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Laminin $\alpha 4$ contributes to airway remodeling and inflammation in asthma

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1 **LAMININ α 4 CONTRIBUTES TO AIRWAY REMODELING AND INFLAMMATION IN ASTHMA**

2

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22 approved manuscript: PP, DW, PR, CB, TP, NtH, MvdB, WT, HM, BD.

23

24 **Running head:** Laminin α 4 in ASM remodeling and inflammation.

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31

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34

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36 **Abstract**

37 Airway inflammation and remodeling are characteristic features of asthma, both contributing to
38 airway hyperresponsiveness (AHR) and lung function limitation. Airway smooth muscle (ASM)
39 accumulation and extracellular matrix deposition are characteristic features of airway remodeling,
40 which may contribute to persistent AHR. Laminins containing the $\alpha 2$ chain contribute to
41 characteristics of ASM remodeling *in vitro* and AHR in animal models of asthma. The role of other
42 laminin chains, including the laminin $\alpha 4$ and $\alpha 5$ chains, which contribute to leukocyte migration in
43 other diseases, is currently unknown. The aim of the current study was to investigate the role of
44 these laminin chains in ASM function and in AHR, remodeling and inflammation in asthma.
45 Expression of both laminin $\alpha 4$ and $\alpha 5$ was observed in the human and mouse ASM bundle. *In vitro*,
46 laminin $\alpha 4$ was found to promote a pro-proliferative, pro-contractile and pro-fibrotic ASM cell
47 phenotype. In line, treatment with laminin $\alpha 4$ and $\alpha 5$ function-blocking antibodies reduced allergen-
48 induced increases in ASM mass in a mouse model of allergen-induced asthma. Moreover,
49 eosinophilic inflammation was reduced by the laminin $\alpha 4$ function-blocking antibody as well. Using
50 airway biopsies from healthy subjects and asthmatic patients, we found inverse correlations
51 between ASM $\alpha 4$ chain expression and lung function and AHR, whereas eosinophil numbers
52 correlated positively with expression of laminin $\alpha 4$ in the ASM bundle. This study for the first time
53 indicates a prominent role for laminin $\alpha 4$ in ASM function and in inflammation, AHR and remodeling
54 in asthma, whereas the role of laminin $\alpha 5$ is more subtle.

55 **Introduction**

56 Asthma is a chronic airway disease, associated with airway inflammation and structural changes in
57 the airway architecture, termed 'remodeling' (13). Both inflammation and remodeling may lead to
58 airway hyperresponsiveness (AHR), defined as an exaggerated obstructive response to various non-
59 specific stimuli (29). Airway remodeling includes increased airway smooth muscle (ASM) mass and
60 contractility, and abnormal extracellular matrix (ECM) turnover resulting in an increased deposition
61 (13). Studies on ECM modifications in asthma revealed an altered airway presence of several
62 laminins (1, 2). Laminins are a group of heterotrimeric proteins comprised of five α , three β and
63 three γ chains (4). Together with collagen IV, nidogens and proteoglycans, laminins constitute the
64 main functional components of basement membranes (BMs). In the healthy lung, laminin $\alpha 2$, $\alpha 3$, $\alpha 5$,
65 $\beta 1-3$, and $\gamma 1-2$ are localized in the BMs beneath the airway epithelium, while laminin $\alpha 4$, $\beta 1-2$ and $\gamma 1$
66 are present in ASM BMs (1, 8, 22, 25, 28, 42). Studies on the expression of the laminin chains in the
67 airways of asthmatics are limited and focused on epithelial BMs. In these BMs, laminin $\alpha 2$, $\alpha 3$, $\alpha 5$,
68 $\beta 1-2$ and $\gamma 1$ were found to be increased (1, 2).

69 Increased ASM mass may be related to switching of the ASM cell between proliferative and
70 contractile phenotypes (47). Exposure to proliferative stimuli induces a proliferative ASM phenotype,
71 associated with increased synthetic function and reduced contractility, whereas removal of mitogens
72 induces a contractile phenotype (47). In addition to their role as physical and mechanical support,
73 laminins may also affect ASM phenotype switching. *In vitro*, laminin-111 (composed of laminin $\alpha 1$,
74 $\beta 1$ and $\gamma 1$) inhibits ASM cell proliferation (9, 14, 18). Moreover, prolonged exposure of ASM cells to
75 insulin and serum deprivation enhances laminin-211 ($\alpha 2\beta 1\gamma 1$) expression, which also inhibits ASM
76 proliferation (15, 40). In addition, laminin-111 prevents growth factor-induced reductions in ASM
77 contractile protein expression and contractility, whereas laminin-211 increases contractile protein
78 expression and contractility (9, 14, 15, 18, 40). Increased laminin-211 expression may also be
79 important *in vivo*, as allergen-induced AHR was not observed in laminin $\alpha 2$ -deficient mice (41).

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

82 Airway inflammation closely relates to the development of variable and persistent AHR and is
83 considered to contribute to the development, progression and maintenance of asthma (24).

84 Laminins are important regulators of immune cell migration (32). Laminin α 4 promotes trans-
85 endothelial migration of leukocytes, whereas laminin α 5 restricts migration. Extravasation of
86 leukocytes from blood vessels, in particular T-lymphocytes, but also monocytes and neutrophils,
87 occurs predominantly at sites of low or absent laminin α 5 expression (20, 33, 49).

88 The role of laminin α 4 and α 5 in abnormal ASM function in asthma has not been investigated yet.
89 Therefore, the aim of the current study was to investigate the expression of laminin α 4 and α 5 in the
90 airways and their role in airway smooth muscle function and airway remodeling and inflammation in
91 asthma.

92 **Materials and methods**

93

94 Additional detail is provided in the online data supplement

95 (<https://figshare.com/s/e1f9577c4c54ed74fa04>).

96

97 *Human subjects and bronchial biopsies*

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

104

105 *Immunohistochemistry human biopsies*

106 Bronchial biopsies were cut into 3- μ m thick sections. Biopsy sections were stained with laminin α 4
107 and α 5 antibodies, horse radish peroxidase-labeled secondary antibodies and diaminobenzidine,
108 followed by a hematoxylin counterstain. Staining intensity was scored in triplicate by two
109 independent observers in a blinded manner with scores ranging from 1-4 (Figure S1). The ASM,
110 epithelium and endothelium were scored individually.

111

112 *Transplantation tissue and immunostaining*

113 Human tracheal sections from lung transplantation donors were used in this study (12). Tissue was
114 collected according to the Research Code of the University Medical Center Groningen and national
115 ethical and professional guidelines. Cryostat sections (4 μ m) were probed with pan-laminin, laminin
116 α 4 or laminin α 5 antibodies. Antibodies were a gift from dr. LM Sorokin of the University of
117 Muenster, Germany. Antibodies were visualized by Alexa-488- or Cy3-labelled secondary antibodies.

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

120

121 *Cell culture and lentiviral shRNA transduction*

122 Two human bronchial smooth muscle cell lines were used, immortalized by stable expression of
123 human telomerase reverse transcriptase (17). Cells were used up to passage 30. Cells were
124 transduced with 3×10^4 infectious units lentiviral shRNA particles per well (2 ml) to knockdown
125 laminin $\alpha 4$ (sc-43147-V) or laminin $\alpha 5$ (sc-43149-V) or with scrambled (control) shRNA lentiviral
126 particles (sc-108080), according to the manufacturer's instructions. Preliminary results indicated this
127 concentration of lentiviral particles to be maximally effective in reducing mRNA expression (data not
128 shown). Stable clones were selected by growing transfected cells in medium containing puromycin.

129

130 *Real-time quantitative RT-PCR*

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

134

135 *Cell proliferation assays*

136 Cell number was determined using both the AlamarBlue conversion assay and cell counting, using a
137 hemocytometer (12). DNA synthesis was determined using the [^3H]-thymidine incorporation assay
138 (12).

