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# LAMININ α4 CONTRIBUTES TO AIRWAY REMODELING AND INFLAMMATION IN ASTHMA

P Prabhala, PhD[1-3]*, DB Wright, PhD[1-3]*, P Robbe, PhD[1-3], C Bitter, MSc[1-3], T Pera, PhD[4], NHT ten Hacken, PhD[2,5], M van den Berge, PhD[2,5], W Timens, PhD[2,6], H Meurs, PhD[1-3], BGJ Dekkers, PhD[1-3,7]

*Both authors contributed equally

[1]*University of Groningen, Department of Molecular Pharmacology, Groningen, The Netherlands*

[2]*University of Groningen, University Medical Center Groningen, Groningen Research Institute for Asthma and COPD, Groningen, The Netherlands*

[3]*University of Groningen, Groningen Research Institute for Pharmacy, Groningen, The Netherlands,*

[4]*Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA*

[5]*University of Groningen, University Medical Center Groningen, Department of Pulmonary Diseases, Groningen, The Netherlands*

[6]*University of Groningen, University Medical Center Groningen, Department of Pathology and Medical Biology, Groningen, The Netherlands*

[7]*University of Groningen, University Medical Center Groningen, Department of Clinical Pharmacy and Pharmacology, Groningen, The Netherlands*

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**Running head:** Laminin α4 in ASM remodeling and inflammation.

**Corresponding author:** Bart GJ Dekkers, Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands, Phone: +31 50 3619434, Fax: +31 50 3614087, e-mail : b.g.j.dekkers@umcg.nl.

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**Abstract**

Airway inflammation and remodeling are characteristic features of asthma, both contributing to airway hyperresponsiveness (AHR) and lung function limitation. Airway smooth muscle (ASM) accumulation and extracellular matrix deposition are characteristic features of airway remodeling, which may contribute to persistent AHR. Laminins containing the α2 chain contribute to characteristics of ASM remodeling *in vitro* and AHR in animal models of asthma. The role of other laminin chains, including the laminin α4 and α5 chains, which contribute to leukocyte migration in other diseases, is currently unknown. The aim of the current study was to investigate the role of these laminin chains in ASM function and in AHR, remodeling and inflammation in asthma. Expression of both laminin α4 and α5 was observed in the human and mouse ASM bundle. *In vitro*, laminin α4 was found to promote a pro-proliferative, pro-contractile and pro-fibrotic ASM cell phenotype. In line, treatment with laminin α4 and α5 function-blocking antibodies reduced allergen-induced increases in ASM mass in a mouse model of allergen-induced asthma. Moreover, eosinophilic inflammation was reduced by the laminin α4 function-blocking antibody as well. Using airway biopsies from healthy subjects and asthmatic patients, we found inverse correlations between ASM α4 chain expression and lung function and AHR, whereas eosinophil numbers correlated positively with expression of laminin α4 in the ASM bundle. This study for the first time indicates a prominent role for laminin α4 in ASM function and in inflammation, AHR and remodeling in asthma, whereas the role of laminin α5 is more subtle.

## Introduction

Asthma is a chronic airway disease, associated with airway inflammation and structural changes in the airway architecture, termed 'remodeling' (13). Both inflammation and remodeling may lead to airway hyperresponsiveness (AHR), defined as an exaggerated obstructive response to various non-specific stimuli (29). Airway remodeling includes increased airway smooth muscle (ASM) mass and contractility, and abnormal extracellular matrix (ECM) turnover resulting in an increased deposition (13). Studies on ECM modifications in asthma revealed an altered airway presence of several laminins (1, 2). Laminins are a group of heterotrimeric proteins comprised of five α, three β and three γ chains (4). Together with collagen IV, nidogens and proteoglycans, laminins constitute the main functional components of basement membranes (BMs). In the healthy lung, laminin α2, α3, α5, β1-3, and γ1-2 are localized in the BMs beneath the airway epithelium, while laminin α4, β1-2 and γ1 are present in ASM BMs (1, 8, 22, 25, 28, 42). Studies on the expression of the laminin chains in the airways of asthmatics are limited and focused on epithelial BMs. In these BMs, laminin α2, α3, α5, β1-2 and γ1 were found to be increased (1, 2).

