURE 2. *A*, inhibition of IaI-Vn binding through RGD peptides, but not PAI-1 or heparin. *, $p < 0.001$ (ANOVA with Bonferroni post-hoc test). *B*, inhibition of IaI-Vn binding through GRGDS peptide, but not control SDGRG peptide. **, $p < 0.01$; *, $p < 0.001$ compared with GRGDS or bovine serum albumin (ANOVA with Bonferroni post-hoc test).

IaI Promotes Cell Adhesion on Vitronectin Substratum—Cell adhesion to the ECM is important for the maintenance of differentiation and function of bronchial epithelia (6). We therefore examined the effect of IaI on cell adhesion on a Vn substratum. We used the BEAS-2B human bronchial epithelial cell line as representative of airway epithelia. We found that IaI enhances cell binding in a dose-dependent fashion, even within the physiological range of IaI concentrations, *i.e.* 50 μg/ml (Fig. 3*A*) and 250 μg/ml (Fig. 3*B*). Interestingly the most pronounced IaI effect appeared to occur in low and medium range Vn concentrations, whereas the increase of adhesion at high Vn concentrations was small and was only seen at the higher IaI concentration (Fig. 3*B*).

IaI Promotes Vitronectin-dependent Epithelial "Wound Healing" in Vitro—We used an *in vitro* cell wounding model to investigate the functional consequences of IaI-Vn binding on cell migration and proliferation. We injured epithelial cells *in vitro* by punching out a circular defect in a confluent culture layer of A549 cells and examined closure of the gap 24 and 48 h later. At these time points, wound closure is a product of cell

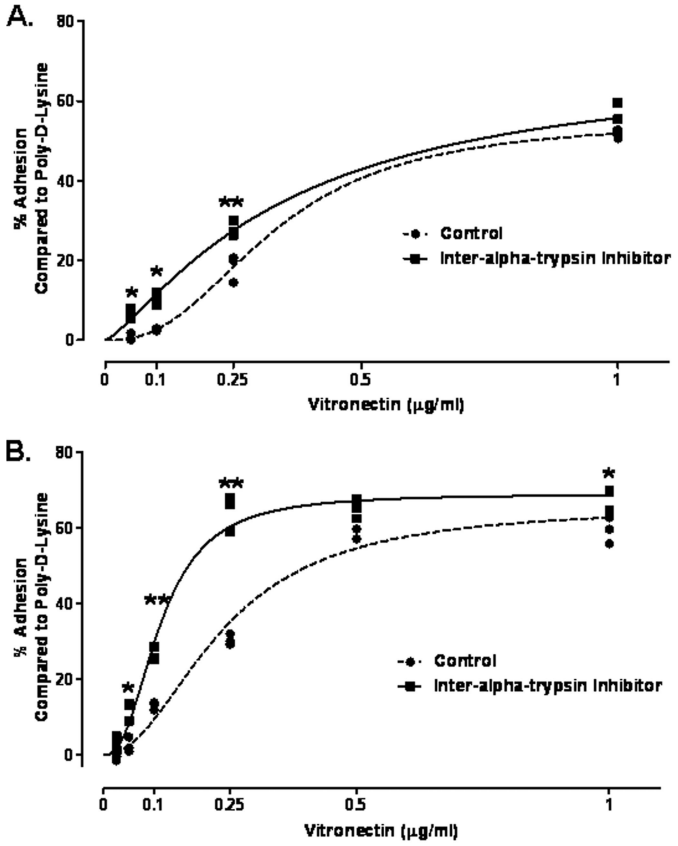

FIGURE 3. **IaI promotes BEAS-2B cell adhesion to Vn substratum.** *A*, 50 μg/ml IaI. *B*, 250 μg/ml IaI. *, $p < 0.001$ (ANOVA with Bonferroni post-hoc test.)