139

140 *Animal provocations*

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

144 Animal provocations were performed in two separate protocols, an acute protocol and a chronic
145 protocol (Figure 3A). For both protocols, animals were sensitized on days 1, 14 and 21 by an
146 intraperitoneal injection of ovalbumin (OVA) together with aluminum hydroxide in saline (7, 36).
147 Animals were exposed to aerosolized 1% OVA or saline for 20 min, on day 28-30 (acute protocol) or
148 days 26, 27, 33, 34, 40, 41, 47 and 48 (chronic protocol). In the acute protocol, animals were
149 exposed to 5% OVA or saline on day 32 (7). To block laminin α 4 and α 5 function, 100 μ g of anti-
150 laminin α 4 or anti-laminin α 5 IgG antibodies, respectively or control IgG antibodies were
151 administered intravenously 1 day prior to the first aerosolized OVA exposure (45). In the chronic
152 protocol, administration was repeated on day 39. Animals were sacrificed and lungs were harvested
153 6 hours (acute protocol) or 24 hours (chronic protocol) after the last OVA exposure. Time points
154 were chosen so as to reflect maximum infiltration of eosinophils after allergen exposure (acute
155 protocol) or to reflect remodeling and chronic inflammation (chronic protocol)(7, 21). ASM was
156 visualized by sm- α -actin staining. Eosinophils were visualized by staining for cyanide resistant
157 endogenous peroxidase activity. Airways within sections were digitally photographed and sm- α -actin
158 staining and eosinophil numbers were quantified using Image J.

159

160 *Statistics*

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

168 **Results**

169

170 *Expression of laminin α 4 and α 5 in human tissue*

171 To investigate the expression of laminin α 4 and α 5 in human airways, tracheal sections from lung
172 transplant donors were stained with immunofluorescent antibodies. Staining with a pan-laminin
173 antibody showed laminins to be present in the BMs of the epithelium, endothelium, airway and
174 vascular smooth muscle, and submucosal glands (Figure 1). Laminin α 4 was observed in the BMs of
175 the airway and vascular smooth muscle and endothelium, but not the epithelium. Laminin α 5 was
176 observed in the BMs of the airway and vascular smooth muscle, epithelium, endothelium and
177 submucosal glands.

178

179 *Regulation of human ASM cell phenotype by laminin α 4 and α 5*

180 Given the important role of the ASM in AHR (13), we subsequently investigated the role of laminin
181 α 4 and α 5 in ASM cell function. Laminin α 4 mRNA is abundantly expressed by human ASM cells
182 ($\Delta C_T=12.1\pm 1.0$; Figure 2A), which is even higher than the abundantly expressed laminin α 2
183 ($\Delta C_T=13.4\pm 1.1$) (40). Laminin α 5 mRNA was expressed much lower ($\Delta C_T=17.5\pm 0.6$). Lentiviral knock-
184 down of laminin α 4 and laminin α 5 significantly reduced expression of the respective laminin chains
185 (Figure 2B-C). In addition, laminin α 4 knock-down increased laminin α 5 mRNA expression. No
186 significant effects were observed on other laminin α chains (Supplemental Figure S2). Laminin α 4
187 knock-down significantly reduced baseline ASM cell proliferation, as indicated by cell number (Figure
188 2D), DNA synthesis and metabolic activity (Supplemental Figure S3). Silencing of laminin α 4 reduced
189 sm- α -actin and fibronectin mRNA expression, while no significant effects were observed on smooth
190 muscle-myosin heavy chain (sm-MHC) and calponin expression (Figure 2E). Protein expression of sm-
191 α -actin, calponin and fibronectin was also reduced in α 4 deficient cells (Figure 2F-H). Silencing of
192 laminin α 5 significantly increased sm-MHC and calponin mRNA expression, however, this increase is

193 not carried through to the protein level. Collectively, these data indicate that laminin $\alpha 4$ may play an
194 important role in triggering a pro-fibrotic, pro-proliferative, and pro-contractile ASM phenotype.

195

196 *Laminin $\alpha 4$ and $\alpha 5$ regulate ASM remodeling in vivo.*

197 To explore the potential role of laminin $\alpha 4$ and $\alpha 5$ in ASM remodeling *in vivo*, we evaluated the
198 effects of function-blocking antibodies in a mouse model of chronic allergic asthma (Figure 3A). This
199 model is characterized by an allergen-induced inflammatory response, AHR and airway remodeling,
200 including increased ASM mass (23). In this model, similar localization profiles were observed for
201 laminin $\alpha 4$ and $\alpha 5$ in lung cryo-sections as for human tissue (Figure 3B). To investigate the role of
202 both laminin chains in ASM remodeling, mice were treated with laminin $\alpha 4$ or $\alpha 5$ function-blocking
203 antibodies. Allergen-induced ASM accumulation induced by repeated allergen challenge, as
204 observed in control IgG-treated animals, was completely prevented by both laminin blocking-
205 antibodies (Figure 3C). No effects were observed in saline-challenged animals. In addition, no effects
206 were observed on ASM mass in the acute model (Figure S4). Collectively, these observations indicate
207 that both laminin $\alpha 4$ and $\alpha 5$ are involved in allergen-induced ASM accumulation induced by
208 repeated allergen challenge.

209

210 *Laminin $\alpha 4$ regulates eosinophil infiltration.*

211 Airway inflammation, particularly influx of eosinophilic granulocytes, closely relates to AHR in allergic
212 asthma (24). As laminin $\alpha 4$ and $\alpha 5$ have been shown to be important in leukocyte migration (32), we
213 next investigated their potential role in eosinophil infiltration in an acute and chronic challenge
214 protocol (Figure 3A). In the acute model, the laminin $\alpha 4$ function-blocking antibody significantly
215 reduced both basal and allergen-induced increase in airway eosinophils (Figure 4A), whereas the
216 laminin $\alpha 5$ function-blocking antibody tended to decrease airway eosinophils ($P=0.07$). In the chronic
217 model, treatment with the $\alpha 4$ antibody significantly reduced allergen-induced airway eosinophilia
218 (Figure 4B). As in the acute model, no significant effect of the $\alpha 5$ antibody was observed. No effects

219 were observed in saline-challenged animals. In both models, no significant effect of the antibodies
220 on eosinophils surrounding the vasculature was observed either (data not shown). Collectively, these
221 findings indicate that in addition to its role in ASM remodeling, laminin $\alpha 4$ also contributes to
222 leukocyte infiltration in a mouse model of asthma.

223

224 *ASM laminin $\alpha 4$ is associated with lung function, AHR and eosinophilia in asthma*

225 To investigate the potential role of laminin $\alpha 4$ and $\alpha 5$ expression in patients, staining intensity was
226 scored in biopsies of healthy subjects and asthmatic patients (Figure S1). Staining patterns in airway
227 biopsies (Figure 5A en 5B) were similar to those observed in tracheal sections (Figure 1). A small, but
228 significant increase in endothelial laminin $\alpha 4$ expression was observed in asthmatic patients
229 compared to healthy controls (Supplemental Table S2). Surprisingly, a significant reduction in ASM
230 laminin $\alpha 4$ and laminin $\alpha 5$ expression was observed in asthmatic patients. For laminin $\alpha 4$, this
231 reduction appeared to be due to an interaction between smoking and asthma, as laminin $\alpha 4$
232 expression was only significantly reduced in smoking asthmatics (Supplemental Figure S5). For
233 laminin $\alpha 5$, the reduction observed in asthmatic patients was independent of smoking. No
234 differences were observed in endothelial or epithelial laminin $\alpha 5$ expression (Supplemental Table
235 S2).

236 To investigate associations of laminin expression with clinical and biochemical parameters,
237 expression was associated with previously published patient characteristics (5). Scores were grouped
238 into low (score 1-2) and high (score 3-4). Interestingly, increased ASM laminin $\alpha 4$ staining in the
239 asthmatic patients was associated with reduced lung function (lower FEV₁ and lower FEV₁/FVC)
240 (Figure 5C-D), increased airway reactivity to adenosine monophosphate (AMP) (Figure 5E) and
241 increased eosinophil numbers (Figure 5F). Similar associations were observed when smoking
242 subjects were excluded from the analysis. Associations per individual staining score (1-4) are
243 available in Supplemental Figure S6. No associations were observed for endothelial laminin $\alpha 4$
244 staining (Supplemental Figure S7) or ASM laminin $\alpha 5$ staining (data not shown). Higher endothelial

245 laminin α 5 staining was associated with increased numbers of macrophages and reduced neutrophil
246 numbers ($P < 0.05$, both; Supplemental Figure S8). No associations were observed with other
247 parameters.