Increased ASM mass may be related to switching of the ASM cell between proliferative and contractile phenotypes (47). Exposure to proliferative stimuli induces a proliferative ASM phenotype, associated with increased synthetic function and reduced contractility, whereas removal of mitogens induces a contractile phenotype (47). In addition to their role as physical and mechanical support, laminins may also affect ASM phenotype switching. *In vitro,* laminin-111 (composed of laminin α1, β1 and γ1) inhibits ASM cell proliferation (9, 14, 18). Moreover, prolonged exposure of ASM cells to insulin and serum deprivation enhances laminin-211 (α2β1γ1) expression, which also inhibits ASM proliferation (15, 40). In addition, laminin-111 prevents growth factor-induced reductions in ASM contractile protein expression and contractility, whereas laminin-211 increases contractile protein expression and contractility (9, 14, 15, 18, 40). Increased laminin-211 expression may also be important *in vivo*, as allergen-induced AHR was not observed in laminin α2-deficient mice (41).

Collectively, these findings suggest that laminins may importantly contribute to airway remodeling and AHR. However, the role of other laminin chains remains to be explored.

Airway inflammation closely relates to the development of variable and persistent AHR and is considered to contribute to the development, progression and maintenance of asthma (24). Laminins are important regulators of immune cell migration (32). Laminin α4 promotes trans-endothelial migration of leukocytes, whereas laminin α5 restricts migration. Extravasation of leukocytes from blood vessels, in particular T-lymphocytes, but also monocytes and neutrophils, occurs predominantly at sites of low or absent laminin α5 expression (20, 33, 49).

The role of laminin α4 and α5 in abnormal ASM function in asthma has not been investigated yet. Therefore, the aim of the current study was to investigate the expression of laminin α4 and α5 in the airways and their role in airway smooth muscle function and airway remodeling and inflammation in asthma.

## Materials and methods

Additional detail is provided in the online data supplement (https://figshare.com/s/e1f9577c4c54ed74fa04).

*Human subjects and bronchial biopsies*

Airway wall biopsies from healthy subjects and patients with current asthma were obtained from two studies (5, 16, 35). Clinical and (immuno)histochemical parameters of the subjects in these studies have been reported (5, 16, 35). All subjects gave written informed consent. Studies were approved by the medical ethics committee of the University Medical Center Groningen. Biopsies used in the current study were selected on the presence of ASM, sections without ASM were excluded. Corresponding clinical characteristics are outlined in Table 1.

*Immunohistochemistry human biopsies*

Bronchial biopsies were cut into 3-µm thick sections. Biopsy sections were stained with laminin α4 and α5 antibodies, horse radish peroxidase-labeled secondary antibodies and diaminobenzidine, followed by a hematoxylin counterstain. Staining intensity was scored in triplicate by two independent observers in a blinded manner with scores ranging from 1-4 (Figure S1). The ASM, epithelium and endothelium were scored individually.

*Transplantation tissue and immunostaining*

Human tracheal sections from lung transplantation donors were used in this study (12). Tissue was collected according to the Research Code of the University Medical Center Groningen and national ethical and professional guidelines. Cryostat sections (4 µm) were probed with pan-laminin, laminin α4 or laminin α5 antibodies. Antibodies were a gift from dr. LM Sorokin of the University of Muenster, Germany. Antibodies were visualized by Alexa-488- or Cy3-labelled secondary antibodies.

Nuclei were labeled with Hoechst-33342. Sections were analyzed using an Olympus AX70 microscope and digital image capture system.

*Cell culture and lentiviral shRNA transduction*

Two human bronchial smooth muscle cell lines were used, immortalized by stable expression of human telomerase reverse transcriptase (17). Cells were used up to passage 30. Cells were transduced with $3x10^4$ infectious units lentiviral shRNA particles per well (2 ml) to knockdown laminin α4 (sc-43147-V) or laminin α5 (sc-43149-V) or with scrambled (control) shRNA lentiviral particles (sc-108080), according to the manufacturer's instructions. Preliminary results indicated this concentration of lentiviral particles to be maximally effective in reducing mRNA expression (data not shown). Stable clones were selected by growing transfected cells in medium containing puromycin.

