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Am J Physiol Lung Cell Mol Physiol 296:763-770, 2009. First published Mar 13, 2009;
doi:10.1152/ajplung.90638.2008

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A severe deficiency of coagulation factor VIIa results in attenuation of the asthmatic response in mice

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Submitted 24 December 2008; accepted in final form 10 March 2009

Shinagawa K, Ploplis VA, Castellino FJ. A severe deficiency of coagulation factor VIIa results in attenuation of the asthmatic response in mice. *Am J Physiol Lung Cell Mol Physiol* 296: L763–L770, 2009. First published March 13, 2009; doi:10.1152/ajplung.90638.2008.—Eosinophil counts in the bronchoalveolar lavage fluid of wild-type (WT) mice increased after ovalbumin (OVA) challenge, a response that was diminished in comparably challenged low-expressing coagulation factor VII (FVII^{TA/TA}) mice. Levels of T helper type 2 (Th2) cytokines, IL-4, IL-5, and IL-13, and eosinophil-attracting chemokines, eotaxin and RANTES, were also lower in the OVA-challenged FVII^{TA/TA} mice. Eosinophils purified from low-FVII mice underwent apoptosis at a faster rate compared with WT eosinophils, and eosinophil migration in response to eotaxin was reduced in eosinophils obtained from FVII^{TA/TA} mice. Airway hyperresponsiveness and mucous layer thickness were reduced in OVA-treated FVII^{TA/TA} mice, and addition of exogenous coagulation factor X (FX) enhanced mucin production in human epithelial NCI-H292 cells. Correspondingly, incubation of FX with NCI-H292 cells resulted in activated (a) FX production, suggesting that the components required for FX activation were present on NCI-H292 cells. These results demonstrate that FVIIa functions in the asthmatic response to an allergen by stimulating lung eosinophilia, airway hyperresponsiveness, and mucin production, this latter effect through its ability to activate FX in conjunction with tissue factor.

coagulation factor X; eosinophilia; T helper type 2 cytokines; chemokines; airway

NUMEROUS REPORTS INDICATE an association of hemostasis pathway components with inflammatory responses. In the case of asthma, some studies indicate that coagulation- and fibrinolysis-related proteins contribute to its pathogenesis. Plasminogen activator inhibitor-1 (PAI-1) levels are increased during asthma and contribute to lung fibrosis (4, 11, 16). Furthermore, activated (a) anticoagulant protein C levels are decreased in asthmatic patients, and inhaled activated protein C diminished the asthmatic response in mice (7, 32). Fibrin deposition and abnormalities in the coagulation and fibrinolytic pathways in the distal airways of the lung significantly contribute to airway hyperresponsiveness (AHR) and airway closure in asthma (31), and extravascular fibrinogen and thrombin have been identified in the sputa of patients with asthma (5, 19). Thrombin activity was increased in bronchoalveolar lavage fluid (BALF) as a result of segmental antigen provocation in asthmatic patients, and BALF from these patients induced proliferation of fibroblasts (29). In addition, we reported that activated coagulation factor X (FXa) is associated with airway remodeling (26). Specifically, we found that FX mRNA levels are increased in lungs of asthmatic mice,

and FXa activity is increased in the BALF of asthmatic mice. A FXa inhibitor ameliorated AHR by decreasing the thickness of the airway wall and by attenuating collagen deposition in the lung, although the number of eosinophils in the BALF of challenged mice was not affected. In NCI-H292 cells, mucin (MUC5AC) production was increased by stimulation with FXa (26).

The tissue factor (TF)/coagulation factor VII (FVII) complex is known to be a physiological pathway for FXa production. Recently, a study demonstrated that FVII is expressed on airway epithelial cells and is associated with epithelial repair (18). In that study, it was found that epithelial cells constitutively expressed TF, and FVII mRNA increased subsequent to mechanical damage in normal human bronchial epithelial (NHBE) cells and 16HBE14o– cells. Although TF and FVIIa exist in the airway, there are no reports indicating whether these proteins function during asthma. Thus the present investigation was undertaken with a focus on examining the FVII/TF system in the airway and assessing the role of FVIIa in the asthmatic response to an allergen. To assist these studies, we advantageously employed mice (FVII^{TA/TA}) that were genetically engineered to produce very low levels of FVII.

MATERIALS AND METHODS

Mice. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Notre Dame. C57BL/6 female FVII^{TA/TA} mice and wild-type (WT) controls (7–10 wk old) were used in this study (23). FVII levels in FVII^{TA/TA} mice were <2% of WT levels.

Induction of eosinophil-based inflammation by intranasal administration of OVA. The mice were immunized and boosted by subcutaneous injection with 10 µg of ovalbumin (OVA; grade V; Sigma-Aldrich, St. Louis, MO) adsorbed onto Al(OH)₃ gel (Serva), and this OVA/alum was administered on days 0 and 14 (26). At day 21, mice were challenged with OVA (50 µg) intranasally. The lungs were lavaged four times with sterile PBS at 6, 12, or 24 h after OVA challenge. The resulting BALF was applied to cytospin slides and stained with Diff-Quick. Total and differential cell counts were determined.

In the chronic model, mice were immunized with OVA/alum and given OVA intranasally 3 days/wk for 2 wk. Mice were killed 24 h after the final OVA challenge.

Extravasation of Evans blue dye into the lung. Mice were injected intravenously with 2 mg/kg Evans blue (EB) immediately after OVA or PBS challenge and killed 24 h later to obtain blood and BALF. EB concentrations in serum and BALF (100-µl aliquots) were determined spectrophotometrically at 620 nm in 96-well microtiter plates.

Inflammatory and coagulation mediators in BALF. IL-4, IL-5, IL-13, IFN-γ, RANTES, and eotaxin levels in BALF supernatants were measured by ELISA using commercial kits (R&D Systems, Minneapolis, MN). Levels of the thrombin-antithrombin complex (TAT) were determined using a TAT ELISA kit [Enzyme Research Laboratories (ERL), South Bend, IN].