248 Previously, T-lymphocyte migration has been shown to be dependent on the laminin α 4/ α 5
249 ratio (49). In line with these observations, a laminin α 4/ α 5 ratio of >1 was associated with reduced
250 lung function, increased AMP responsiveness and increased eosinophil numbers in asthma patients
251 (Figure S9).

252 **Discussion**

253 In the current study, we demonstrate for the first time that laminin $\alpha 4$ may contribute importantly
254 to the abnormal ASM function in asthma. *In vitro*, high expression of laminin $\alpha 4$ by ASM cells was
255 found and knock-down of this laminin reduced proliferation, contractile protein expression and ECM
256 production, processes which are involved in airway remodeling, AHR and lung function decline in
257 asthma. Accordingly, in asthmatic patients, ASM laminin $\alpha 4$ expression was correlated with AHR,
258 reduced lung function and increased eosinophil numbers and in an animal model *in vivo*, laminin $\alpha 4$ -
259 blocking antibodies reduced allergen-induced ASM remodeling and inflammation. Although no
260 obvious effects were observed for laminin $\alpha 5$ silencing *in vitro*, *in vivo* blockade of laminin $\alpha 5$
261 prevented allergen-induced ASM increases.

262 Airway remodeling is a characteristic feature of chronic asthma and contributes to persistent
263 AHR. Increased ECM deposition is an important characteristic of airway remodeling (13). Various
264 ECM proteins, including collagens and fibronectin, are increased in the epithelial BM of asthmatic
265 patients (6, 31). In addition, increased expression of several laminins, including laminin $\alpha 5$, have
266 been reported (1, 2). In the current study no increase in epithelial laminin $\alpha 5$ expression was
267 observed. This may be explained by the parameter analyzed. In the current study, laminin staining
268 intensity was scored, whereas in the previous study the thickness of the stained BMs was quantified
269 (2). In the present study, intensity scoring was chosen as this method can also be used for other
270 compartments, including the ASM. Increased ECM presence, including fibronectin and elastin, has
271 also been shown in the ASM of asthmatic patients, which is related to airway function (3, 34).
272 Another study showed no differences in the fractional area of ECM components in the ASM of
273 asthmatic subjects (51). In that study, however, an inverse correlation was found between the
274 fractional area of (pan-)laminin in the ASM bundle and FEV₁ reversibility (51). Remarkably, we show
275 that both laminin $\alpha 4$ and $\alpha 5$ staining is reduced in the ASM of asthmatics. However, although
276 laminin $\alpha 4$ expression was reduced, there were significant associations between ASM laminin $\alpha 4$
277 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 α 4 and lung function and AHR could be related to laminin
281 α 4-induced ASM cell phenotype changes. *In vitro* laminin α 4 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 α 4-
286 blocking antibody prevented allergen-induced ASM increase in a mouse model of asthma,
287 supporting a role for laminin α 4 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 α 5 *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 promoters 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 α 5 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 promoters
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 α 5 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 α 5. 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 α 4 is ubiquitously localized
317 in the endothelial BMs, while laminin α 5 distribution is patchy (49). Extravasation of T-lymphocytes,
318 neutrophils and monocytes occurs predominantly at sites expressing no or low laminin α 5 (20, 43,
319 49), indicating that laminin α 5 may restrict, whereas laminin α 4 may promote extravasation. In the
320 current study, we found that expression of endothelial laminin α 4 is increased in asthmatic patients,
321 which could promote inflammatory cell infiltration (19). In line, inhibition of laminin α 4 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 α 5-blocking antibody treated mice.

325 The relative expression of laminin α 4 in relation to laminin α 5 has been shown to regulate
326 α ₆ β ₁ integrin-dependent T cell migration across laminin α 4 matrices (49). Migration was maximal in
327 the absence of laminin α 5 and decreased with increasing proportions of laminin α 5, indicating that
328 not only the absolute expression of laminin α 4, but also the balance between laminin α 4 and laminin
329 α 5 is important in these processes (49). In our studies, knock-down of laminin α 4 resulted in an

330 increased laminin $\alpha 5$ mRNA expression in ASM cells. These findings are in line with previous studies
331 in laminin $\alpha 4$ knock-out mice showing an ubiquitous expression of laminin $\alpha 5$ along the vessels,
332 whereas expression of this laminin was patchy and lower in wild-type littermates (49). Although an
333 increased laminin $\alpha 5$ expression could contribute to the observed effects of the laminin $\alpha 4$ knock-
334 down in ASM cells, this is not very likely as we did not observe significant effects of laminin $\alpha 5$
335 knock-down on proliferation or contractile protein in these cells. An effect of an altered laminin $\alpha 4$
336 to laminin $\alpha 5$ (laminin $\alpha 4/\alpha 5$ ratio) could, which has previously been found to be relevant for
337 lymphocyte migration across vascular laminins (49), on ASM function cannot be ruled out. In line, in
338 our biopsy studies a laminin $\alpha 4/\alpha 5$ ratio in the ASM bundle greater than 1 was associated with
339 clinical characteristics in asthma.

340 Increased laminin $\alpha 4$ expression in ASM was associated with increased numbers of
341 eosinophils in asthmatic patients. Although regulation of laminin expression is poorly described,
342 endothelial expression of laminin $\alpha 4$ has been shown to be strongly upregulated by pro-
343 inflammatory stimuli, such as lipopolysaccharide, interleukin- 1β and tumor necrosis factor- α (33),
344 which may indicate that the increased ASM laminin $\alpha 4$ expression is the result of inflammation.

345 Recently, a number of studies have shown that components of the laminin-integrin signaling
346 axis may contribute to ASM abnormalities in asthma. Expression of CD151, a 4-transmembrane
347 glycoprotein which associates with laminin-binding integrins, has been shown to be increased in the
348 ASM of asthmatic patients (30). In these studies, CD151 was shown to associate with the laminin-
349 binding $\alpha 7B$ integrin and was required for G protein-coupled receptor-induced calcium mobilization
350 in human ASM cells and AHR in a mouse model of asthma. Similarly, expression of integrin $\alpha 7$ was
351 shown to be increased in the ASM bundle of asthmatic patients (37). This integrin has previously
352 been shown to be associated with a contractile ASM phenotype and knock-down of this integrin
353 prevented the induction of a contractile phenotype (39). Moreover, this integrin was shown to be
354 involved in ASM survival (41), suggesting that the laminin-integrin signaling axis may be involved in
355 both increased ASM contractility as well as increased ASM mass, through reduced apoptosis. These

356 effects have thus far been attributed to the laminin α 2 chain, as induction of a contractile,
357 hypoproliferative ASM phenotype was associated with increased expression of this laminin chain
358 (15, 40). Moreover, allergen-induced AHR was not observed in laminin α 2 deficient mice (41). These
359 effects of laminin α 2 in ASM cells are inhibited by the laminin β 1 competing peptide Tyr-Ile-Gly-Ser-
360 Arg (YIGSR) (15, 40, 41). In contrast to expectations from these in vitro studies, in vivo treatment
361 with YIGSR attenuated the allergen-induced increase in ASM mass and enhanced ASM contractile
362 protein expression and - contractility, both in control and in allergen-challenged animals (11). In the
363 current study, we show that in addition to laminin α 2, laminin α 4 also plays an important role in
364 ASM abnormalities, providing a potential explanation for the contrasting observations with the
365 YIGSR peptide in the in vivo model (11).

366 In conclusion, our results suggest that laminin α 4 is involved in airway remodeling and
367 inflammation in asthma, which may contribute to lung function limitation and AHR. The role of
368 laminin α 5 in these processes is less apparent and requires further investigation. On the basis of
369 these results, laminin α 4 and/or its integrin ligand(s) may represent a novel target for the treatment
370 of inflammation and airway remodeling in asthma.

371

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375 Lydia Sorokin (University of Muenster, Germany) for valuable discussion.