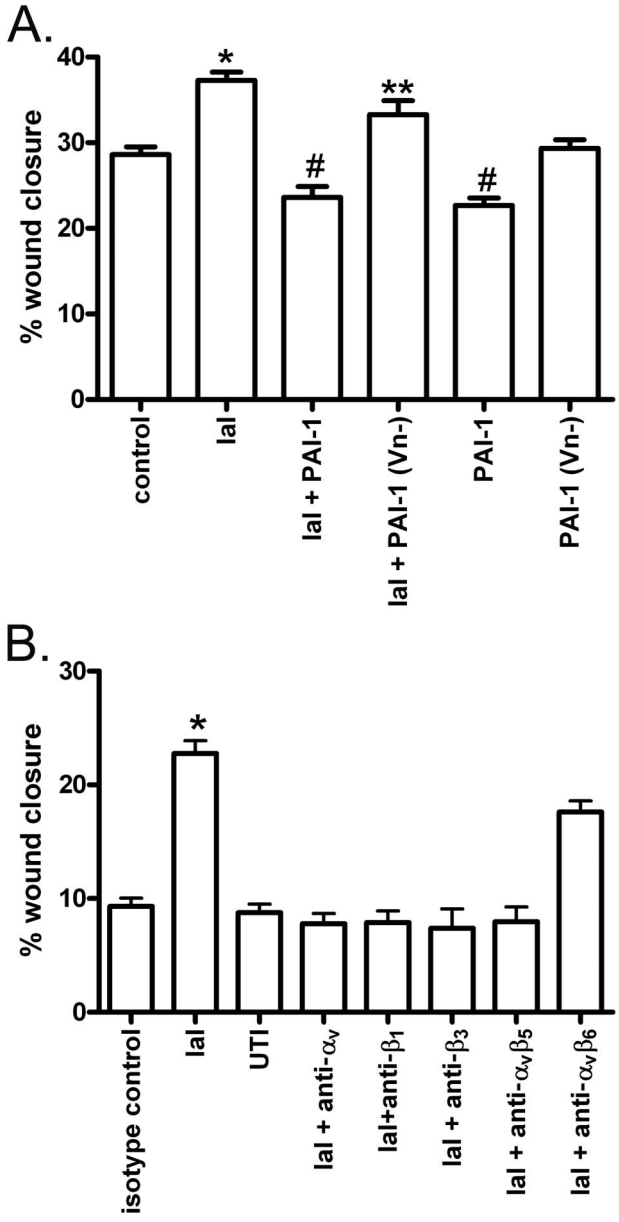

FIGURE 4. *A*, IaI significantly enhances *in vitro* wound closure, but its effect is inhibited by PAI-1 in a Vn-specific fashion. *, $p < 0.001$ compared with control and PAI-1. **, $p < 0.001$ compared with PAI-1, and $p < 0.05$ compared with control. #, $p < 0.001$ compared with control. (ANOVA with Tukey honestly significant difference). *B*, the IaI effect on wound closure can be blocked through antibodies that inhibit Vn-specific integrins. *, $p < 0.001$ compared with IaI+α_v, IaI+β_1, IaI+β_3, and IaI+α_v β_5, and $p < 0.05$ compared with IaI+$\alpha_v\beta_6$. (ANOVA with Bonferroni post-hoc test.)

proliferation and migration. We found that IaI significantly increased wound closure compared with control medium, which was supplemented with 0.1% fetal bovine serum (Fig. 4*A*). Because PAI-1 is known to inhibit Vn-dependent lung epithelial migration and adhesion through steric competition with integrin binding to the RGD site (9), we examined whether IaI-stimulated wound healing could be inhibited by PAI-1. Indeed, incubation of cells in media supplemented with both IaI and PAI-1 completely abolished IaI-dependent gains in wound closure and decreased closure to an even lower level than that observed in the control medium. However, when we supplemented the medium with IaI and a PAI-1 variant that is deficient in Vn binding, we detected no statistical difference in wound closure compared with IaI treatment alone. Collectively, these data suggest that the IaI effect in this *in vitro* model of wound closure is mediated through vitronectin.

