



HAL
open science

Platelet activation and aggregation promote lung inflammation and influenza virus pathogenesis

- Vuong Ba Lê, Jochen G. Schneider, Yvonne Boergeling, Fatma Berri, Mariette Ducatez, Jean-Luc Guerin, Iris Adrian, Elisabeth Errazuriz-Cerda, Sonia Frasquilho, Laurent Antunes, et al.

► **To cite this version:**

- Vuong Ba Lê, Jochen G. Schneider, Yvonne Boergeling, Fatma Berri, Mariette Ducatez, et al.. Platelet activation and aggregation promote lung inflammation and influenza virus pathogenesis. *American Journal of Respiratory and Critical Care Medicine*, 2015, 191 (7), pp.804-819. <10.1164/rccm.201406-1031OC>. <hal-02637130>

HAL Id: hal-02637130

<https://hal.inrae.fr/hal-02637130v1>

Submitted on 11 Sep 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

1 **Platelet activation and aggregation promote lung inflammation and influenza virus**
2 **pathogenesis**

3

4 Vuong Ba Lê^{1*}, Jochen G. Schneider^{2,3*}, Yvonne Boergeling⁴, Fatma Berri¹, Mariette Ducatez^{5,6},
5 Jean-Luc Guerin^{5,6}, Iris Adrian³, Elisabeth Errazuriz-Cerda⁷, Sonia Frascuilho⁸, Laurent Antunes⁸,
6 Bruno Lina¹, Jean-Claude Bordet⁹, Martine Jandrot-Perrus¹⁰, Stephan Ludwig⁴, Béatrice Riteau^{1,9}

7

8 ¹EA4610, Lyon, France; ²Luxembourg Centre for Systems Biomedicine, Esch-Sur-Alzette,
9 Luxembourg; ³Saarland University Medical Center, Homburg/Saar, Germany; ⁴Institute Molecular
10 Virology, ZMBE, Münster, Germany; ⁵UMR 1225, IHAP, INRA Toulouse, France; ⁶INP, ENVT,
11 Toulouse France; ⁷Centre Commun d'Imagerie Quantitative Lyon Est (CIQLE), SFR Santé Lyon-Est,
12 University of Lyon, France.; ⁸IBBL, Integrated BioBank of Luxembourg, For Next Generation
13 Healthcare, Luxembourg; ⁹Unité d'Hémostase Clinique, Lyon, France; ¹⁰INSERM UMR_S1148, Paris
14 Diderot, CHU Xavier Bichat, Paris, France; ⁹INRA Nouzilly, France

15 * VBL and JGS contributed equally to this work as co-first authors.

16 **Corresponding author: Beatrice Riteau:** E-mail: beatrice.riteau@laposte.net.

17 **Author contributions:** VBL, JGS, JCB, MJP, SL, and BR designed the experiments. VBL,
18 YB, FB, IA, EEC, SF, and LA performed the experiments. FB, JGS, SL, and BL critically
19 read the manuscript. VBL, MJP and BR wrote the manuscript. **Support:** BR and MJP
20 acquired funding from ANR (ANR-13-BSV3-0011, HemoFlu) and JGS from DFG
21 (SCH682/3-1), EU CIG303682, and FNR CORE Itgb3VascIn. **Short Head:** Platelet
22 dysfunction during influenza; **Classification:** 10.15 Treatment.

23 **Commentary:** Our research shows that platelets play a key role in the pathogenesis of
24 influenza-induced acute lung injury. These findings may have an impact on the development
25 of novel drugs for the treatment of these diseases.

26

27 **Abstract**

28

29 **Rationale:** The hallmark of severe influenza virus infections is excessive inflammation of the
30 lungs. Platelets are activated during influenza, but their role in influenza virus pathogenesis
31 and inflammatory responses is unknown.

32 **Objectives:** To determine the role of platelets during influenza A virus (IAV) infections and
33 propose new therapeutics against influenza.

34 **Methods:** We used targeted gene deletion approaches and pharmacological interventions to
35 investigate the role of platelets during influenza virus infection in mice.