376 **References**

- 377 1. **Altraja A, Laitinen A, Virtanen I, Kampe M, Simonsson BG, Karlsson SE, Hakansson L, Venge**
378 **P, Sillastu H, Laitinen LA.** Expression of laminins in the airways in various types of asthmatic
379 patients: a morphometric study. *Am J Respir Cell Mol Biol* 15: 482–488, 1996.
- 380 2. **Amin K, Janson C, Seveus L, Miyazaki K, Virtanen I, Venge P.** Uncoordinated production of
381 Laminin-5 chains in airways epithelium of allergic asthmatics. *RespirRes* 6: 110, 2005.
- 382 3. **Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, Ferreira DS, Mulder A, Gomes HA,**
383 **Fernezlian SM, James A, Mauad T.** Extracellular matrix components and regulators in the
384 airway smooth muscle in asthma. *Eur Respir J* 32: 61–69, 2008.
- 385 4. **Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, Engel J,**
386 **Engvall E, Hohenester E, Jones JC, Kleinman HK, Marinkovich MP, Martin GR, Mayer U,**
387 **Meneguzzi G, Miner JH, Miyazaki K, Patarroyo M, Paulsson M, Quaranta V, Sanes JR, Sasaki**
388 **T, Sekiguchi K, Sorokin LM, Talts JF, Tryggvason K, Uitto J, Virtanen I, von der MK, Wewer**
389 **UM, Yamada Y, Yurchenco PD.** A simplified laminin nomenclature. *Matrix Biol* 24: 326–332,
390 2005.
- 391 5. **Broekema M, Timens W, Vonk JM, Volbeda F, Lodewijk ME, Hylkema MN, Ten Hacken NH,**
392 **Postma DS.** Persisting remodeling and less airway wall eosinophil activation in complete
393 remission of asthma. *Am J Respir Crit Care Med* 183: 310–316, 2011.
- 394 6. **Burgess JK, Mauad T, Tjin G, Karlsson JC, Westergren-Thorsson G.** The extracellular matrix -
395 the under-recognized element in lung disease? *J Pathol* 240: 397–409, 2016.
- 396 7. **Cieslewicz G, Tomkinson A, Adler A, Duez C, Schwarze J, Takeda K, Larson KA, Lee JJ, Irvin**
397 **CG, Gelfand EW.** The late, but not early, asthmatic response is dependent on IL-5 and
398 correlates with eosinophil infiltration. *J Clin Invest* 104: 301–308, 1999.
- 399 8. **Coraux C, Meneguzzi G, Rousselle P, Puchelle E, Gaillard D.** Distribution of laminin 5, integrin
400 receptors, and branching morphogenesis during human fetal lung development. *Dev Dyn* 225:
401 176–185, 2002.

- 402 9. **Dekkers BGJ, Bos IS, Gosens R, Halayko AJ, Zaagsma J, Meurs H.** The integrin-blocking
403 peptide RGDS inhibits airway smooth muscle remodeling in a guinea pig model of allergic
404 asthma. *Am J Respir Crit Care Med* 181: 556–565, 2010.
- 405 10. **Dekkers BGJ, Bos IS, Gosens R, Halayko AJ, Zaagsma J, Meurs H.** Inhibition of Airway Smooth
406 Muscle Remodeling in an Animal Model of Chronic Asthma by the Integrin-Blocking Peptide
407 RGDS. *Am. J. Respir. Crit. Care Med. An Off. J. Am. Thorac. Soc. Med. Sect. Am. Lung Assoc.*
408 179: A5600, 2010.
- 409 11. **Dekkers BGJ, Bos IS, Halayko AJ, Zaagsma J, Meurs H.** The laminin beta1-competing peptide
410 YIGSR induces a hypercontractile, hypoproliferative airway smooth muscle phenotype in an
411 animal model of allergic asthma. *Respir Res* 11: 170, 2010.
- 412 12. **Dekkers BGJ, Bos IS, Zaagsma J, Meurs H.** Functional consequences of human airway smooth
413 muscle phenotype plasticity. *Br J Pharmacol* 166: 359–367, 2012.
- 414 13. **Dekkers BGJ, Maarsingh H, Meurs H, Gosens R.** Airway structural components drive airway
415 smooth muscle remodeling in asthma. *Proc Am Thorac Soc* 6: 683–692, 2009.
- 416 14. **Dekkers BGJ, Schaafsma D, Nelemans SA, Zaagsma J, Meurs H.** Extracellular matrix proteins
417 differentially regulate airway smooth muscle phenotype and function. *Am J Physiol Lung Cell*
418 *Mol Physiol* 292: L1405–L1413, 2007.
- 419 15. **Dekkers BGJ, Schaafsma D, Tran T, Zaagsma J, Meurs H.** Insulin-induced Laminin Expression
420 Promotes a Hypercontractile Airway Smooth Muscle Phenotype. *Am J Respir Cell Mol Biol* 41:
421 494–504, 2009.
- 422 16. **Draijer C, Boorsma CE, Robbe P, Timens W, Hylkema MN, Ten Hacken NH, van den Berge M,**
423 **Postma DS, Melgert BN.** Human asthma is characterized by more IRF5+ M1 and CD206+ M2
424 macrophages and less IL-10+ M2-like macrophages around airways compared with healthy
425 airways. *J Allergy Clin Immunol* 140: 280-283.e3, 2017.
- 426 17. **Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni**
427 **AS, Zaagsma J, Halayko AJ.** Role of caveolin-1 in p42/p44 MAP kinase activation and

- 428 proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 291: L523–
429 L534, 2006.
- 430 18. **Hirst SJ, Twort CH, Lee TH.** Differential effects of extracellular matrix proteins on human
431 airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* 23: 335–
432 344, 2000.
- 433 19. **Jeffery PK.** Remodeling and inflammation of bronchi in asthma and chronic obstructive
434 pulmonary disease. *Proc Am Thorac Soc* 1: 176–183, 2004.
- 435 20. **Kenne E, Soehnlein O, Genove G, Rotzius P, Eriksson EE, Lindbom L.** Immune cell recruitment
436 to inflammatory loci is impaired in mice deficient in basement membrane protein laminin
437 alpha4. *J Leukoc Biol* 88: 523–528, 2010.
- 438 21. **Kistemaker LEM, Bos ST, Mudde WM, Hylkema MN, Hiemstra PS, Wess J, Meurs H,
439 Kerstjens HAM, Gosens R.** Muscarinic M₃ Receptors Contribute to Allergen-Induced Airway
440 Remodeling in Mice. *Am J Respir Cell Mol Biol* 50: 690–698, 2014.
- 441 22. **Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, Burgeson RE, Champliand MF.**
442 Characterization and expression of the laminin gamma3 chain: a novel, non-basement
443 membrane-associated, laminin chain. *J Cell Biol* 145: 605–618, 1999.
- 444 23. **McMillan SJ, Lloyd CM.** Prolonged allergen challenge in mice leads to persistent airway
445 remodelling. *Clin Exp Allergy* 34: 497–507, 2004.
- 446 24. **Meurs H, Gosens R, Zaagsma J.** Airway hyperresponsiveness in asthma: lessons from in vitro
447 model systems and animal models. *Eur Respir J* 32: 487–502, 2008.
- 448 25. **Nguyen NM, Senior RM.** Laminin isoforms and lung development: all isoforms are not equal.
449 *Dev Biol (Basel)* 294: 271–279, 2006.
- 450 26. **Nguyen TT, Ward JP, Hirst SJ.** beta1-Integrins mediate enhancement of airway smooth
451 muscle proliferation by collagen and fibronectin. *Am J Respir Crit Care Med* 171: 217–223,
452 2005.
- 453 27. **Peng Q, Lai D, Nguyen TT, Chan V, Matsuda T, Hirst SJ.** Multiple beta 1 integrins mediate