Our previous results indicated that IaI binds Vn at the RGD site. Based on IaI biology, this binding can only be achieved by the vWA domain of the IaI heavy chains. We therefore tested the effect of UTI, which consists of the chondroitin sulfate chain and the light chain bikunin but lacks heavy chains, on wound closure. When used in equimolar concentration to IaI, UTI did not have any effect on wound closure compared with the control (Fig. 4*B*). Furthermore, the effect of IaI was abolished when we incubated the cells with blocking antibodies to the Vn-specific integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Fig. 4*B*). Interestingly, the blocking antibody to $\alpha_v\beta_6$ did not completely block the IaI effect, suggesting that either the antibody lacks efficiency or that $\alpha_v\beta_6$ does not play a major role in epithelial migration and recovery in this model. Incubation with blocking antibodies in the absence of IaI resulted in similar wound closure as the controls (not shown). In conclusion, our findings suggest that IaI improves wound closure in this *in vitro* model of epithelial injury and that this IaI effect is mediated by and predicated upon cell integrin-Vn binding.

IaI Promotes Epithelial Wound Healing in Vivo—IaI is found in relatively high concentrations in mammalian serum (12) and can therefore quickly reach sites of tissue injury and exert its effects there. We investigated the role of IaI deficiency in epithelial recovery after *in vivo* injury utilizing the naphthalene