36 **Measurements and Main Results:** Lungs of infected mice were massively infiltrated by
37 aggregates of activated platelets. Platelet activation promoted IAV pathogenesis. Activating
38 protease-activated receptor 4 (PAR4), a platelet receptor for thrombin that is crucial for
39 platelet activation, exacerbated influenza-induced acute lung injury and death. In contrast,
40 deficiency in the major platelet receptor glycoprotein IIIa (GPIIIa) protected mice from death
41 caused by influenza viruses, and treating the mice with a specific GPIIb/IIIa antagonist,
42 eptifibatid, had the same effect. Interestingly, mice treated with other anti-platelet
43 compounds (antagonists of PAR4, MRS 2179, and clopidogrel) were also protected from
44 severe lung injury and lethal infections induced by several influenza strains.

45 **Conclusions:** The intricate relationship between hemostasis and inflammation has major
46 consequences in influenza virus pathogenesis, and anti-platelet drugs might be explored to
47 develop new anti-inflammatory treatment against influenza virus infections.

48 **Key words:** Lung injury, novel drugs, Flu pathogenesis, pneumonia, platelets.

49

50 **Introduction**

51 Influenza is one of the most common infectious diseases in humans, occurring as sporadic
52 pandemic and seasonal epidemic outbreaks, leading to significant **numbers of fatalities**.
53 Influenza pathogenesis is a complex process involving both viral determinants and the
54 immune system (1-3). During severe influenza, dysregulation of cytokine production
55 contributes to **lung** damage, possibly leading to organ failure and death (4-6). The
56 endothelium, which lines the interior surface of **the** blood vessels, is thought to orchestrate the
57 crescendo in cytokine accumulation, although the mechanism involved is not fully **understood**
58 (7).

59

60 Upon endothelial injury, platelets are recruited by inflamed endothelial cells, **where they**
61 **adhere and are activated** (8). Simultaneously, the family of protease-activated receptors
62 (PARs) mediates **platelet** activation by thrombin. PAR4 is strictly required for **platelet**
63 activation in mice, as **mouse platelets** do not express PAR1. In contrast, both PAR1 and PAR4
64 are important for platelet activation in humans. These events lead to **a** conformational change
65 **in** the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor for fibrinogen that bridges platelets,
66 leading to their aggregation and a reinforcement of their activation. Importantly, platelet
67 activation is strongly associated with enhanced inflammatory responses. Activated platelets
68 release potent inflammatory molecules and play a key role in leukocyte recruitment (9).
69 Platelet activation is finely tuned, but its dysfunction is pathogenic and contributes to
70 inflammatory disorders (10, 11). **Thus**, uncontrolled platelet activation could contribute to the
71 pathogenesis of IAV infections by fueling a harmful inflammatory response in the respiratory
72 tract. However, the role of platelets in the context of IAV infection has never been
73 investigated. In the present study, using pharmacological and gene deletion approaches, we

74 investigated the role of platelets in IAV pathogenesis *in vivo*. We found that during severe
75 influenza A virus infection in mice, platelet activation worsens the severity of lung injury.
76