- 454 enhancement of human airway smooth muscle cytokine secretion by fibronectin and type I
455 collagen. *J Immunol* 174: 2258–2264, 2005.
- 456 28. **Petajaniemi N, Korhonen M, Kortesmaa J, Tryggvason K, Sekiguchi K, Fujiwara H, Sorokin L,**
457 **Thornell LE, Wondimu Z, Assefa D, Patarroyo M, Virtanen I.** Localization of laminin alpha4-
458 chain in developing and adult human tissues. *J Histochem Cytochem* 50: 1113–1130, 2002.
- 459 29. **Postma DS, Kerstjens HA.** Characteristics of airway hyperresponsiveness in asthma and
460 chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158: S187–S192, 1998.
- 461 30. **Qiao Y, Tam JKC, Tan SSL, Tai YK, Chin CY, Stewart AG, Ashman L, Sekiguchi K, Langenbach**
462 **SY, Stelmack G, Halayko AJ, Tran T, Melbourne Epidemiological Study of Childhood Asthma**
463 **group.** CD151, a laminin receptor showing increased expression in asthmatic patients,
464 contributes to airway hyperresponsiveness through calcium signaling. *J Allergy Clin Immunol*
465 139: 82-92.e5, 2017.
- 466 31. **Roche WR, Beasley R, Williams JH, Holgate ST.** Subepithelial fibrosis in the bronchi of
467 asthmatics. *Lancet* 1: 520–524, 1989.
- 468 32. **Simon T, Bromberg JS.** Regulation of the Immune System by Laminins. *Trends Immunol* 38:
469 858–871, 2017.
- 470 33. **Sixt M, Engelhardt B, Pausch F, Hallmann R, Wendler O, Sorokin LM.** Endothelial cell laminin
471 isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain
472 barrier in experimental autoimmune encephalomyelitis. *J Cell Biol* 153: 933–946, 2001.
- 473 34. **Slats AM, Janssen K, Van SA, van der Plas DT, Schot R, van den Aardweg JG, de Jongste JC,**
474 **Hiemstra PS, Mauad T, Rabe KF, Sterk PJ.** Expression of smooth muscle and extracellular
475 matrix proteins in relation to airway function in asthma. *J Allergy Clin Immunol* 121: 1196–
476 1202, 2008.
- 477 35. **Telenga ED, Oudkerk M, van Ooijen PMA, Vliegenthart R, ten Hacken NHT, Postma DS, van**
478 **den Berge M.** Airway wall thickness on HRCT scans decreases with age and increases with
479 smoking. *BMC Pulm Med* 17: 27, 2017.

- 480 36. **Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK.** An improved murine model of
481 asthma: selective airway inflammation, epithelial lesions and increased methacholine
482 responsiveness following chronic exposure to aerosolised allergen. *Thorax* 53: 849–56, 1998.
- 483 37. **Teoh CM, Tan SSL, Langenbach SY, Wong AH, Cheong DHJ, Tam JKC, New CS, Tran T.** Integrin
484 $\alpha 7$ expression is increased in asthmatic patients and its inhibition reduces Kras protein
485 abundance in airway smooth muscle cells. *Sci Rep* 9: 9892, 2019.
- 486 38. **Thompson RD, Noble KE, Larbi KY, Dewar A, Duncan GS, Mak TW, Nourshargh S.** Platelet-
487 endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and
488 cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement
489 membrane. *Blood* 97: 1854–60, 2001.
- 490 39. **Tran T, Ens-Blackie K, Rector ES, Stelmack GL, McNeill KD, Tarone G, Gerthoffer WT, Unruh
491 H, Halayko AJ.** Laminin-binding Integrin $\{\alpha\}7$ is Required for Contractile Phenotype
492 Expression by Human Airway Myocyte. *Am J Respir Cell Mol Biol* 37: 668–680, 2007.
- 493 40. **Tran T, McNeill KD, Gerthoffer WT, Unruh H, Halayko AJ.** Endogenous laminin is required for
494 human airway smooth muscle cell maturation. *RespirRes* 7: 117, 2006.
- 495 41. **Tran T, Teoh CM, Tam JK, Qiao Y, Chin CY, Chong OK, Stewart AG, Harris T, Wong WS, Guan
496 SP, Leung BP, Gerthoffer WT, Unruh H, Halayko AJ.** Laminin drives survival signals to
497 promote a contractile smooth muscle phenotype and airway hyperreactivity. *FASEB J* 27:
498 3991–4003, 2013.
- 499 42. **Virtanen I, Laitinen A, Tani T, Paakko P, Laitinen LA, Burgeson RE, Lehto VP.** Differential
500 expression of laminins and their integrin receptors in developing and adult human lung. *Am J
501 Respir Cell Mol Biol* 15: 184–196, 1996.
- 502 43. **Wang S, Voisin MB, Larbi KY, Dangerfield J, Scheiermann C, Tran M, Maxwell PH, Sorokin L,
503 Nourshargh S.** Venular basement membranes contain specific matrix protein low expression
504 regions that act as exit points for emigrating neutrophils. *J Exp Med* 203: 1519–1532, 2006.
- 505 44. **Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, Olson EN.** Myocardin and

- 506 ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature*
507 428: 185–189, 2004.
- 508 45. **Warren KJ, Iwami D, Harris DG, Bromberg JS, Burrell BE.** Laminins affect T cell trafficking and
509 allograft fate. *J Clin Invest* 124: 2204–2218, 2014.
- 510 46. **Wright DB, Meurs H, Dekkers BGJ.** Integrins: therapeutic targets in airway
511 hyperresponsiveness and remodelling? *Trends Pharmacol Sci* 35: 567, 2014.
- 512 47. **Wright DB, Trian T, Siddiqui S, Pascoe CD, Johnson JR, Dekkers BG, Dakshinamurti S, Bagchi**
513 **R, Burgess JK, Kanabar V, Ojo OO.** Phenotype modulation of airway smooth muscle in
514 asthma. *Pulm Pharmacol Ther* 26: 42–49, 2013.
- 515 48. **Wright DB, Trian T, Siddiqui S, Pascoe CD, Ojo OO, Johnson JR, Dekkers BG, Dakshinamurti**
516 **S, Bagchi R, Burgess JK, Kanabar V.** Functional phenotype of airway myocytes from asthmatic
517 airways. *Pulm Pharmacol Ther* 26: 95–104, 2013.
- 518 49. **Wu C, Ivars F, Anderson P, Hallmann R, Vestweber D, Nilsson P, Robenek H, Tryggvason K,**
519 **Song J, Korpos E, Loser K, Beissert S, Georges-Labouesse E, Sorokin LM.** Endothelial
520 basement membrane laminin alpha5 selectively inhibits T lymphocyte extravasation into the
521 brain. *Nat Med* 15: 519–527, 2009.
- 522 50. **Wu Y, Huang Y, Herring BP, Gunst SJ.** Integrin-linked kinase regulates smooth muscle
523 differentiation marker gene expression in airway tissue. *Am J Physiol Lung Cell Mol Physiol*
524 295: L988–L997, 2008.
- 525 51. **Yick CY, Ferreira DS, Annoni R, von der Thusen JH, Kunst PW, Bel EH, Lutter R, Mauad T,**
526 **Sterk PJ.** Extracellular matrix in airway smooth muscle is associated with dynamics of airway
527 function in asthma. *Allergy* 67: 552–559, 2012.

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.

534 **Figure legends**

535 **Figure 1** *Laminin $\alpha 4$ and $\alpha 5$ are expressed in human airways.* Stainings were performed on
536 tracheal sections were obtained from one lung transplantation donor. (A) Localization of pan-laminin
537 (green) in the basement membranes of the endothelium (Endo), epithelium (Epi), submucosal glands
538 (SG) and airway smooth muscle (ASM). (B) Localization of laminin $\alpha 4$ (green) in the basement
539 membrane of the endothelium and airway smooth muscle. (C) Expression of laminin $\alpha 5$ (red) in the
540 basement membrane of the epithelium, endothelium, submucosal glands and airway smooth
541 muscle. Pictures were taken at a 400 \times magnification. Blue: nuclei.

542

543 **Figure 2** *Laminin $\alpha 4$ is involved in the induction of a pro-fibrotic, pro-proliferative, and pro-*
544 *contractile ASM phenotype in vitro.* (A) Laminin mRNA expression by human ASM cells. (B) Laminin
545 $\alpha 4$ mRNA expression is reduced by lentiviral shRNA directed against this laminin. No effects were
546 observed for lentiviral shRNA directed against laminin $\alpha 5$. (C) Laminin $\alpha 5$ mRNA expression is
547 reduced by lentiviral shRNA directed against this laminin. Lentiviral shRNA directed against laminin
548 $\alpha 4$ increased laminin $\alpha 5$ mRNA expression. (D) Cell number is reduced in laminin $\alpha 4$ deficient cells.
549 (E) Regulation of sm-MHC, calponin, sm- α -actin and fibronectin mRNA expression in laminin $\alpha 4$ and
550 $\alpha 5$ deficient cells. (F-H) Protein expression of sm- α -actin, calponin and fibronectin is reduced in
551 laminin $\alpha 4$ deficient cells. Data represent means \pm SEM of 4-12 experiments. * $P < 0.05$, ** $P < 0.01$,
552 *** $P < 0.001$ compared to scrambled shRNA transfected cells.