*Real-time quantitative RT-PCR*

Real-time quantitative RT-PCR was performed using standard techniques. These data were analyzed using the comparative cycle threshold ($C_T$) method. The amount of target gene was normalized to GAPDH. The specific primers used are listed in Supplemental Table S1.

*Cell proliferation assays*

Cell number was determined using both the AlamarBlue conversion assay and cell counting, using a hemocytometer (12). DNA synthesis was determined using the [$^3$H]-thymidine incorporation assay (12).

*Animal provocations*

Inbred female BALB/c mice were obtained from Charles River. All animal care and experimental procedures complied with the animal protection and welfare guidelines, were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Animal provocations were performed in two separate protocols, an acute protocol and a chronic protocol (Figure 3A). For both protocols, animals were sensitized on days 1, 14 and 21 by an intraperitoneal injection of ovalbumin (OVA) together with aluminum hydroxide in saline (7, 36). Animals were exposed to aerosolized 1% OVA or saline for 20 min, on day 28-30 (acute protocol) or days 26, 27, 33, 34, 40, 41, 47 and 48 (chronic protocol). In the acute protocol, animals were exposed to 5% OVA or saline on day 32 (7). To block laminin α4 and α5 function, 100 μg of anti-laminin α4 or anti-laminin α5 IgG antibodies, respectively or control IgG antibodies were administered intravenously 1 day prior to the first aerosolized OVA exposure (45). In the chronic protocol, administration was repeated on day 39. Animals were sacrificed and lungs were harvested 6 hours (acute protocol) or 24 hours (chronic protocol) after the last OVA exposure. Time points were chosen so as to reflect maximum infiltration of eosinophils after allergen exposure (acute protocol) or to reflect remodeling and chronic inflammation (chronic protocol)(7, 21). ASM was visualized by sm-α-actin staining. Eosinophils were visualized by staining for cyanide resistant endogenous peroxidase activity. Airways within sections were digitally photographed and sm-α-actin staining and eosinophil numbers were quantified using Image J.

*Statistics*

Animal and cell data represents means±SEM. For human experiments, data are presented as medians. Comparisons between two groups were made using Student's unpaired *t*-test (normally distributed data) or a Mann–Whitney *U*-test (non-parametric equivalent). Comparisons between three or more groups were performed using a one-way ANOVA, followed by Tukey's post-hoc test (normally distributed data) or Kruskal–Wallis *H*-test followed by Dunn's post-hoc test (non-normally distributed data). Correlations were calculated by non-parametric Spearman correlations. A value of $P<0.05$ was considered statistically significant. Analyses were performed with GraphPad Prism.

## Results

*Expression of laminin α4 and α5 in human tissue*

To investigate the expression of laminin α4 and α5 in human airways, tracheal sections from lung transplant donors were stained with immunofluorescent antibodies. Staining with a pan-laminin antibody showed laminins to be present in the BMs of the epithelium, endothelium, airway and vascular smooth muscle, and submucosal glands (Figure 1). Laminin α4 was observed in the BMs of the airway and vascular smooth muscle and endothelium, but not the epithelium. Laminin α5 was observed in the BMs of the airway and vascular smooth muscle, epithelium, endothelium and submucosal glands.

*Regulation of human ASM cell phenotype by laminin α4 and α5*

Given the important role of the ASM in AHR (13), we subsequently investigated the role of laminin α4 and α5 in ASM cell function. Laminin α4 mRNA is abundantly expressed by human ASM cells ($\Delta C_T$=12.1±1.0; Figure 2A), which is even higher than the abundantly expressed laminin α2 ($\Delta C_T$=13.4±1.1) (40). Laminin α5 mRNA was expressed much lower ($\Delta C_T$=17.5±0.6). Lentiviral knock-down of laminin α4 and laminin α5 significantly reduced expression of the respective laminin chains (Figure 2B-C). In addition, laminin α4 knock-down increased laminin α5 mRNA expression. No significant effects were observed on other laminin α chains (Supplemental Figure S2). Laminin α4 knock-down significantly reduced baseline ASM cell proliferation, as indicated by cell number (Figure 2D), DNA synthesis and metabolic activity (Supplemental Figure S3). Silencing of laminin α4 reduced sm-α-actin and fibronectin mRNA expression, while no significant effects were observed on smooth muscle-myosin heavy chain (sm-MHC) and calponin expression (Figure 2E). Protein expression of sm-α-actin, calponin and fibronectin was also reduced in α4 deficient cells (Figure 2F-H). Silencing of laminin α5 significantly increased sm-MHC and calponin mRNA expression, however, this increase is