77 **METHODS**

78

79 **Reagents**

80 A549 cells and MDCK cells were purchased from ATCC (Molsheim Cedex, France). IAV
81 A/PR/8/34 virus (H1N1), A/HK/1/68 (H3N2) and A/NL/602/2009 (H1N1) (ATCC) were gifts
82 from G.F. Rimmelzwaan (Erasmus, Netherlands). The highly pathogenic avian
83 A/FPV/Bratislava/79 (H7N7) strain was from the Institute of Molecular Virology, Münster,
84 Germany. The following reagents were used: DAPI (Life Technologies, Paris, France), Alexa
85 Fluor® secondary antibodies (Life Technologies), eptifibatide (Integrilin®, GlaxoSmithKline,
86 Marly-le-Roi, France), Clopidogrel (Santa Cruz Biotechnology, Heidelberg, Germany), MRS
87 2179 (Tocris Bioscience, Bristol, United Kingdom), PAR4 antagonist pepducin p4pal-10
88 (Polypeptide Laboratories, Strasbourg, France), PAR4 agonist peptide (AYPGKF-NH₂,
89 Bachem, Weil-am-Rhein, Germany), PAR4 control peptide (YAPGKF-NH₂, Bachem)
90 monoclonal anti-neutrophil Ly6G (Cedarlane, Tebu-bio, Le Perray en Yvelines, France),
91 polyclonal anti-platelet CD41 (Bioss, Woburn, USA), monoclonal anti-viral HA (Santa Cruz
92 Biotechnology, Heidelberg, Germany), monoclonal anti-IAV NP (gift from GF.
93 Rimmelzwaan), monoclonal anti-p-Selectin FITC-conjugated (Emfret, Eibelstadt, Germany),
94 monoclonal anti-CD41/61 PE-conjugated (Emfret); Vectastain® ABC kit (Vector
95 Laboratories, Burlingame, USA), 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector
96 Laboratories), ketamine/xylazine anesthesia (Virbac, Bayer HealthCare, Carros, France),
97 May-Grünwald and Giemsa solutions (Merck, Darmstadt, Germany), hematoxylin and eosin
98 solutions (Diapath, Martinengo, Italy), and enzyme-linked immunosorbent assay (ELISA) kits
99 for mouse IL-6, IL-1 β , MIP-2 (PromoCell GmbH, Heidelberg, Germany), IFN- α , IFN- γ ,
100 RANTES (R&D Systems, Lille, France), serotonin (BlueGene, Shanghai, China),
101 thromboxane B2 (TXB2; Elabscience, Wuhan, China) and sP-selectin (Qayee-Bio, Shanghai,

102 China). Total protein was evaluated by using the Coomassie Bradford Protein assay kit
103 (Thermo Scientific, Massachusetts, USA).

104

105 **Mice**

106 Experiments were performed in accordance with the Guide for the Care and Use of
107 Laboratory Animals of la Direction des Services Vétérinaires (DSV), the French regulations
108 to which our animal care and protocol adhered. The license authority was issued by the DSV
109 and Lyon University (accreditation 78-114). Protocols were approved by the Committee on
110 Ethics of Animal Experiments of Lyon University (Permit BH2008-13).

111

112 Female, 7-week-old BALB/c mice were used for H7N7 virus infections. Otherwise, 6-week-
113 old C57BL/6 female mice (Charles River Laboratories, Arbresle, France) and GPIIIa^{-/-} mice
114 or wild-type littermates on a C57BL/6 background were used in this study. For the latter,
115 heterozygous mice were crossed, and WT and KO offspring (males and females) were used.
116 Polymerase chain reaction of tail-tip genomic DNA was performed (12) to determine the
117 absence or presence of the GPIIIa gene. Infection experiments were performed as previously
118 described (13). Mice were anesthetized with ketamine/xylazine (42.5/5 mg/kg) and inoculated
119 intranasally with IAV, in a volume of 20 µl. Eptifibatid was injected intraperitoneally (500
120 µg/kg or 10 µg/200 µl per mouse of ~20 g body weight) every 3 days until the end of the
121 experiment. MRS 2179 was dissolved in saline buffer and administered once intravenously
122 (50 mg/kg) on day 0. Clopidogrel dissolved in saline buffer was injected intraperitoneally (30
123 mg/kg) every day until the end of the experiment. For PAR4 stimulation experiments, mice
124 were anesthetized every day for 3 days. On the first day, the anesthetized mice were infected
125 intranasally in the presence or absence of PAR4-AP or control peptide (100 µg/mouse, in a
126 volume of 20 µl). Intranasal peptide treatments were also repeated on days 2 and 3 after

127 infection. For PAR4 antagonist treatment, pepducin p4pal-10 was given intraperitoneally (0.5
128 mg/kg) two days post-infection, and treatments were repeated on the next two days.

129

130 Upon inoculation, the survival rates were followed. Alternatively, mice were sacrificed at
131 prefixed time points to perform BAL or harvest lungs. ELISA was performed according to the
132 manufacturers' instructions. Virus titers were assessed as previously described (14). Lungs
133 were also harvested for histology and immunohistochemistry as previously described (15).