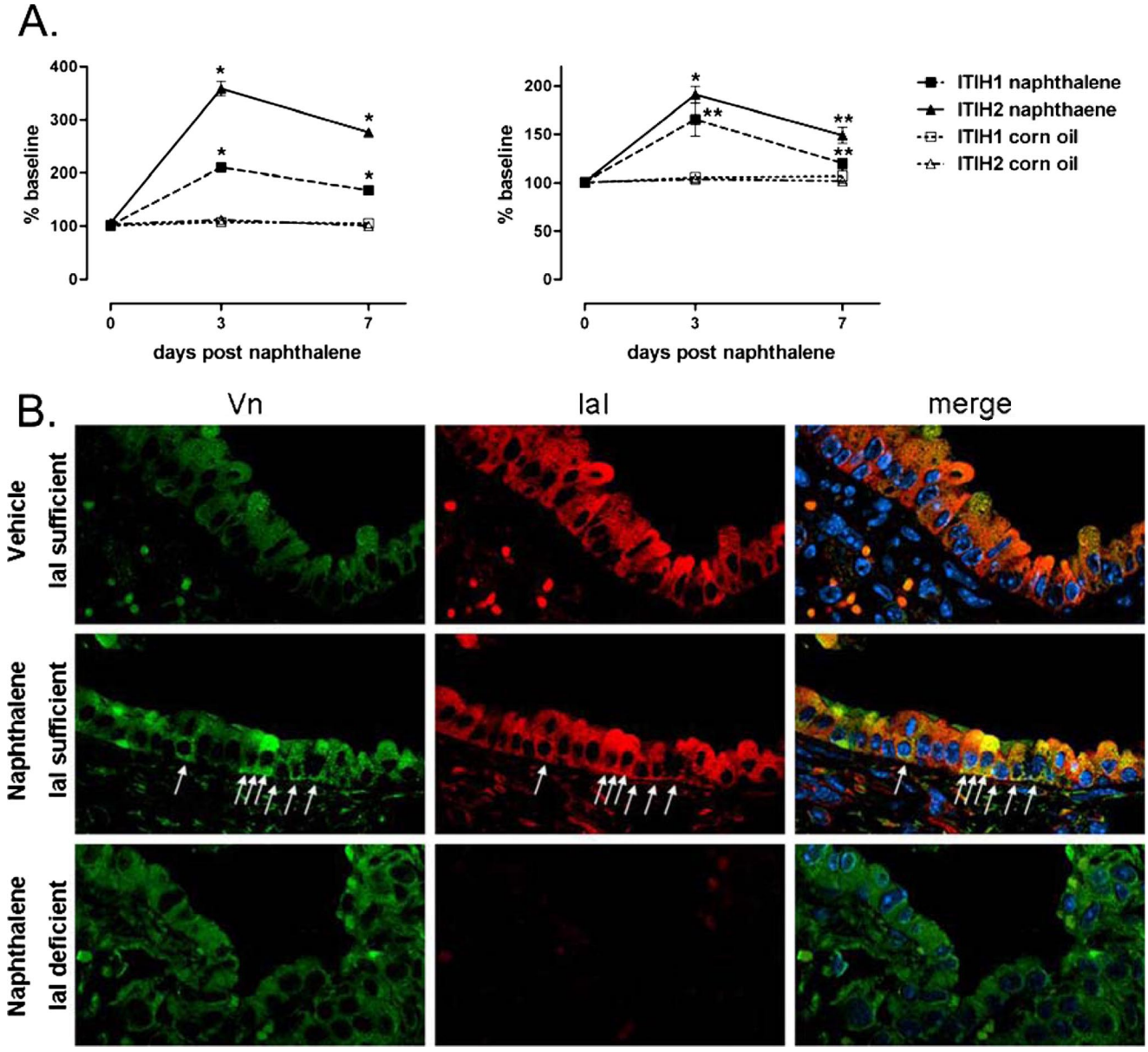

FIGURE 5. *A*, IaI heavy chain expression in liver (*left panel*) and lung (*right panel*) after naphthalene injury. Significant up-regulation of ITIH1 and ITIH2 is seen in naphthalene-treated mice but not in control-treated mice (*, $p < 0.001$; **, $p < 0.05$, Student's *t* test). *B*, co-localization of IaI and vitronectin in airway epithelium after naphthalene lung injury. Vitronectin is depicted in *green* in the *left-hand panels*, IaI is depicted in *red* in the *middle panels*, and co-localization appears in *yellow* in merged *right-hand panels*. *Top panels*, no significant co-localization is seen in airways of naïve mice. *Middle panels*, vitronectin and IaI co-localize in naphthalene-exposed wild-type mice (*arrows*). *Bottom panels*, IaI-deficient mice have no visible IaI expression in the lung, and no co-localization is detected.

model of lung injury. In this model, naphthalene generates isolated bronchial epithelial injury through specific cytotoxicity on bronchial Clara cells, which resolves within 7–21 days (21). First we investigated the expression pattern of IaI heavy chains after acute naphthalene injury. We found that IaI heavy chain 1 and 2 mRNA expression increased in the liver and somewhat less in the lung after naphthalene injury (Fig. 5*A*). We then investigated the localization pattern of Vn and IaI after acute naphthalene injury. We found that IaI and Vn are up-regulated and appear to co-localize particularly on the basal surface of bronchial epithelia after naphthalene injury (Fig. 5*B*). We then examined the effect of IaI on epithelial recovery by using bikunin-deficient mice, which lack the ability to process heavy chains and therefore are also deficient in circulating IaI. Wild-type and heterozygote (IaI-sufficient) littermates were used as the control group. We did not observe any difference between wild-type and heterozygote mice at base line or after naphthalene injury, and in the following we will report these mice as being "IaI-sufficient" to avoid confusion. We found that IaI promotes epithelial recovery in the naphthalene model of bronchial epithelial injury (Fig. 6). Seven days after naphthalene injection, both large (Fig. 6*C*) and small (Fig. 6*D*) airways of IaI-sufficient mice demonstrated an orderly epithelial cell pattern. In contrast, IaI-deficient mice showed dysregulated recovery, with a loss of columnar epithelium, vacuolated cells, and epithelial squamous metaplasia in large and small airways (Fig. 6, *E* and *F*). There were significantly more pathological-appearing cells in IaI-deficient mice after naphthalene injury (Fig. 6*G*).

