# Plasminogen is an Important Regulator in the Pathogenesis of a Murine Model of Asthma

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## Online Data Supplement

## Materials and Methods

### Animals

*Plg*$^{-/-}$ mice were developed and characterized previously (E1). Genotyping for Plg was determined by PCR of genomic DNA as described (E1). Mice were housed in microisolation cages on a 12-hour day/night cycle, fed a regular chow diet and given water *ad libitum*. Experimental mice were 6-10 weeks of age, of mixed gender, and appeared healthy during the course of the experiments, not yet showing weight loss or other adverse symptoms observed in older *Plg*$^{-/-}$ mice (E1,E2).

### Leukocyte Recruitment

BAL was performed as described (E3) by infusing 1 ml of PBS via a tracheal cannula. The BAL was centrifuged (700 g, 5 min, 4°C), the fluid was aliquoted and frozen at -70 °C for cytokine and MMP measurements. After hypotonic lysis of erythrocytes in the pellet, the BAL cells were resuspended in 1 ml PBS and counted using a hemocytometer. Differentials were performed in BAL cells by cytocentrifugation of $10^3$ cells/ml onto glass slides, staining with Wright stain (EM Science, Gibbstown, NJ) and counting microscopically. The percentage of different leukocyte populations was determined in duplicate by counting 200 cells in randomly selected fields at 2X magnification at which eosinophils are not visible.

### Histology

Histological changes were examined in the right lungs 3 h after OVA or PBS treatment on day 21. After BAL, the lungs mice were perfused free of blood, infused with

Histochoice as a fixative (Amresco, Cleveland, OH) and the airway ligated. After 24 h, the lungs were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H and E), Masson Trichrome, and PAS. Images of tissue sections stained with Mason's Trichrome or PAS were acquired at 20X using a Q-Imaging Retiga EXi CCD camera (Burnaby, BC, Canada). Using ImagePro Plus (Media Cybernetics, Carlsbad, CA) representative images were chosen to generate color-cube based profiles for the Trichrome "blue" or PAS "magenta" areas well as a separate profile for the entire tissue area. Percent Trichrome or PAS staining was calculated by dividing the sum of the blue or magenta pixels by total pixel area.

**Cytometric Bead Array (CBA) Assay**

Cytometric bead array, which allows the simultaneous measurement of several cytokines, was used to quantify cytokine protein expression in the BAL. Capture beads (10 μl) for IL-2, IL-4, IL-5, IFN-γ, and TNF-α were mixed with 50 μl of sample or mouse cytokine standard dilutions. Mouse Th1/Th2 PE detection reagent (50 μl) was added and incubated in the dark at 22°C for 2h. Wash buffer provided by the manufacturer (1ml) was added. After centrifugation, pellets were resuspended in 300 μl Wash buffer and analyzed by flow cytometry in a BD FACScan. Cytokine levels were determined against standard curves generated for each cytokine utilizing the BD CBA software.

**TGF-β1, JE/MCP-1 and IL-13 assays**

The levels of the growth factor and cytokines were quantified in BAL from mice of both genotypes. TGF-β1 was assayed using the immunoassay kit (MB100) from R&D

Systems, Inc. (Minneapolis, MN). Assays were performed with and without acidification of the BAL to measure total and active TGF-β1, respectively. For JE/MCP-1 and IL-13, ELISA kits from R&D Systems, Inc. (Minneapolis, MN) were used. All assays detect the murine antigens, and the assays were performed according to the manufacturers' instructions using standard curves constructed with the purified proteins for quantitation.

**Zymographic and Western Blot Analysis of MMPs**

MMP-2 and MMP-9 in BAL were detected by gelatin zymography and Western blot. Defined volumes of BAL were subjected to SDS-PAGE (4.5% stacking/10% separating gel with 1 mg/ml gelatin for zymography only) under non-reducing conditions (zymography) or reducing conditions (Western blot). For gelatin zymography, after electrophoresis, gels were soaked in 2.5% triton X-100 (in 50 mM Tris, pH 7.6) with gentle shaking at 22°C for 60 min with one change. Gels were rinsed 3 times 50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$ and incubated at 37°C for 16-20 h. The gels were stained with Coomasie Brilliant Blue for 1 h. Gelatinase activities appeared as clear bands against a blue background. The gels were then scanned and the black and white image inverted. Human MMP-2/MMP-9 and mouse MMP-9 zymography standards were used as positive controls (Chemicon International, Temecula, CA). Molecular weights were estimated using the kaleidoscope prestained protein standards (Bio Rad, Hercules, CA). For Western blots, after electrophoresis, proteins were transferred overnight to a PDVF membrane. The membrane was blocked with Blotto, incubated with rabbit anti-mouse MMP-2 or MMP-9 antibodies (H-76, sc-10736 and H-129, sc-10737 respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS-

Tween. Membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG (Amersham Biosciences Corp., Piscataway, NJ), washed again and developed using a chemiluminescence kit (Amersham Biosciences Corp.).

**Results:**

**Reduction of pulmonary inflammation due to lack of plasminogen is not restricted by strain background.**

All of the experiments reported above used *Plg* mice of a mixed 129/C57Bl/6 background. To determine if the effects of plasminogen deficiency were strain-dependent, we also studied leukocyte recruitment and lung histology in OVA or PBS-treated littermate mice that had been backcrossed for 7 generations into a C57BL/6 background. As was observed in the mixed 129/C57Bl/6 background, OVA challenge induced a marked increase in leukocyte recruitment to the lungs in the C57Bl/6 background compared to the saline treated WT controls. Compared to WT mice in the C57BL/6 background, $Plg^{+/-}$ or $Plg^{-/-}$ displayed a similar markedly reduced recruitment of lymphocytes [35.9 ± 9.5 (n=6) vs. 6.4 ± 4.0 (n=7) and 4.9 ± 0.9 (n=5), respectively] and eosinophils (620.5 ± 69.4 vs. 152.3± 72.4 and 4.5 ± 1.1) in response to OVA as observed in the mixed background. Histological analysis of lung sections from OVA-treated $Plg^{+/+}$ C57BL/6 mice also showed increased leukocytosis in perivascular and peribronchial areas (Figure 6A), collagen deposition (Figure 6B), goblet cell hyperplasia and mucus production, and this increase was reduced or absent in the C57BL/6 mice with plasminogen deficiency (not shown). Taken together, these data show that the

reduced pulmonary inflammation observed in OVA-treated mice with reduced plasminogen occurs in two different genetic backgrounds. In comparing the response of the WT mice in the two backgrounds, the extent of leukocyte recruitment into the BAL was similar in the two strains, but the leukocytosis in perivascular and peribronchial areas was less extensive in the C57BL/6 mice (compare Figure 6A to Figure 3B).

**References**

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E2. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev* 1995; 9:794-807.

E3. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol* 2000; 157:177-187.