Eosinophil purification and transmigration assay. Eosinophils were purified to >98% of total cells from BALF of OVA-challenged

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mice as described previously (25, 26). Transmigration of eosinophils through basement membrane components was evaluated in 24-well BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA; Ref. 6). For these experiments, eosinophils (0.25 ml of 1.0×10^6 cells/ml) were incubated with or without IL-5 (10 ng/ml) for 30 min at 37°C , placed in the upper chamber of the Matrigel Invasion Chamber, and incubated for 18 h. Eotaxin was used as a chemotactic factor and added separately (all in 0.75 ml) to the lower chambers. At the conclusion of the incubation, cells in both the upper and lower chambers were obtained and counted on a hemocytometer. Under each set of conditions, the percent transmigration was calculated by dividing the number of cells in the lower chamber by the number of cells in the lower chamber of a control invasion chamber without the Matrigel membrane $\times 100$.

Apoptosis assay. Eosinophils (5.0×10^5 cells/ml in 0.1 ml) were incubated with IL-5 (100 pg/ml ; PeproTech, Rocky Hill, NJ) for 24 h, and the number of cells that underwent apoptosis was assessed using the Guava EasyCyte Caspase 3/7 Kit and the Guava PCA system (Guava Technologies, Hayward, CA). The kit utilizes two stains, fluorescent-labeled inhibitor of caspases (FLICA) and propidium iodide (PI), to quantitate the percentage of apoptotic cells. FLICA covalently binds to active caspase 3/7 and was used to stain healthy cells (FLICA $-$, PI $-$) and early-phase apoptotic cells (FLICA $+$ /PI $-$). PI was used to stain late-phase apoptotic cells (FLICA $+$ /PI $+$) and dead cells (FLICA $-$ /PI $+$).

Quantitative RT-PCR. Quantitative RT-PCR (Q-RT-PCR) was performed as previously described (26). Total RNA was isolated from lungs by the RNeasy total RNA purification kit (Qiagen, Valencia, CA). Q-RT-PCR for evaluation of the crossing points for each reaction of each gene was carried out with the primers and probes listed in Table 1 using an ABI Prism 7500 Sequence Detector.

AHR. AHR was measured at 24 h after the last intranasal antigen challenge as previously reported (25, 26). Tracheostomized mice were placed on a Harvard ventilator and inserted inside a whole body plethysmograph (Buxco Electronics, Troy, NY), and lung resistance (RL) to inhaled methacholine ($1\text{--}50 \text{ mg/ml}$; Sigma-Aldrich) was measured.

Histochemistry and measurement of airway wall thickness. Lungs were obtained 24 h after the final OVA administration, fixed with periodate-lysine-paraformaldehyde (PLP), embedded in paraffin, sectioned at a thickness of $4 \mu\text{m}$, and stained with Elastica van Gieson for measurements of airway wall thickness. Each treatment group contained five to nine stained lung sections from each mouse (25, 26). Cross sections of airways were used for measuring the airway wall

thickness (26). FVII immunostaining was performed with rabbit anti-human FVII polyclonal antibody (in-house, $2.9 \mu\text{g/ml}$) as the primary antibody and anti-rabbit IgG as a negative control. Biotinylated swine anti-rabbit IgG (Dako, Carpinteria, CA) was used as a secondary antibody, and detection was achieved with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA).

Mucin production from NCI-H292 cells. To quantify mucin production, human pulmonary mucoepidermoid carcinoma NCI-H292 cells [CRL-1848; American Type Culture Collection (ATCC)] were seeded at 1×10^5 cells/ml in 96-well plates in RPMI 1640 medium/10% FBS/antibiotics, and the MUC5AC content in the cells was measured as described previously (26). After confluency, the cells were washed twice with PBS, and the medium was changed to FBS-free RPMI 1640/antibiotics. After 24 h, the cells were stimulated for 48 h with FX (ERL), FXa (ERL), FVIIa (ERL), and EGF (PeproTech) and fixed with formalin. The cells were incubated overnight with mouse anti-human MUC5AC (45M1; Lab Vision, Fremont, CA) and then washed and incubated with biotinylated rabbit anti-mouse IgG (Vector Laboratories). Detection was achieved using the ABC-AP kit and the substrate, *p*-nitrophenyl phosphate (Vector Laboratories). Maximal production of MUC5AC was obtained from cells stimulated with $1 \mu\text{g/ml}$ EGF.

Assay of FXa activity. Human bronchial epithelial BEAS-2B cells (ATCC CRL-9609) or NCI-H292 cells were cultured at 1×10^5 cells/ml in RPMI 1640 medium/10% FBS/antibiotics. After confluency, the cells were washed with PBS and incubated for 3 h in serum-free RPMI 1640 media. Following this, 30 U/ml FX (ERL) was added for times ranging from 10 min to 24 h, and FXa was measured in the supernatants. FXa activity was determined utilizing a chromogenic assay with substrate, S-2222 (Chromogenix, Lexington, MA). Human FXa ($0.001\text{--}1 \text{ U/ml}$) was used as the standard.

Data analysis. Data were analyzed using the Student's *t*-test and are represented as the means $\pm$ SE. ANOVA with Dunnett's test for multiple comparisons was also used for determining significance. $P < 0.05$ was considered statistically significant.

RESULTS

Assessment of inflammatory cells in BALF of OVA-treated mice. Allergen-induced asthmatic responses of low-expressing ($\sim 2\%$ of WT) mice (FVII^{TA/TA}) were compared with WT mice after immunization with OVA/alum and intranasal chal-

Table 1. Sequences of primers and probes used for Q-RT-PCR

Gene	Primer/Probe Sequences	Gene Positions	Amplicon Sizes, bp
<i>h/mRPL19</i> (NM_009078)			
Forward	ATGTATCACAGCCTGTACCTG	355–375	233
Probe*	TTTCGTGCTTCCTTGGTCTTAGACCT	487–512	
Reverse	TTCTTGGTCTCCTCCTCCTTG	567–587	
<i>mTF</i> (NM_010171)			
Forward	CTCCTCCTCCAGGTGATCG	1267–1285	99
Probe†	AGGCATTCCAGAGAAAGCGTTTAAATTTAACTTGG	1293–1326	
Reverse	GGGTTGCCACTCCAAAATTG	1346–1365	
<i>mFVII</i> (NM_010172)			
Forward	TGTCCTGCAAACCAAAAGTTGA	512–533	101
Probe†	TTTTCTACAACAGGTATTCTCCACACGGGTA	535–566	
Reverse	CACGTTGCCTCCCACAATG	594–612	
<i>hMUC5AC</i> (XM_001130382)			
Forward	TACTCCACAGACTGCACCAACTG	1272–1294	129
Probe†	TGTGCTTGAGGTGCCCACTTCTCAA	1352–1377	
Reverse	CGTGTATTGCTTCCCGTCAA	1381–1400	