not carried through to the protein level. Collectively, these data indicate that laminin α4 may play an important role in triggering a pro-fibrotic, pro-proliferative, and pro-contractile ASM phenotype.

*Laminin α4 and α5 regulate ASM remodeling in vivo.*

To explore the potential role of laminin α4 and α5 in ASM remodeling *in vivo*, we evaluated the effects of function-blocking antibodies in a mouse model of chronic allergic asthma (Figure 3A). This model is characterized by an allergen-induced inflammatory response, AHR and airway remodeling, including increased ASM mass (23). In this model, similar localization profiles were observed for laminin α4 and α5 in lung cryo-sections as for human tissue (Figure 3B). To investigate the role of both laminin chains in ASM remodeling, mice were treated with laminin α4 or α5 function-blocking antibodies. Allergen-induced ASM accumulation induced by repeated allergen challenge, as observed in control IgG-treated animals, was completely prevented by both laminin blocking-antibodies (Figure 3C). No effects were observed in saline-challenged animals. In addition, no effects were observed on ASM mass in the acute model (Figure S4). Collectively, these observations indicate that both laminin α4 and α5 are involved in allergen-induced ASM accumulation induced by repeated allergen challenge.

*Laminin α4 regulates eosinophil infiltration.*

Airway inflammation, particularly influx of eosinophilic granulocytes, closely relates to AHR in allergic asthma (24). As laminin α4 and α5 have been shown to be important in leukocyte migration (32), we next investigated their potential role in eosinophil infiltration in an acute and chronic challenge protocol (Figure 3A). In the acute model, the laminin α4 function-blocking antibody significantly reduced both basal and allergen-induced increase in airway eosinophils (Figure 4A), whereas the laminin α5 function-blocking antibody tended to decrease airway eosinophils ($P$=0.07). In the chronic model, treatment with the α4 antibody significantly reduced allergen-induced airway eosinophilia (Figure 4B). As in the acute model, no significant effect of the α5 antibody was observed. No effects

were observed in saline-challenged animals. In both models, no significant effect of the antibodies on eosinophils surrounding the vasculature was observed either (data not shown). Collectively, these findings indicate that in addition to its role in ASM remodeling, laminin α4 also contributes to leukocyte infiltration in a mouse model of asthma.

*ASM laminin α4 is associated with lung function, AHR and eosinophilia in asthma*

To investigate the potential role of laminin α4 and α5 expression in patients, staining intensity was scored in biopsies of healthy subjects and asthmatic patients (Figure S1). Staining patterns in airway biopsies (Figure 5A en 5B) were similar to those observed in tracheal sections (Figure 1). A small, but significant increase in endothelial laminin α4 expression was observed in asthmatic patients compared to healthy controls (Supplemental Table S2). Surprisingly, a significant reduction in ASM laminin α4 and laminin α5 expression was observed in asthmatic patients. For laminin α4, this reduction appeared to be due to an interaction between smoking and asthma, as laminin α4 expression was only significantly reduced in smoking asthmatics (Supplemental Figure S5). For laminin α5, the reduction observed in asthmatic patients was independent of smoking. No differences were observed in endothelial or epithelial laminin α5 expression (Supplemental Table S2).