553

554 **Figure 3** *Laminin $\alpha 4$ and $\alpha 5$ are involved in airway smooth muscle accumulation in vivo.* (A)
555 Experimental animal procedures. Female BALB/c were sensitized to ovalbumin (OVA) on Days 1, 14,
556 and 21. For the acute protocol, mice were challenged with saline or 1% OVA aerosols for 20 minutes
557 on days 28-30. On day 32, animals were exposed to saline or 5% OVA and sacrificed 6 hours
558 thereafter. For the chronic protocol, animals were exposed to saline or 1% OVA aerosols twice
559 weekly from days 26 to 48. Mice were sacrificed 24 hours after the last challenge. Laminin $\alpha 4$ or $\alpha 5$

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 α 4 and α 5 staining
562 in mouse lung tissue after repeated ovalbumin challenge. Localization of laminin α 4 (green) in the
563 basement membrane (BM) of the alveoli (AV) and airway smooth muscle (ASM). Expression of
564 laminin α 5 (red) in the BM of the alveoli, epithelium (Epi) and ASM. Pictures were taken at 400 \times
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 \pm SEM of 3-6
568 animals.

569

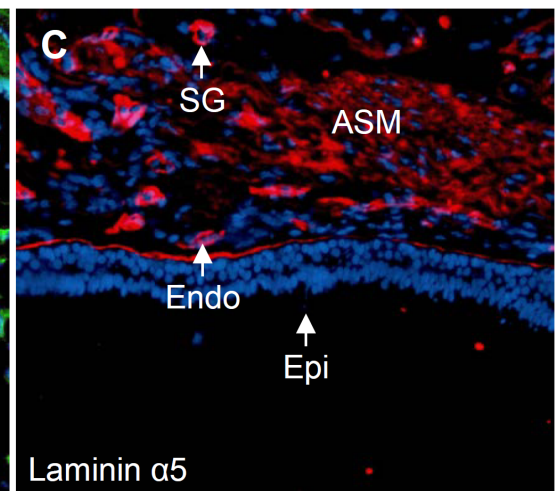
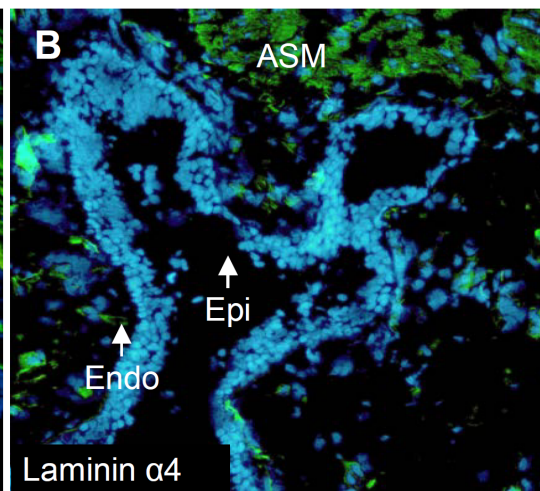
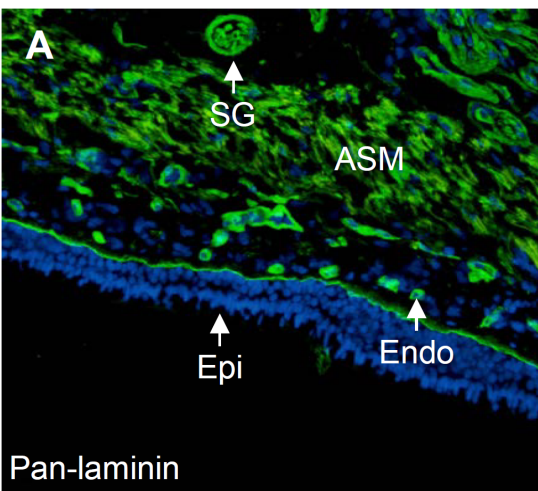
570 **Figure 4** *Basal airway eosinophil numbers and allergen-induced increases in eosinophils are*
571 *inhibited by laminin α 4 blocking antibodies. (A) Effects of laminin function-blocking antibodies on*
572 *acute allergen-induced airway infiltration of eosinophils. (B) Effects of laminin function-blocking*
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 \pm SEM of 3-6 animals. BM: basement membrane.*