Probes were labeled at the 5' end with 6-FAM and at the 3' end with *black hole quencher-1 (BHQ-1) or †5-carboxytetramethylrhodamine (5-TAMRA). Q-RT-PCR, quantitative RT-PCR.

challenge with OVA. The number of inflammatory cells in BALF was determined at times of 0, 6, 12, and 24 h after OVA challenge. Eosinophil infiltration in WT mice was detected as early as 12 h and was highly increased at 24 h. In FVII^{TA/TA} mice, eosinophil infiltration was significantly attenuated at 24 h compared with WT mice (Fig. 1A). In mice challenged with OVA for 24 h, the total cell counts increased in WT mice to a greater extent than in FVII^{TA/TA} mice (Fig. 1B). In addition to the increases in eosinophils, the number of neutrophils was increased in 24-h-challenged mice, but these differences between WT and FVII^{TA/TA} mice were not significant at this time point. The numbers of macrophages and lymphocytes were also not different between the two genotypes at the 24-h time point. The extravasation of EB dye from blood to BALF was significantly increased in OVA-challenged WT mice compared with PBS-challenged mice (Fig. 1, C and D), showing blood component leakage into asthmatic lungs resulting from allergen challenge. In OVA-challenged FVII^{TA/TA} mice, EB levels in BALF were significantly less than those from OVA-challenged WT mice.

Inflammatory mediators in BALF of OVA-treated mice. Cytokine levels in BALF were obtained using ELISA. Levels of T helper type 2 (Th2) cytokines, viz., IL-4, IL-5, and IL-13,

were substantially decreased in FVII^{TA/TA} mice after OVA challenge compared with OVA-challenged WT mice (Fig. 2, A and C). Th1 cytokine IFN- γ levels were similar between WT and FVII^{TA/TA} mice (data not shown). The levels of the eosinophil-attracting chemokines, eotaxin and RANTES, were decreased in FVII^{TA/TA} mice compared with OVA-challenged WT mice (Fig. 2, D and E). TAT levels were significantly lower in FVII^{TA/TA} mice than in WT mice (Fig. 2F).

Migration and survival of eosinophils. Migration of eosinophils from the plasma to the lung is a critical characteristic of asthma. To investigate the effect of FVIIa on eosinophil migration, eosinophils were purified from the BALF of OVA-challenged mice, and transmigration of the isolated eosinophils was measured using Matrigel Invasion Chambers. Eosinophils migrated to the lower chamber after stimulation by eotaxin. In concert with the in vivo experiments, FVII^{TA/TA}-eosinophil migrations were significantly decreased compared with WT eosinophils (Fig. 3A).

As eosinophil survival is also an important factor for eosinophilia, eosinophils obtained from WT and FVII^{TA/TA} mice were incubated with IL-5 (100 pg/ml) for 24 h, after which apoptosis was assessed using caspase 3/7 and PI as markers of apoptosis. The viability of eosinophils obtained from