To investigate associations of laminin expression with clinical and biochemical parameters, expression was associated with previously published patient characteristics (5). Scores were grouped into low (score 1-2) and high (score 3-4). Interestingly, increased ASM laminin α4 staining in the asthmatic patients was associated with reduced lung function (lower $FEV_1$ and lower $FEV_1$/FVC) (Figure 5C-D), increased airway reactivity to adenosine monophosphate (AMP) (Figure 5E) and increased eosinophil numbers (Figure 5F). Similar associations were observed when smoking subjects were excluded from the analysis. Associations per individual staining score (1-4) are available in Supplemental Figure S6. No associations were observed for endothelial laminin α4 staining (Supplemental Figure S7) or ASM laminin α5 staining (data not shown). Higher endothelial

laminin α5 staining was associated with increased numbers of macrophages and reduced neutrophil numbers ($P$<0.05, both; Supplemental Figure S8). No associations were observed with other parameters.

Previously, T-lymphocyte migration has been shown to be dependent on the laminin α4/α5 ratio (49). In line with these observations, a laminin α4/α5 ratio of >1 was associated with reduced lung function, increased AMP responsiveness and increased eosinophil numbers in asthma patients (Figure S9).

## Discussion

In the current study, we demonstrate for the first time that laminin α4 may contribute importantly to the abnormal ASM function in asthma. *In vitro*, high expression of laminin α4 by ASM cells was found and knock-down of this laminin reduced proliferation, contractile protein expression and ECM production, processes which are involved in airway remodeling, AHR and lung function decline in asthma. Accordingly, in asthmatic patients, ASM laminin α4 expression was correlated with AHR, reduced lung function and increased eosinophil numbers and in an animal model *in vivo*, laminin α4-blocking antibodies reduced allergen-induced ASM remodeling and inflammation. Although no obvious effects were observed for laminin α5 silencing *in vitro*, *in vivo* blockade of laminin α5 prevented allergen-induced ASM increases.

Airway remodeling is a characteristic feature of chronic asthma and contributes to persistent AHR. Increased ECM deposition is an important characteristic of airway remodeling (13). Various ECM proteins, including collagens and fibronectin, are increased in the epithelial BM of asthmatic patients (6, 31). In addition, increased expression of several laminins, including laminin α5, have been reported (1, 2). In the current study no increase in epithelial laminin α5 expression was observed. This may be explained by the parameter analyzed. In the current study, laminin staining intensity was scored, whereas in the previous study the thickness of the stained BMs was quantified (2). In the present study, intensity scoring was chosen as this method can also be used for other compartments, including the ASM. Increased ECM presence, including fibronectin and elastin, has also been shown in the ASM of asthmatic patients, which is related to airway function (3, 34). Another study showed no differences in the fractional area of ECM components in the ASM of asthmatic subjects (51). In that study, however, an inverse correlation was found between the fractional area of (pan-)laminin in the ASM bundle and $FEV_1$ reversibility (51). Remarkably, we show that both laminin α4 and α5 staining is reduced in the ASM of asthmatics. However, although laminin α4 expression was reduced, there were significant associations between ASM laminin α4 expression and lung function and AHR within the group of asthmatic patients. This paradoxical

278 observation might be related to an interaction between asthma and smoking. The mechanisms
279 behind this interaction, however, are currently unknown and warrant further investigation.

280 The association between laminin α4 and lung function and AHR could be related to laminin
281 α4-induced ASM cell phenotype changes. *In vitro* laminin α4 knock-down reduced ASM cell
282 proliferation, contractile protein expression and fibronectin expression. These findings are in line
283 with observations showing that ECM proteins are important regulators of ASM phenotype switching.
284 Monomeric collagen I and fibronectin induce a proliferative phenotype, whereas laminin-111 and
285 laminin-211 inhibit phenotype switching (9, 12, 14, 15, 18, 26). *In vivo*, treatment with a laminin α4-
286 blocking antibody prevented allergen-induced ASM increase in a mouse model of asthma,
287 supporting a role for laminin α4 in ASM abnormalities in asthma. The reduction in ASM mass may,
288 however, also be (partly) indirect due to inhibition of eosinophil infiltration. Surprisingly, although
289 only limited effects were observed for laminin α5 *in vitro, in vivo* blockade of this laminin fully
290 prevented allergen-induced ASM accumulation. The mechanisms involved remain to be established.