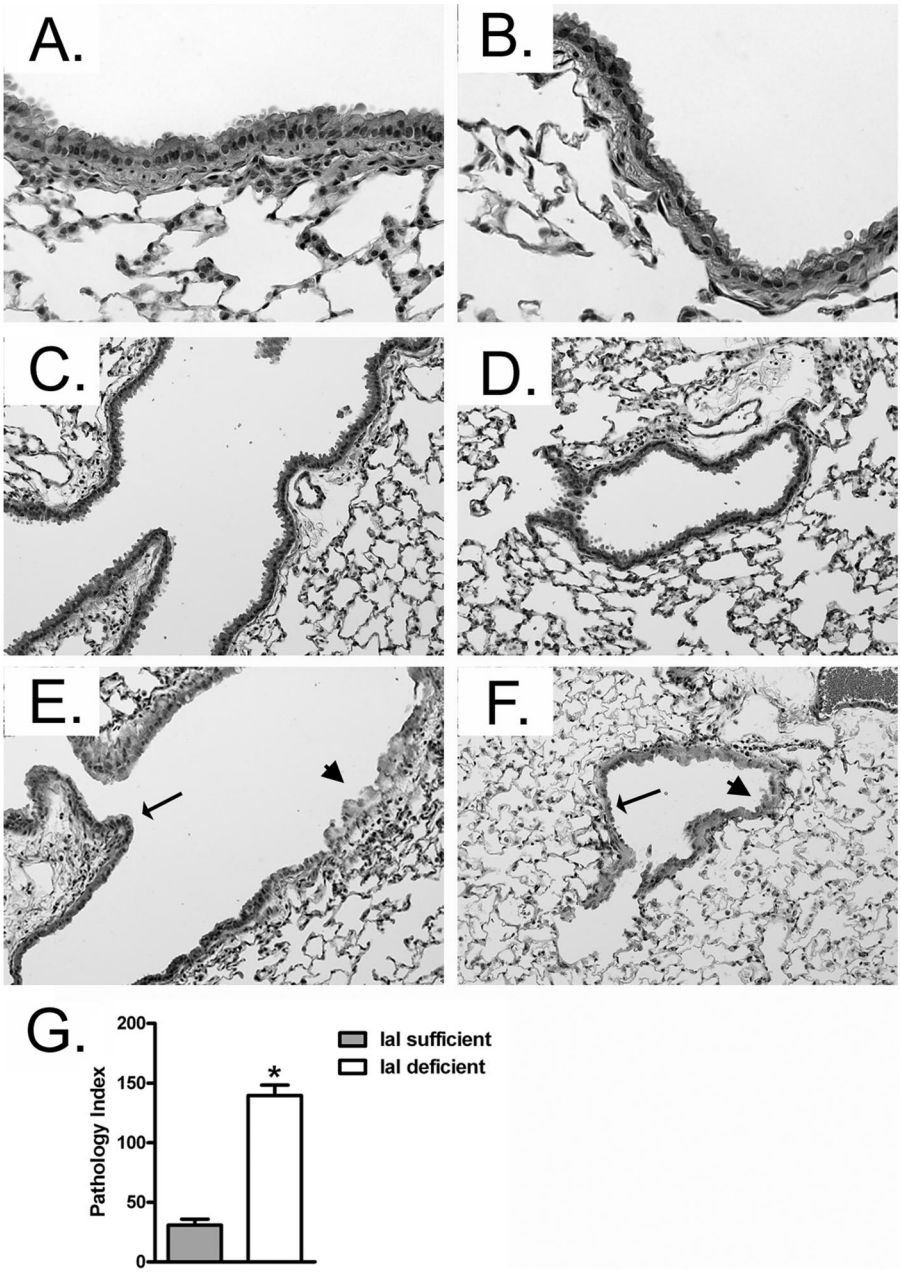

FIGURE 6. **IaI deficiency leads to abnormal epithelial recovery 7 days after naphthalene lung injury.** No difference in airway histology in naïve IaI-sufficient (*A*) and deficient (*B*) mice (HE staining, 400× magnification). Orderly epithelial cell pattern in large (*C*) and small (*D*) airways of IaI-sufficient mice. Loss of columnar epithelium (*arrowheads*) and abnormal nuclei (*arrows*) in large (*E*) and small (*F*) airways of IaI-deficient mice (HE staining, 200× magnification). *G*, IaI-deficient mice have significantly more abnormal cells per length of basal membrane than IaI-sufficient mice. *, $p < 0.0001$ compared with IaI-sufficient mice (Student's *t* test) $n = 4–6$ mice/group.