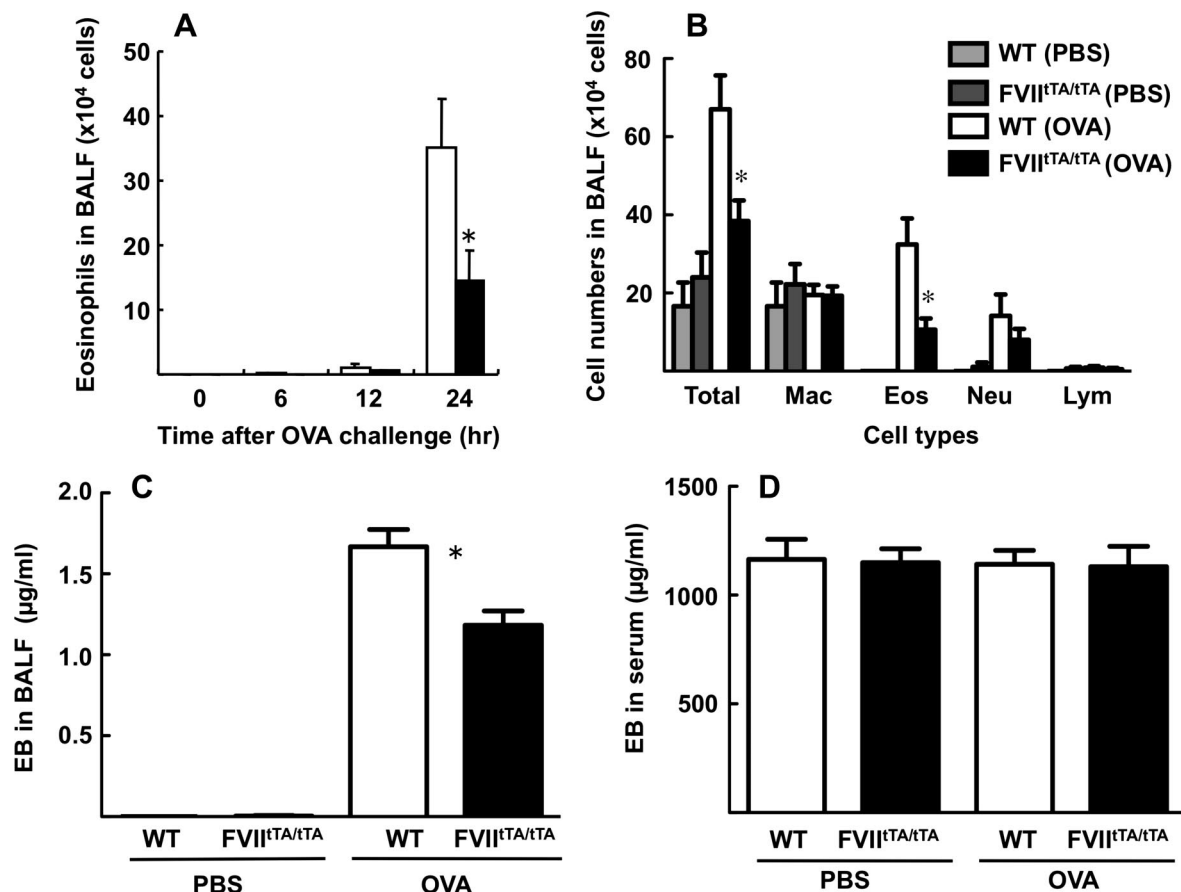


Fig. 1. Inflammatory cells in bronchoalveolar lavage fluid (BALF) of ovalbumin (OVA)-challenged mice. A: eosinophil counts in BALF from mice challenged with OVA intranasally. Lavage was performed at the indicated times after OVA challenge in wild-type (WT; white bars) and low-expressing coagulation factor VII (FVII^{TA/TA}; black bars) mice. BALF cells were counted and centrifuged onto microscope slides using a cytopsin. The preparations were stained with Diff-Quick to obtain differential leukocyte counts. B: total, macrophage (Mac), eosinophil (Eos), neutrophil (Neu), and lymphocyte (Lym) counts in BALF. BALF samples were obtained 24 h after OVA challenge. The data represent the means $\pm$ SE ($n = 9$). * $P < 0.05$ vs. OVA-challenged WT mice. C and D: extravasation of Evans blue (EB) dye from blood to the BALF. BALF (C) and serum (D) samples were obtained 24 after OVA or PBS challenge. The data represent the means $\pm$ SE ($n = 6$). * $P < 0.05$ vs. PBS-challenged WT mice.

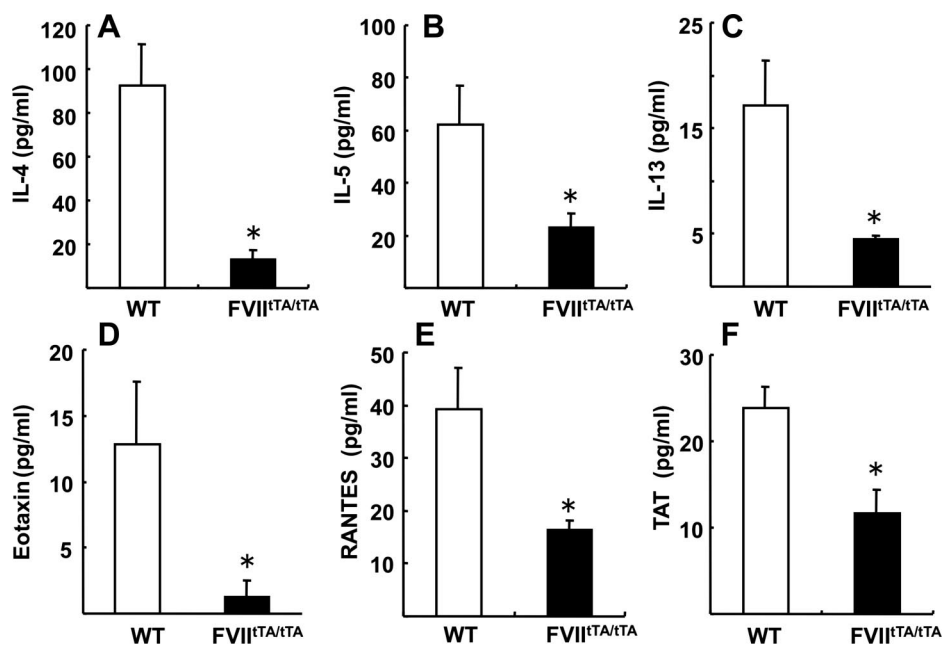


Fig. 2. Cytokine levels in the BALF of OVA-exposed mice. IL-4 (A), IL-5 (B), IL-13 (C), eotaxin (D), RANTES (E), and thrombin-antithrombin complex (TAT; F) levels in BALF were obtained from WT and FVII^{TΔ/TΔ} mice 12 h after OVA challenge. The data represent the means $\pm$ SE ($n = 5-7$) for each group. * $P < 0.05$ of OVA-treated FVII^{TΔ/TΔ} mice vs. similarly treated WT mice.

FVII^{TΔ/TΔ} mice was diminished compared with eosinophils obtained from WT mice, with most cells in late stages of apoptosis at the time period chosen (Fig. 3B). The apoptosis differences between WT and FVII^{TΔ/TΔ} mice are small but are in a direction that can be contributory to the mechanism of the viability differences noted.

Assessment of inflammatory cells in BALF of multiple OVA-treated mice. Single OVA challenge is an appropriate model for assessing the numbers of migrated inflammatory cells but is not suitable for determining this event in chronic asthma. Therefore, mice were immunized with OVA/alum and challenged with OVA intranasally for 0–3 wk (3 times per week). The number of inflammatory cells in BALF was determined 24 h after final OVA challenge. Eosinophil numbers in BALF peaked at 2 wk but then declined despite continuous OVA exposure throughout 3 wk (Fig. 4A). Since this phenomenon was the same as reported previously (26), we analyzed mice challenged for 2 wk for further analysis. In FVII^{TΔ/TΔ} mice, eosinophil counts in BALF were significantly attenuated in 1- and 2-wk post-OVA exposure compared with WT mice (Fig. 4A).

mRNA levels of coagulation FVII in OVA-treated mice. Lungs were removed from OVA-treated C57BL/6 mice after 2-wk exposure to OVA, and the mRNA was extracted. The levels of transcripts encoding FVII and TF were analyzed by Q-RT-PCR and compared with PBS-exposed mice (Fig. 4, B and C, respectively). The mRNA levels of FVII in PBS-challenged FVII^{TΔ/TΔ} mice were lower compared with PBS-challenged WT mice (Fig. 4B). The mRNA levels of FVII in OVA-challenged WT mice were significantly increased, by $\sim 20\times$, compared with the PBS WT and PBS FVII^{TΔ/TΔ} controls (Fig. 4B). FVII mRNA levels remained low in OVA-challenged FVII^{TΔ/TΔ} mice and were not significantly different from those values in PBS-treated FVII^{TΔ/TΔ} mice. Furthermore, the mRNA of TF in WT mice increased $\sim 2\times$ compared with PBS-treated WT mice but was not different from the WT control in PBS or OVA-challenged FVII^{TΔ/TΔ} mice (Fig. 4C).