291 Increased expression of contractile proteins has been observed in biopsies from asthmatic
292 donors compared to non-asthmatic donors and may contribute to AHR (48). Expression of smooth
293 muscle specific genes has been shown to be dependent on the binding of serum response factor
294 (SRF) to the CArG [$CC(A/T)_6GG$] box found in the promotors of contractile proteins, including sm-
295 MHC and calponin (44). In line with an increased SRF binding, we found that expression of sm-MHC
296 and calponin mRNA was increased in laminin α5 deficient cells. Increased expression of contractile
297 proteins, including sm-MHC and calponin mRNA, has also been shown in tracheal smooth muscle
298 tissue treated with antisense oligodeoxynucleotides directed against Integrin-linked kinase (ILK) (50).
299 Reduced expression of ILK in these tissues resulted in an increased binding of SRF to the promotors
300 of sm-MHC and calponin providing a potential link between integrins and contractile protein
301 expression. Silencing of ILK resulted in an increased protein expression of sm-MHC, but not of
302 calponin (50). In cultured ASM cells expression of sm-MHC is very low (17), therefore we were
303 unable to quantify the effect laminin α5 knock-down on sm-MHC protein expression. Expression of

304 sm-MHC could thus be increased. Future studies using alternative culture systems should address
305 whether sm-MHC protein expression requires laminin α5. Conversely, increased expression of
306 calponin mRNA did not result in an increased protein expression, which is also in line with previous
307 studies (50). Reasons for this discrepancy between mRNA and protein expression may be due to a
308 number of factors, including differences in protein degradation, posttranslational mechanisms which
309 regulate protein expression, differential expression of co-factors and/or amount of protein synthesis
310 relative to basal protein expression (50).

311 Inflammation is a characteristic feature of asthma and contributes to both acute and
312 persistent AHR (24). Acute AHR is transient and associated with episodic airway inflammation,
313 whereas persistent AHR is associated with airway remodeling in response to recurrent airway
314 inflammation (24). During extravasation, leukocytes cross the endothelial layer and penetrate the
315 underlying BM. This step appears to be rate-limiting as leukocytes accumulate at the BM (38).
316 Penetration of the BM depends on its composition (20, 33, 49). Laminin α4 is ubiquitously localized
317 in the endothelial BMs, while laminin α5 distribution is patchy (49). Extravasation of T-lymphocytes,
318 neutrophils and monocytes occurs predominantly at sites expressing no or low laminin α5 (20, 43,
319 49), indicating that laminin α5 may restrict, whereas laminin α4 may promote extravasation. In the
320 current study, we found that expression of endothelial laminin α4 is increased in asthmatic patients,
321 which could promote inflammatory cell infiltration (19). In line, inhibition of laminin α4 using
322 function-blocking antibodies prevented allergen-induced increases in eosinophil infiltration, both in
323 the acute and chronic mouse model. In contrast to our expectations, no increase in inflammatory
324 cell migration was observed in laminin α5-blocking antibody treated mice.

325 The relative expression of laminin α4 in relation to laminin α5 has been shown to regulate
326 $\alpha_6\beta_1$ integrin-dependent T cell migration across laminin α4 matrices (49). Migration was maximal in
327 the absence of laminin α5 and decreased with increasing proportions of laminin α5, indicating that
328 not only the absolute expression of laminin α4, but also the balance between laminin α4 and laminin
329 α5 is important in these processes (49). In our studies, knock-down of laminin α4 resulted in an

increased laminin α5 mRNA expression in ASM cells. These findings are in line with previous studies in laminin α4 knock-out mice showing an ubiquitous expression of laminin α5 along the vessels, whereas expression of this laminin was patchy and lower in wild-type littermates (49). Although an increased laminin α5 expression could contribute to the observed effects of the laminin α4 knock-down in ASM cells, this is not very likely as we did not observe significant effects of laminin α5 knock-down on proliferation or contractile protein in these cells. An effect of an altered laminin α4 to laminin α5 (laminin α4/α5 ratio) could, which has previously been found to be relevant for lymphocyte migration across vascular laminins (49), on ASM function cannot be ruled out. In line, in our biopsy studies a laminin α4/α5 ratio in the ASM bundle greater than 1 was associated with clinical characteristics in asthma.