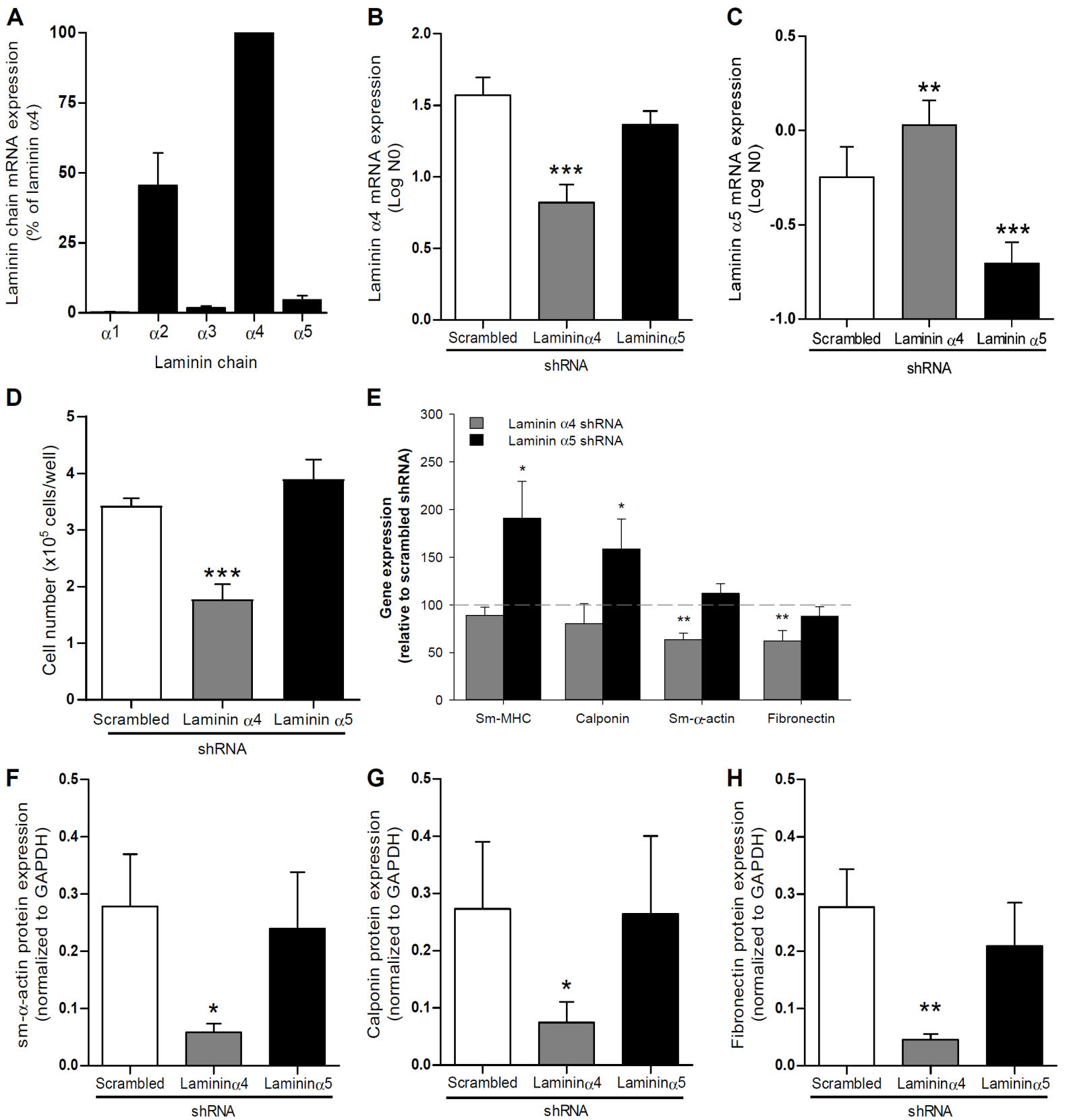
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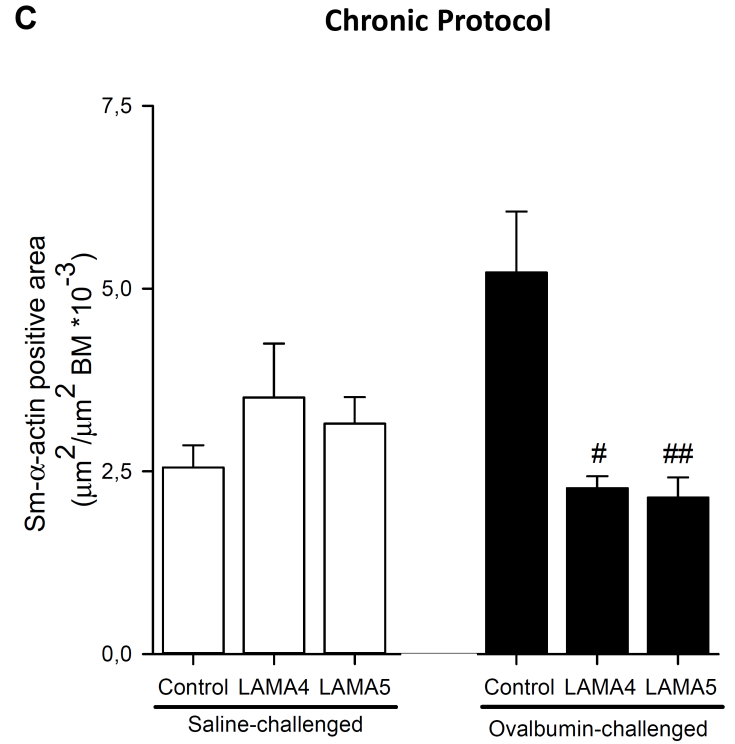
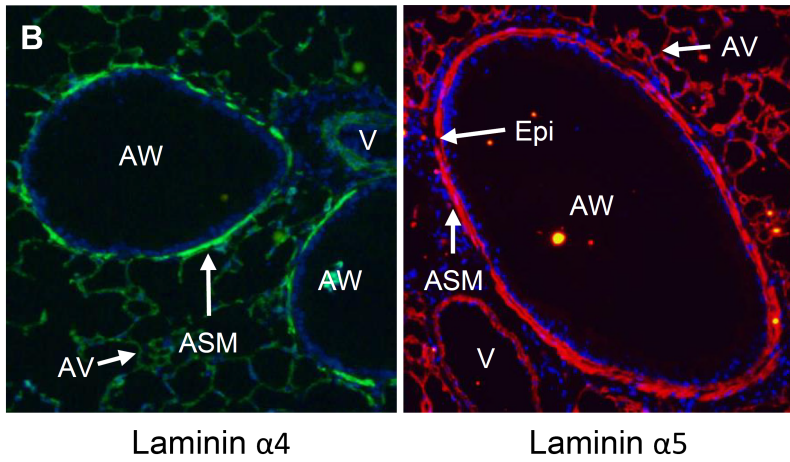
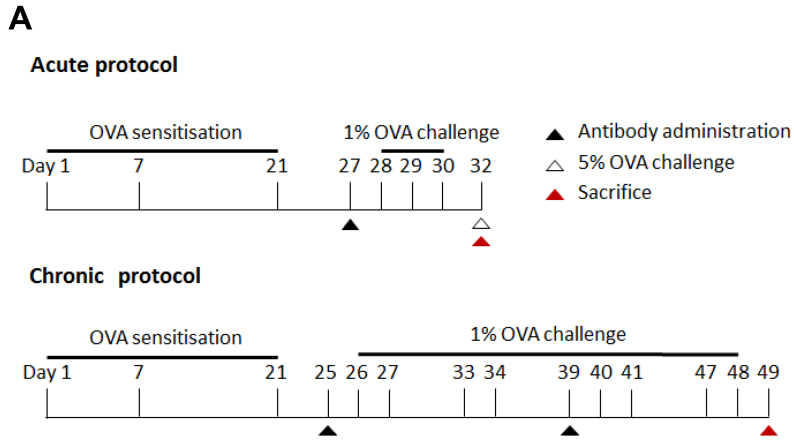
577 **Figure 5** *Laminin α 4 scoring is associated with clinical characteristics of asthmatic patients.*
578 *(A,B) Representative photographs of staining for (A) laminin α 4 and (B) laminin α 5 in ASM,*
579 *epithelium and endothelium in biopsy sections. (C,D) High (score 3-4) laminin α 4 staining is*
580 *associated with reduced lung function of asthmatic patients expressed as both (C) FEV₁ %predicted*
581 *and (D) FEV₁/FVC %predicted. (E) High (score 3-4) laminin α 4 staining is associated with increased*
582 *airway reactivity of asthmatic patients to adenosine monophosphate (AMP). (F) High (score 3-4)*
583 *laminin α 4 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*

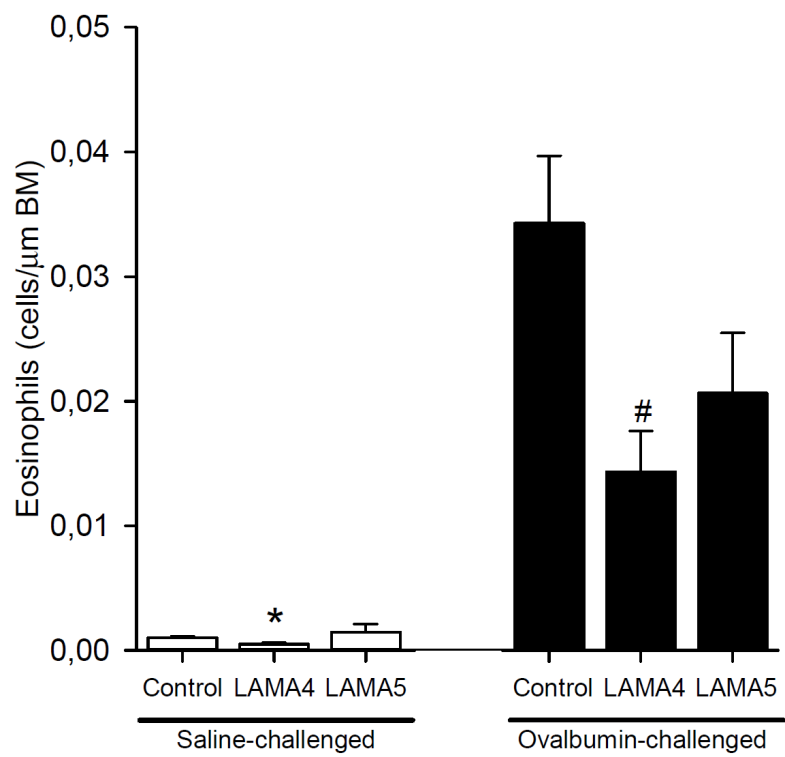
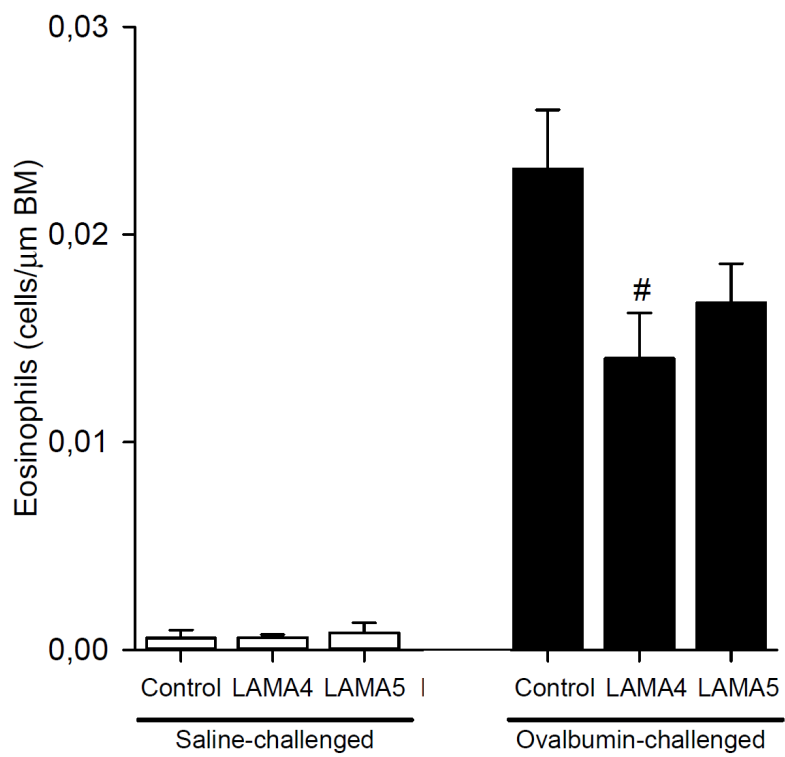
585 in Figs C-F. $**P<0.01$, $***P<0.001$. Examples of staining intensity scores 1-4 are shown in
586 supplemental Figure S1.