Histological analysis of coagulation factors in lung. Histological analyses were performed on lung tissues of mice that were challenged with OVA for 2 wk. The mucous layer of these mice was thickened compared with PBS-challenged mice. For the immunohistochemical analysis of FVII, lungs were removed 24 h after the final challenge. FVII was expressed in bronchial epithelial cells in both PBS- and OVA-treated WT mice (Fig. 4, D and E), with more intense staining in the OVA-treated lungs.

AHR in FVII^{TΔ/TΔ} mice. Respiratory functions in FVII^{TΔ/TΔ} mice were assessed 24 h after the final antigen provocation in 2-wk antigen-treated mice. The RL of OVA-challenged WT mice was significantly higher than that of PBS-challenged WT mice after exposure to increasing concentrations of aerosolized methacholine (Fig. 5A). In FVII^{TΔ/TΔ} mice, the RL in OVA-treated mice was increased compared with PBS-challenged FVII^{TΔ/TΔ} mice. However, the response to methacholine was significantly attenuated in OVA-challenged FVII^{TΔ/TΔ} mice compared with WT mice.

Measurements of airway-wall thickness in chronically OVA-exposed mice. To quantify the thickness of the airway wall, cross-sectional areas were obtained using computer graphic analysis of Elastica van Gieson-stained sections as reported previously (25, 26). In WT mice, the thickness of the mucous layer was increased by $\sim 2.5\times$ by 2 wk of OVA challenge compared with that of a control PBS challenge (Fig. 5B). In FVII^{TΔ/TΔ} mice, the cross-sectional areas of the mucous layers were increased significantly in OVA-challenged mice. However, the mucous layers in OVA-challenged FVII^{TΔ/TΔ} mice were significantly less compared with OVA-challenged WT mice. On the other hand, the thickness of the smooth muscle layer was not altered by OVA challenge in both WT and FVII^{TΔ/TΔ} mice (Fig. 5C).

Mucin production in NCI-H292 cells. To assess regulation of mucin production in a simplified system, cells from a human mucin-producing cell line (NCI-H292) were seeded on 96-well plates, and mucin production was measured after stimulation with FX or FXa (Fig. 6). MUC5AC production was signifi-

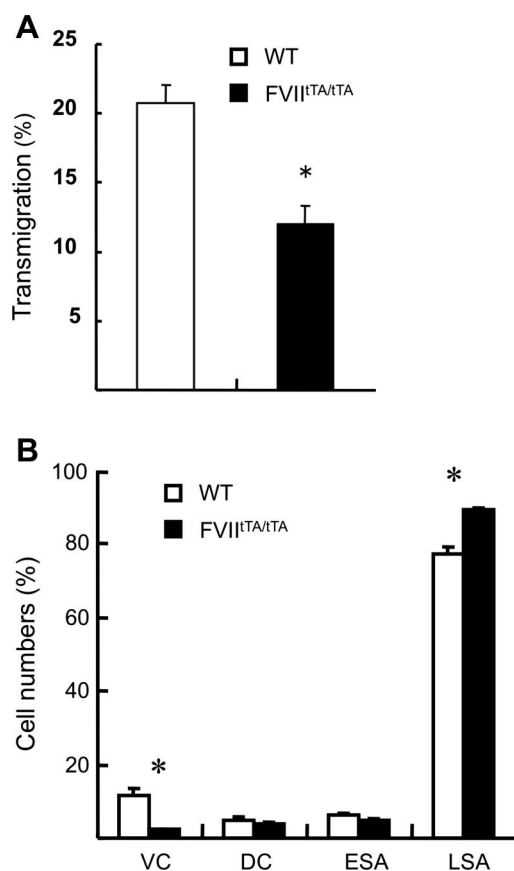


Fig. 3. Eosinophil migration and survival. **A:** the effect of activated (a) FVII in eotaxin-induced eosinophil migration through Matrigel basement membrane was examined. Eosinophils obtained from WT (white bars) or FVII^{tTA/tTA} mice (black bars) were preincubated with IL-5, added to the upper chamber, and incubated for 18 h at 37°C. The data represent the means $\pm$ SE ($n = 4$) for each group. * $P < 0.05$ vs. vehicle. **B:** survival of eosinophils after OVA challenge. Eosinophil apoptosis was determined using the Guava EasyCyte Caspase 3/7 Kit and the Guava PCA system. Fluorescent-labeled inhibitor of caspases (FLICA) and propidium iodide (PI) were used to stain the apoptotic cells. Eosinophil viability was assessed by the percentage of FLICA (–) and PI (–) cells. * $P < 0.05$ vs. WT mice. VC, viable cells (FLICA–, PI–); DC, dead cells (FLICA–, PI+); ESA, early stage apoptosis (FLICA+, PI–); LSA, late stage apoptosis (FLICA+, PI+).

cantly increased in a dose-dependent manner by addition of either FX or FXa. The concentration needed for induction of MUC5AC by FXa was $>0.03 \mu\text{M}$, which is lower than the plasma concentration of FX. On the other hand, MUC5AC was not increased by stimulation with $1 \mu\text{M}$ FVIIa, a concentration $>100\times$ higher than the plasma concentration of FVII.

Expression and function of FVII and TF in lung epithelial cell lines. Because FVII stained positive in immunohistochemical analyses of lung tissue, the mRNA levels of FVII and TF in lung epithelial cell lines were analyzed by Q-RT-PCR. FVII expression was analyzed by Q-RT-PCR and detected in NCI-H292 cells but was undetectable in BEAS-2B cells (data not shown). Contrarily, TF expression was detected in both cell lines (data not shown). Furthermore, prothrombin (FII) and FX expression were not detected in either of the cell lines (data not shown).