DISCUSSION

Inter-α-trypsin inhibitor is found in high concentrations in mammalian plasma; however, its exact function is unclear. Anti-inflammatory activity of the light chain and hyaluronan binding activity of the heavy chains has been described (12), and we have recently shown that IaI can inhibit complement activation (22) and promote hyaluronan-induced angiogenesis in lung injury (23). As a plasma component, IaI can reach all sites of tissue injury via extravasation, and because of its abundance, IaI can be a potent modifier of inflammatory and reparative processes. In this paper we provide evidence for a novel function of IaI, namely binding to the ECM component vitronectin and modifying Vn-dependent epithelial repair after injury. This report suggests a new spectrum of potential interactions for IaI in tissue inflammation and repair and suggests a global function of IaI as a "tissue healing" factor.

The effect of IaI-Vn interactions on cell behavior after injury seems to be multifaceted. We show *in vitro* that IaI promotes wound closure in this cell injury model at 24 and 48 h after injury. Closure of the cell layer defect at these time points depends mostly on cell migration and adhesion. Indeed we also show that IaI promotes cell adhesion on a Vn substratum. However, our *in vivo* data also suggest that IaI promotes cell proliferation, because IaI-deficient mice have significantly less BrdUrd incorporation after naphthalene injury than their IaI-sufficient littermates. It is known that cell adhesion to the ECM promotes proliferation and differentiation and that conversely cell detachment from the ECM substratum can cause cell death, termed anoikis (24). We therefore believe that IaI may affect cell proliferation and cell migration after injury, at least partly through its Vn binding properties.

Because Vn affects not only epithelial adhesion and migration but also epithelial proliferation, we examined whether IaI deficiency also had an effect on bronchial proliferative potential at 3 days after naphthalene injury. We found that IaI-sufficient mice had more BrdUrd incorporation in bronchial nuclei (Fig. 7*A*) than IaI-deficient mice (Fig. 7*B*) at 3 days after naphthalene treatment. This difference was statistically significant at 3 days after naphthalene treatment, whereas in untreated mice IaI status had no effect (Fig. 7*C*). Collectively, our findings indicate that IaI is important for epithelial recovery *in vivo*, and the IaI effect is at least partly mediated through a positive effect on cell proliferation.

Epithelial recovery after injury is an intricately regulated process. Both *in vivo* and *in vitro* studies have shown that a number of matrix metalloproteases (*e.g.* matrix metalloproteases 7 and 9), cytokines (*e.g.* interleukin-8), and growth factors (*e.g.* epidermal growth factor, insulin-like growth factor) are released after epithelial injury (25). These findings collec-