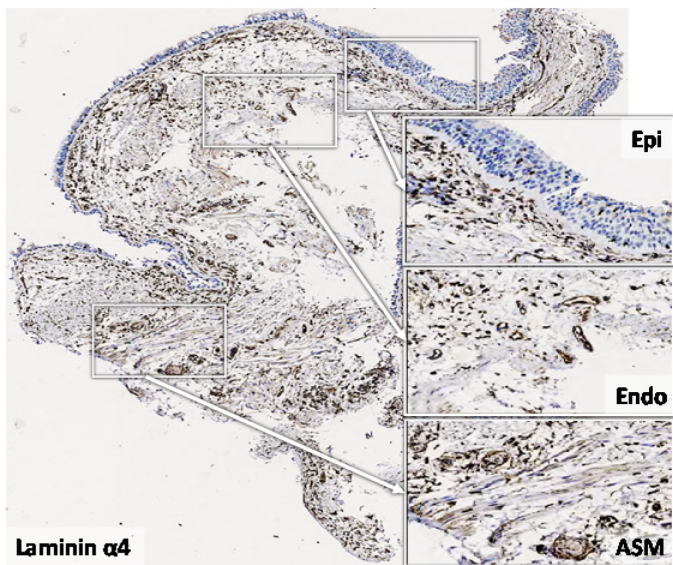
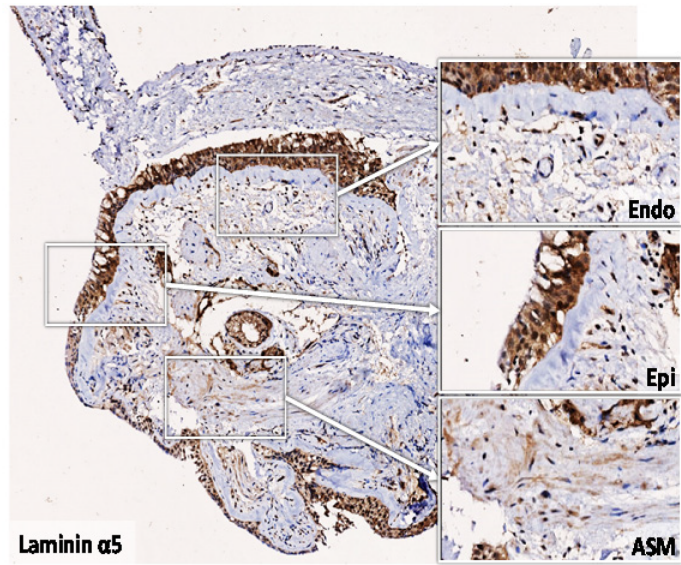
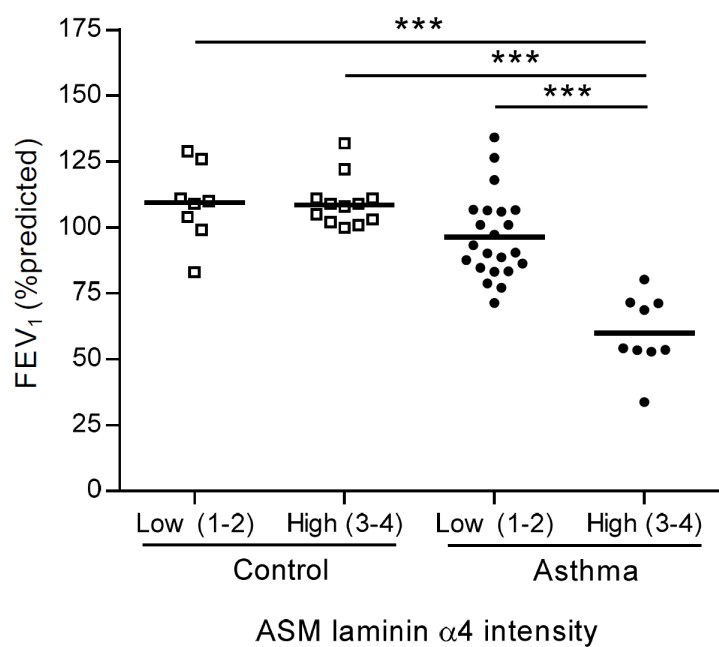
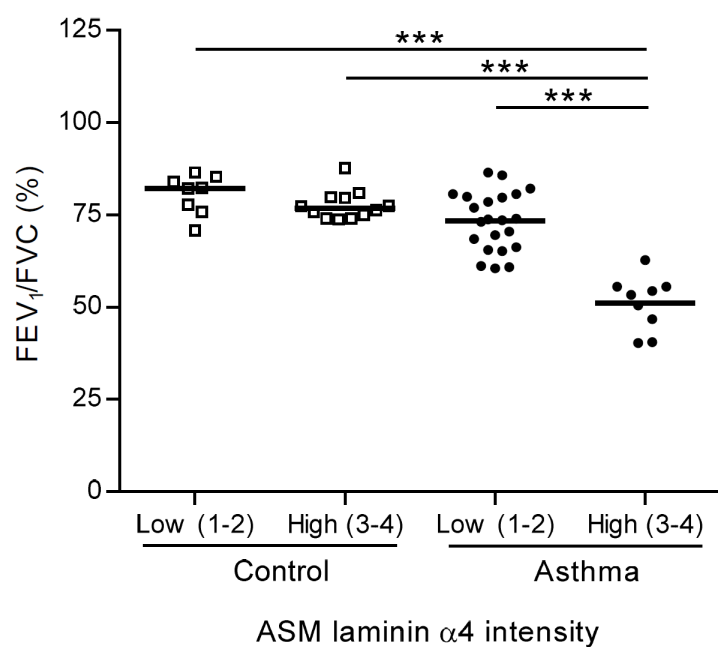
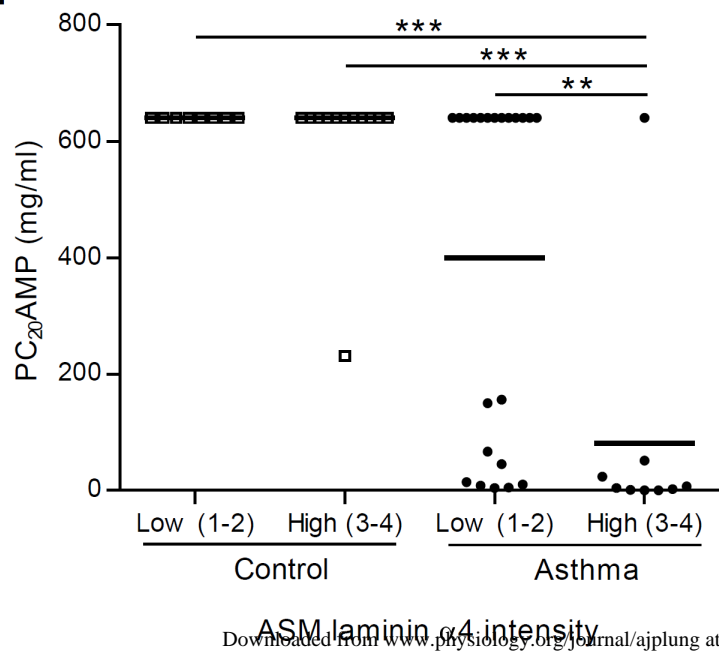
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A**B**

A**B****C****D****E****F**