To confirm whether FVIIa/TF was functional in NCI-H292 cells, the presence of FXa, generated from activation of FX, was measured after incubation of FX with NCI-H292 cells at

various times. FXa activity was detected in NCI-H292 cells after 3-h incubation, and its activity was maintained to 6 h (Fig. 7A). On the other hand, MUC5AC mRNA levels were increased at 6 h but not at 3 h, suggesting MUC5AC synthesis was induced after the activation of FXa. Neither FXa activity (Fig. 7B) nor MUC5AC were present in BEAS-2B cells after the addition of FX. However, FXa activity was observed when both FX and FVIIa were coincubated in BEAS-2B cells (data not shown), suggesting that TF was functional in BEAS-2B cells and confirming the functional absence of FVII in these cells.

DISCUSSION

We have taken advantage of a mouse line that genetically expresses very low levels of FVII to study the role of this protein in a number of spontaneous and challenge phenotypes, in this case, the asthmatic response to an allergen. At the outset, we found that levels of other coagulation factors, e.g., FXII, FXI, FIX, and FVIII, in plasma of FVII^{tTA/tTA} mice were also slightly lower compared with WT mice (23). Although the reduction of these factors was much milder than FVII, and certainly no lower than a heterozygous deficiency state of these genes, which are generally much less responsive to inflammatory challenge models, e.g., sepsis, it is nonetheless possible that other coagulation factors are important for asthmatic responses to allergens. Our working assumption in the current communication then is that the only difference between WT and FVII^{tTA/tTA} mice that affect this phenotype is in the levels of FVII.

Hepatocytes are the predominant source of FVII production under normal physiological conditions, although FVII is also expressed by monocytes/macrophages in inflammation (3, 30). On the other hand, TF is constitutively expressed in cells of the adventitial and medial layers of the vessel wall and rapidly initiates coagulation subsequent to vascular injury (22). TF is also expressed on monocytes (20) and eosinophils (14), where it is associated with cell migration, and on lung epithelial cells (9). More recently, a report showed that FVII is expressed on airway epithelial cells and is important in epithelial repair (18). In this latter study, it was found that epithelial cells constitutively expressed TF, and FVII mRNA increased subsequent to scrape damage in NHBE cells and 16HBE14o– cells. In the present study, FVII and TF were detected in NCI-H292 cells. These extrinsic coagulation factors were functional since FX activation was observed in NCI-H292 cells, resulting in generation of FXa. These data suggest that locally produced coagulation factors are active and may function in the lung. On the other hand, there is a possibility that the coagulation factors from plasma sources may affect lung inflammation in addition to those locally produced. The extravasation of EB from plasma to BALF was seen in OVA-challenged mice but was not detected in PBS-challenged WT or FVII^{tTA/tTA} mice. Therefore, it is possible that blood proteins can emigrate to lung tissue during asthma. However, the concentration of EB in the BALF was only one seven-hundredth of that in serum. Whether this is sufficient to produce pathophysiologically important levels of coagulation factors is possible but seems unlikely.

Our (26) previous work showed that FXa induced mucin production, and there is a possibility that FVIIa affects mucin