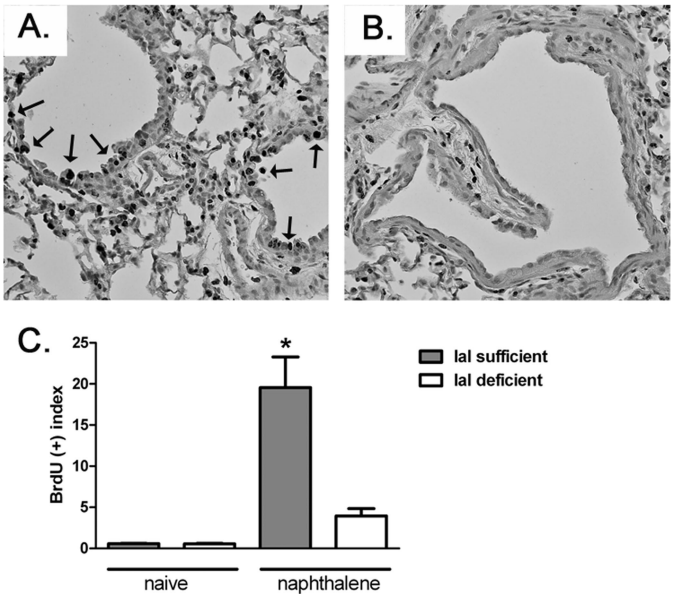

FIGURE 7. **IaI deficiency leads to decreased epithelial proliferation 3 days after naphthalene lung injury.** *A*, significant BrdUrd staining in epithelial layer of an IaI-sufficient mouse. *B*, absent BrdUrd staining in an airway of an IaI-deficient mouse. *C*, quantification of BrdUrd-positive cells in airways in naïve mice and 3 days after naphthalene lung injury. There are significantly more BrdUrd-incorporating cells in IaI-sufficient mice than IaI-deficient mice. *, $p < 0.01$ compared with IaI-deficient mice (Student's *t* test). $n = 5–7$ mice/group.

tively indicate a significant involvement of ECM in the repair process. However, the composition of ECM itself is altered in response to injury. Beyond the degradation caused by metalloprotease and other enzymatic activity, injury-related vascular leakage causes plasma factors such as Vn to extravasate to the ECM. Our findings complement this theoretical framework and suggest that IaI aids epithelial recovery after injury through Vn binding.

The stoichiometry of *in vivo* IaI-Vn interactions cannot be conclusively gleaned from the present experiments. We demonstrated that UTI does not bind Vn and does not affect wound closure after *in vitro* injury, suggesting that the heavy chains are necessary for the IaI-Vn interactions. IaI heavy chains are released into the extracellular space from the IaI molecule and locally bind hyaluronan (12). The heavy chain switches its binding from chondroitin sulfate (IaI molecule) to hyaluronan (in the extracellular matrix) via a transesterification reaction involving its C-terminal aspartates (26). In this fashion, the heavy chain molecule is tethered on the C-terminal side to hyaluronan but otherwise appears to extend away from hyaluronan in electron microscopical images (27) and would therefore be able to bind an RGD-containing protein through its vWA domain. Indeed, our calorimetry experiments suggested that IaI and vitronectin bind at one site, which is the predicted finding if we postulate a vWA-RGD interaction. However, this may not completely mirror the matrix conditions *in vivo*. It was recently shown that Vn forms multiplexes with PAI-1, which can contain multiple Vn molecules (10). We therefore speculate that IaI or its heavy chains may act as an intermediary agent, connecting and possibly stabilizing Vn on the hyaluronan matrix. IaI could therefore act as the "glue" connecting Vn to the hyaluronan lattice and allowing Vn to more efficiently interact with cell receptors. Indeed, in our cell adhesion experiments, the most pronounced effect of IaI was demonstrable at low and intermediate concentrations of Vn, indicating that IaI acts as a modifier rather than direct ligand in cell-matrix binding. The vWA domain of IaI heavy chains contains the functionally important metal ion-dependent adhesion site. Metal ion-dependent adhesion sites were recently shown to stabilize integrin binding to RGD domains under force (19). IaI heavy chains could thus not only fix Vn on the hyaluronan lattice but further enhance the effect of Vn on cellular integrin receptors. It is interesting that for at least some RGD-containing peptides, the hyaluronan receptor CD44 and integrins appear to act synergistically for cell growth or migration (28, 29), an effect that might be explained if hyaluronan acts as a carrier for ECM proteins via IaI.

In conclusion, we demonstrate that IaI can bind the ECM protein vitronectin and promote lung epithelial repair after injury in both *in vitro* and *in vivo* models. This finding constitutes a novel role for IaI and suggests an expanded role for IaI as a ubiquitous tissue repair mediator. Further experiments, currently underway in our laboratory, will better define the mechanism of IaI binding to RGD-containing ECM proteins and hopefully expand our understanding of the function of this abundant but often overlooked plasma component.

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