Increased laminin α4 expression in ASM was associated with increased numbers of eosinophils in asthmatic patients. Although regulation of laminin expression is poorly described, endothelial expression of laminin α4 has been shown to be strongly upregulated by pro-inflammatory stimuli, such as lipopolysaccharide, interleukin-1β and tumor necrosis factor-α (33), which may indicate that the increased ASM laminin α4 expression is the result of inflammation.

Recently, a number of studies have shown that components of the laminin-integrin signaling axis may contribute to ASM abnormalities in asthma. Expression of CD151, a 4-transmembrane glycoprotein which associates with laminin-binding integrins, has been shown to be increased in the ASM of asthmatic patients (30). In these studies, CD151 was shown to associate with the laminin-binding α7B integrin and was required for G protein-coupled receptor-induced calcium mobilization in human ASM cells and AHR in a mouse model of asthma. Similarly, expression of integrin α7 was shown to be increased in the ASM bundle of asthmatic patients (37). This integrin has previously been shown to be associated with a contractile ASM phenotype and knock-down of this integrin prevented the induction of a contractile phenotype (39). Moreover, this integrin was shown to be involved in ASM survival (41), suggesting that the laminin-integrin signaling axis may be involved in both increased ASM contractility as well as increased ASM mass, through reduced apoptosis. These

effects have thus far been attributed to the laminin α2 chain, as induction of a contractile, hypoproliferative ASM phenotype was associated with increased expression of this laminin chain (15, 40). Moreover, allergen-induced AHR was not observed in laminin α2 deficient mice (41). These effects of laminin α2 in ASM cells are inhibited by the laminin β1 competing peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) (15, 40, 41). In contrast to expectations from these in vitro studies, in vivo treatment with YIGSR attenuated the allergen-induced increase in ASM mass and enhanced ASM contractile protein expression and - contractility, both in control and in allergen-challenged animals (11). In the current study, we show that in addition to laminin α2, laminin α4 also plays an important role in ASM abnormalities, providing a potential explanation for the contrasting observations with the YIGSR peptide in the in vivo model (11).

In conclusion, our results suggest that laminin α4 is involved in airway remodeling and inflammation in asthma, which may contribute to lung function limitation and AHR. The role of laminin α5 in these processes is less apparent and requires further investigation. On the basis of these results, laminin α4 and/or its integrin ligand(s) may represent a novel target for the treatment of inflammation and airway remodeling in asthma.

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528 **Tables**

529

530 **Table 1** Clinical characteristics

| | Control subjects | Asthmatic patients |
|---|---|---|
| Number | 20 | 32 |
| Gender (M/F) | 11/9 | 12/20 |
| Age (years)† | 43 (19-65) | 52 (19-71) |
| Current smoking, n (%) | 9 (45) | 6 (19)* |
| Atopy, n (%) | 5 (25) | 24 (67)*** |
| β-agonist use, n (%) | 0 | 18 (56) |
| ICS use, n (%) | 0 | 15 (47) |
| $FEV_1$ (% pred)† | 109 (83-132) | 87 (34-134)*** |
| $FEV_1$/FVC (%)† | 78 (71-88) | 67 (40-86)*** |
| Reversibility (%)† | 2.7 (-7.4-8.2) | 8.7 (1.3-38.4)*** |
| $PC_{20}$AMP (mg/ml)† | >320 (231.1 to >320) | 8.7 (0.01 to >320)*** |

531 †Data are presented as median (range). Atopy is defined as the ratio of the concentration of specific

532 IgE's in patient serum relative to the concentration of specific IgE's in control serum >1 (5). *$P$<0.05,

533 ***$P$<0.001 versus control subjects.

**Figure legends**

**Figure 1** *Laminin α4 and α5 are expressed in human airways.* Stainings were performed on tracheal sections were obtained from one lung transplantation donor. (A) Localization of pan-laminin (green) in the basement membranes of the endothelium (Endo), epithelium (Epi), submucosal glands (SG) and airway smooth muscle (ASM). (B) Localization of laminin α4 (green) in the basement membrane of the endothelium and airway smooth muscle. (C) Expression of laminin α5 (red) in the basement membrane of the epithelium, endothelium, submucosal glands and airway smooth muscle. Pictures were taken at a 400× magnification. Blue: nuclei.