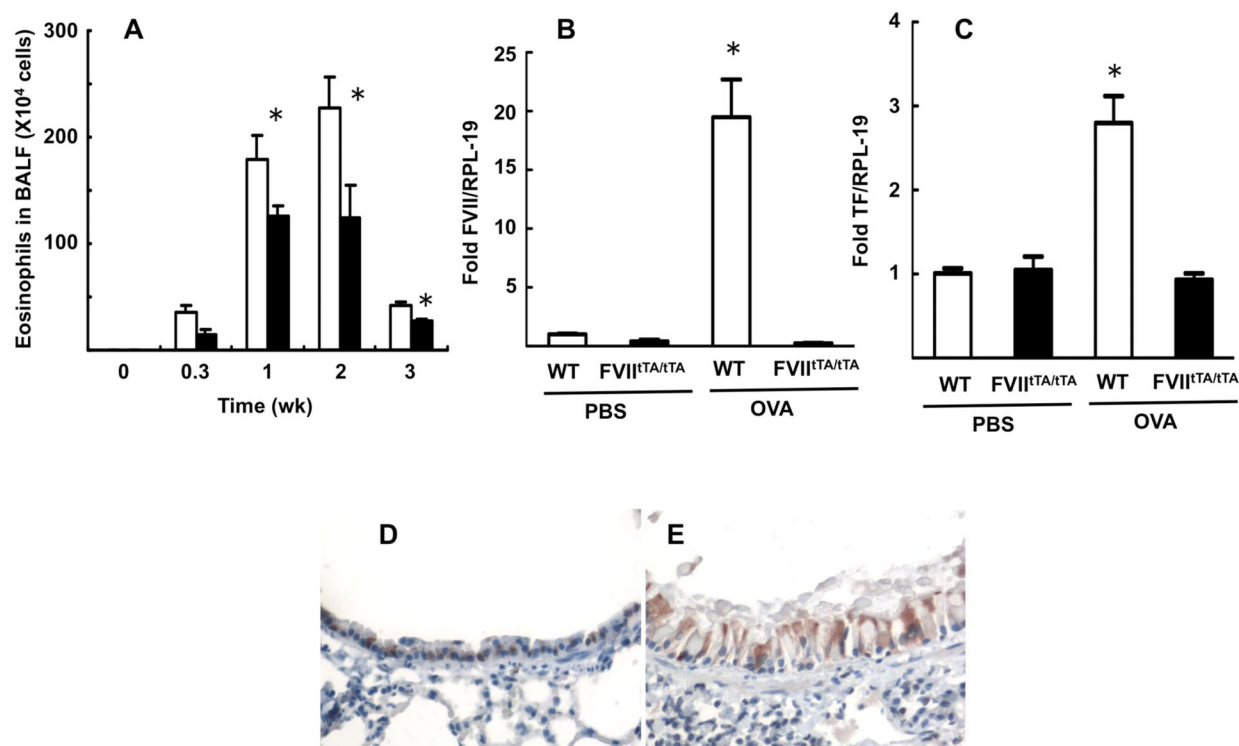


Fig. 4. Eosinophil counts in BALF of multiple challenged mice. A: mice were challenged with OVA for the indicated times (3 times per week), and then lavage was performed 24 h after final OVA challenge in WT (white bars) and FVII^{TA/TA} (black bars) mice. BALF cells were counted and centrifuged onto microscope slides using a cytospin. The preparations were stained with Diff-Quick to obtain differential leukocyte counts. The data represent the means $\pm$ SE ($n = 5-6$). * $P < 0.05$ vs. OVA-challenged WT mice. B and C: quantitative RT-PCR (Q-RT-PCR) analysis of FVII (B) or tissue factor (TF; C) mRNA in lungs obtained from OVA-challenged WT (black bars; $n = 7$) and FVII^{TA/TA} mice (white bars; $n = 7$) at 2 wk of treatment vs. PBS-treated WT (white bars; $n = 5$) and FVII^{TA/TA} mice (black bars; $n = 5$). The values indicate the fold difference relative to the ribosomal protein L19 (RPL19) gene, presented as the means $\pm$ SE. * $P < 0.05$ vs. PBS-treated mice. D and E: immunostaining of FVII in whole lung sections from mice challenged with OVA for 2 wk. The photomicrographs are representative of lungs removed from PBS-challenged (D) and OVA-challenged (E) WT mice. Original magnification, $\times 400$.

production in the lung. However, whereas FVIIa itself did not induce mucin production (Fig. 6), the thickness of mucous layer was decreased in OVA-challenged FVII^{TA/TA} mice compared with OVA-challenged WT mice. Thus we conclude that FVIIa, in collaboration with TF, enhances mucin production by activating FX to FXa. Since activation of FX to FXa was observed in NCI-H292 cells, local activation of FXa

appears to be important for mucin production. This is in concert with our (26) previous work showing that FX is produced by lung macrophages, and its expression was increased in macrophages obtained from OVA-challenged mice. In addition, FX is produced in the lung during wounding (18). Because epithelial damage is observed in asthmatic patients (12), we deduce that FX is produced in bronchial epithelial

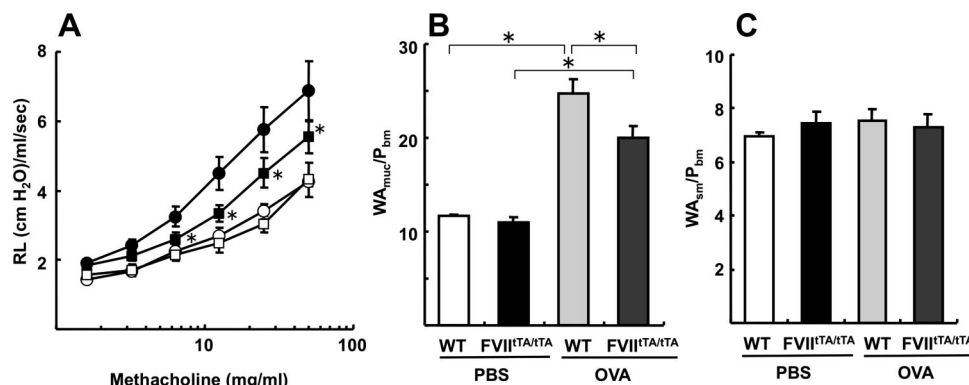


Fig. 5. The effect of FVII on airway parameters in OVA-treated mice. A: airway hyperresponsiveness (AHR) measured as lung resistance in WT and FVII^{TA/TA} mice. AHR was induced by methacholine inhalation (1–50 mg/ml) and monitored 24 h after the final OVA challenge. Unchallenged (PBS) WT (○) and FVII^{TA/TA} (□) mice and OVA-challenged WT (●) and FVII^{TA/TA} mice (■) were used. Data represent the means $\pm$ SE ($n = 5-7$). * $P < 0.05$ vs. OVA-challenged WT. B and C: airway wall thickness in bronchi of mice challenged with OVA for 2 wk. Values for the cross-sectional areas of the mucous layer (B) and the smooth muscle cell layer (C) are presented as the means $\pm$ SE ($n = 5-7$) for each group. * $P < 0.05$ vs. PBS-treated mice. WA_{muc}, wall area of the mucous layer; WA_{sm}, wall area of the smooth muscle layer; P_{bm}, the basement membrane perimeter.

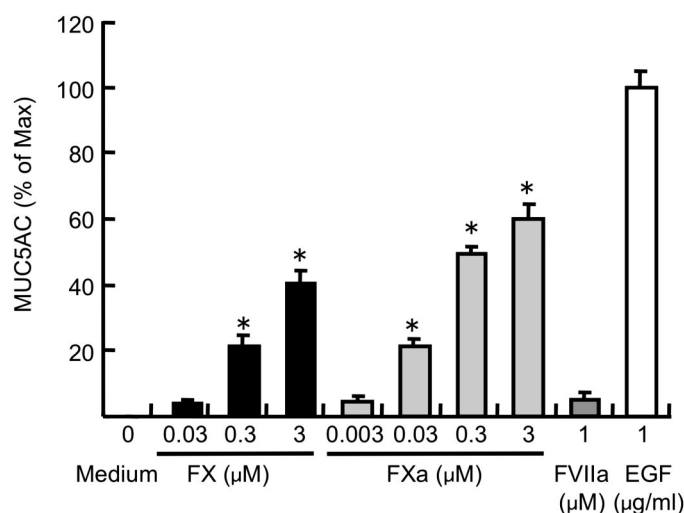


Fig. 6. Mucin (MUC5AC) production in NCI-H292 cells. NCI-H292 cells were cultured in 96-well plates and stimulated with FX (black bars), FXa (light gray bars), FVIIa (dark gray bar), or EGF (white bar) at the indicated concentrations for 48 h. MUC5AC production was determined as the percentage relative to maximum (Max) expression (stimulated with EGF at 1 μ g/ml). * P < 0.05 vs. medium alone.

cells after OVA challenge. Therefore, it is likely that, in vivo, FX is activated by FVIIa and TF on the surface of mucin producing cells, thereby allowing FXa to activate the cells to produce mucin. In support of this concept, the thickness of mucous layer was decreased in OVA-challenged FVII^{TA/TA} mice compared with OVA-challenged WT mice and thereby remedied the AHR. From these and our (26) previous results, evidence has emerged showing that production of coagulation factors is important for mucin production and for airway remodeling and that the extrinsic coagulation system plays an important role in this regard.

In addition to mucin production, FVII was found to be involved in eosinophilia. Specifically, in FVII^{TA/TA} mice, the number of eosinophils in asthmatic BALF was diminished compared with WT mice. One explanation for this observation is that the eosinophil-attractant chemokines, eotaxin and RANTES, were reduced in the lungs of the low-FVII mice. The recruitment of eosinophils into inflamed tissue is largely regulated by chemokines, and many reports indicate that CCR3-reactive chemokines are associated with eosinophilia in asthma (1, 8, 15). Our data suggest that a decrease of eotaxin and RANTES in FVII^{TA/TA} mice resulted in a reduction in eosinophil levels in the lung. The decrease of these chemokines provides one explanation of why eosinophils were lower in FVII^{TA/TA} mice, but other mechanisms uncovered in this work also contribute to this effect. One such discovery is that eosinophils purified from FVII^{TA/TA} mice underwent more rapid apoptosis compared with WT mice. In some eosinophilic inflammatory diseases, reduced eosinophil apoptosis has been observed, and this mechanism may contribute to increased eosinophil counts (27). An additional factor to be considered is the direct effect of FVII(a) on eosinophil migration. In the present study, the migration of eosinophils isolated from FVII^{TA/TA} mice in response to eotaxin was diminished compared with eosinophils obtained from WT mice. Reports exist showing that TF is expressed on eosinophils, and its expression is augmented by stimulation with

granulocyte/macrophage colony-stimulating factor (GM-CSF) or platelet-activating factor (PAF) (14). Furthermore, using neutralizing antibodies against TF, it has been shown that TF is associated with the migration of eosinophils. Therefore, the TF/FVIIa system appears to be heavily associated with eosinophil migration. All of these effects, viz., diminished eosinophil-attracting chemokines, enhanced apoptosis of eosinophils, and reduced migration of eosinophils, in a FVII-deficient state can contribute to lowered eosinophil counts in BALF of asthmatic FVII-deficient mice.

A number of coagulation factors are activators of protease-activated receptors (PARs) (10, 17). Several reports indicate that PAR-2 is involved in inflammatory lung reactions (2, 10, 17, 21, 24). PAR-2 is expressed on eosinophils, and its activation leads to the generation of superoxide anion (2). In mice overexpressing PAR-2, eosinophil infiltration in lung is increased under asthmatic challenge, and AHR is increased (24), whereas in PAR-2-deficient mice, the number of eosinophils is reduced in the BALF, and AHR is diminished (24, 28). In addition, PAR-2 is expressed on lung epithelial cells and contributes to mucin production (13). The results obtained in these reports are consistent with our current findings such as decreases of eosinophilic inflammation and mucin production

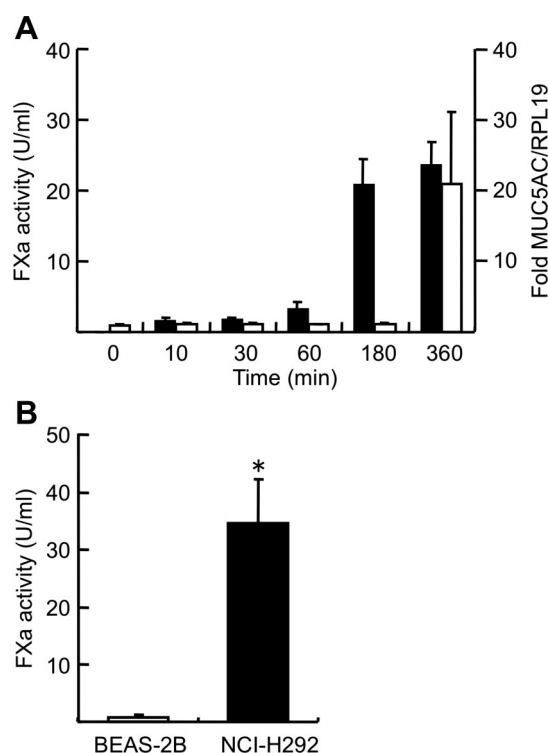


Fig. 7. Activation of FX by endogenous extrinsic coagulation pathway factors in bronchial cell lines. A: FXa activity (black bars) and MUC5AC mRNA (white bars) in NCI-H292 cells. Cells were cultured in 12-well plates and placed under FCS-free conditions for 3 h after confluency. The cells were then incubated with FX (30 U/ml) for the indicated times, and supernatants were assayed for FXa activity. The cells were harvested for measuring mRNA levels of MUC5AC and for FXa activity, determined by a S-2222-based chromogenic assay. B: FXa activity in human bronchial epithelial BEAS-2B cells (white bars) and NCI-H292 cells (black bars). Both cell types were cultured in 12-well plates, and cells were placed under FCS-free conditions for 3 h after confluency. The cells were then incubated with FX (30 U/ml) for 24 h, and supernatants were obtained for measuring FXa activity. Means $\pm$ SE, n = 4–6 for each group; * P < 0.05 vs. BEAS-2B cells.

in low-FVII mice. Therefore, we further suggest that FVIIa can affect the asthmatic response via activating PAR-2-dependent signaling.

Lastly, we propose that FVIIa is produced in the lung and complexes with TF to promote activation of FX to FXa, which then exacerbates asthmatic responses such as AHR. One of the mechanisms by which AHR is attenuated in low-FVII mice is via reduction of airway wall thickness, largely due to reduced mucin production in these mice. In addition, FVIIa stimulates migration of eosinophils to the lung and in this way participates in the exacerbation of asthma, since it is known that eosinophils produce cytotoxic proteins, e.g., major basic protein (MBP) and eosinophil cationic protein (ECP), and these proteins are known to increase AHR. These results further implicate hemostasis system components in the asthmatic response to allergens and specifically suggest that specific targeted inhibitors of FVIIa and/or FXa may be of assistance in attenuation of asthma.

ACKNOWLEDGMENTS

We thank Deborah L. Donahue for work with the mouse studies, Melanie DeFord for assistance with cell culture, J. Andrew Martin for help with the RT-PCR measurements, and Mayra J. Sandoval-Cooper for assistance with histology.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-073750.

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