**Figure 2** *Laminin α4 is involved in the induction of a pro-fibrotic, pro-proliferative, and pro-contractile ASM phenotype in vitro*. (A) Laminin mRNA expression by human ASM cells. (B) Laminin α4 mRNA expression is reduced by lentiviral shRNA directed against this laminin. No effects were observed for lentiviral shRNA directed against laminin α5. (C) Laminin α5 mRNA expression is reduced by lentiviral shRNA directed against this laminin. Lentiviral shRNA directed against laminin α4 increased laminin α5 mRNA expression. (D) Cell number is reduced in laminin α4 deficient cells. (E) Regulation of sm-MHC, calponin, sm-α-actin and fibronectin mRNA expression in laminin α4 and α5 deficient cells. (F-H) Protein expression of sm-α-actin, calponin and fibronectin is reduced in laminin α4 deficient cells. Data represent means±SEM of 4-12 experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to scrambled shRNA transfected cells.

**Figure 3** *Laminin α4 and α5 are involved in airway smooth muscle accumulation in vivo*. (A) Experimental animal procedures. Female BALB/c were sensitized to ovalbumin (OVA) on Days 1, 14, and 21. For the acute protocol, mice were challenged with saline or 1% OVA aerosols for 20 minutes on days 28-30. On day 32, animals were exposed to saline or 5% OVA and sacrificed 6 hours thereafter. For the chronic protocol, animals were exposed to saline or 1% OVA aerosols twice weekly from days 26 to 48. Mice were sacrificed 24 hours after the last challenge. Laminin α4 or α5

560 function-blocking antibodies or control IgG antibodies were administered on day 27 (acute protocol)
561 or days 25 and 39 (chronic protocol). (B) Representative photographs of laminin α4 and α5 staining
562 in mouse lung tissue after repeated ovalbumin challenge. Localization of laminin α4 (green) in the
563 basement membrane (BM) of the alveoli (AV) and airway smooth muscle (ASM). Expression of
564 laminin α5 (red) in the BM of the alveoli, epithelium (Epi) and ASM. Pictures were taken at 400×
565 magnification. AW: airway, V: vessel. Blue: nuclei. (C) Treatment with laminin function-blocking
566 antibodies prevented allergen-induced ASM accumulation in the chronic model. $^{\#}P<0.05$, $^{\#\#}P<0.01$
567 compared with IgG-treated, ovalbumin-challenged controls. Data represent means±SEM of 3-6
568 animals.
569
570 **Figure 4** *Basal airway eosinophil numbers and allergen-induced increases in eosinophils are*
571 *inhibited by laminin α4 blocking antibodies.* (A) Effects of laminin function-blocking antibodies on
572 acute allergen-induced airway infiltration of eosinophils. (B) Effects of laminin function-blocking
573 antibodies on chronic allergen-induced airway infiltration of eosinophils. $^{*}P<0.05$ compared to with
574 IgG-treated, saline-challenged controls. $^{\#}P<0.05$ compared with IgG-treated, ovalbumin-challenged
575 controls. Data represent means±SEM of 3-6 animals. BM: basement membrane.
576
577 **Figure 5** *Laminin α4 scoring is associated with clinical characteristics of asthmatic patients.*
578 (A,B) Representative photographs of staining for (A) laminin α4 and (B) laminin α5 in ASM,
579 epithelium and endothelium in biopsy sections. (C,D) High (score 3-4) laminin α4 staining is
580 associated with reduced lung function of asthmatic patients expressed as both (C) $FEV_1$ %predicted
581 and (D) $FEV_1$/FVC %predicted. (E) High (score 3-4) laminin α4 staining is associated with increased
582 airway reactivity of asthmatic patients to adenosine monophosphate (AMP). (F) High (score 3-4)
583 laminin α4 staining is associated with increased numbers of EPX-positive eosinophils in the airway
584 biopsies of asthmatic patients. Results from 20 control subjects and 31 asthmatic patients are shown

585 in Figs C-F. **$P$<0.01, ***$P$<0.001. Examples of staining intensity scores 1-4 are shown in
586 supplemental Figure S1.
587