134

135 **Evaluation of hemorrhagic foci by histopathological analysis**

136 Lungs from mice inoculated with A/PR/8/34 virus (250 PFU/mouse) with or without
137 eptifibatide treatment were fixed in 10% neutral buffered formalin and embedded in paraffin.
138 Then, 4–6 µm sections were cut and stained with hematoxylin and eosin (H&E) to evaluate
139 histopathological changes. Staining was performed by incubation of the lung sections with
140 Harris hematoxylin for 6 min, running tap water for 1 min, eosin Y for 10 min, 70% ethanol
141 for 1 min, 95% ethanol for 1 min, 100% ethanol for 1 min and two rinses in 100% xylene for
142 1 min. Histology and injury scoring were performed by a blinded investigator who analyzed
143 the samples and determined the levels of injury according to a semiquantitative scoring
144 system (counting inflammatory infiltration, vascular congestion, hemorrhage, fibrin deposits
145 and epithelial cell apoptosis).

146

147 **Microscopy**

148 For ultrastructural analysis, lung tissues were cut into 1-mm³ pieces, fixed in 2%
149 glutaraldehyde at 4°C, washed in 0.2 M cacodylate-HCl buffer containing 0.4 M saccharose
150 and post-fixed in 0.3 M cacodylate-HCl buffer containing 2% osmium tetroxide for 1 hour.
151 After dehydration in a graded alcohol series, tissue samples were impregnated with a 75%

152 Epon A/25% Epon B/1.7% DMP30 mixture. Tissue embedding entailed polymerization at
153 60°C for 72 hours. Then, 70-nm sections were cut using an ultramicrotome (Leica
154 Microsystems), mounted on 200-mesh copper grids coated with 1:1,000 polylysine, stabilized
155 for 24 hours and contrasted with uranyl acetate/citrate. Sections were examined using a
156 transmission electron microscope (JEOL 1400, Japan) at 80 kV equipped with an Orius
157 SC600 camera (Gatan, France). Immunogold staining was performed using the anti-HA
158 antibody followed by 10 nm gold-conjugated secondary antibody, as previously described
159 (16). As a control of HA labelling, we used IAV particles that we recently purified (17).

160

161 **Fluorescence microscopy experiments**

162 Cells from the BAL were centrifuged at 1,800 rpm for 5 minutes at room temperature and
163 suspended in phosphate buffer saline (PBS) at a concentration of 5×10^5 /ml. Then, 100 μ l of
164 the solution was used to centrifuge the cells onto coverslips (1,000 rpm for 5 minutes), using a
165 Shandon Cytospin 4 centrifuge. The slides were then dipped in a box containing methanol and
166 kept at -20°C for fixation and permeabilization. After 10 minutes, cells were extensively
167 washed with PBS to remove the fixative. Cells were then incubated with primary antibodies to
168 CD41 and viral HA for 1 hour at room temperature. Revelation was performed using Alexa
169 Fluo® (Life Technologies) secondary antibodies for 1 hour at room temperature. Cells were
170 also counterstained with DAPI for 15 minutes at room temperature. Images were analyzed
171 using a Leica TCS SP5 confocal system (Leica Microsystems).

172

173 **Evaluation of platelet and leukocyte numbers**

174 Platelets were counted using the Vet ABC™ Hematology Analyzer (SCIL) using the mouse
175 smart card 7030. The automated cell counter differentiates mouse platelets based on their size

176 in multiple sample fluids. Leukocytes and neutrophils in the BAL were visualized by May-
177 Grünwald Giemsa stained cytopsin preparations, as previously performed (13).

178

179 **Flow cytometry of blood platelets**

180 Blood was collected in ACD buffer by cardiac puncture. CD41-positive cells and platelet
181 activation in whole blood were evaluated using FITC-conjugated P-selectin and PE-
182 conjugated CD41/CD61 antibodies, as previously described (18, 19).

183

184 **Statistical analysis**

185 The Kaplan-Meier test was used for survival rates. The Mann-Whitney test was used for two-
186 group comparisons of mean percentages in the flow cytometry experiments, lung virus titers,
187 ELISA and total protein quantifications. One-way ANOVA for non-parametric measures
188 (Kruskal-Wallis) was used for multiple-group comparisons in dose-responses or kinetics
189 experiments. Dunn's multiple comparison test was employed as a post hoc test using NI as a
190 control. Probabilities $*p < 0.05$ was considered statistically significant.

191

192 **RESULTS**

193

194 **Platelet recruitment to the lungs upon IAV infection**

195 Platelet recruitment to the lungs was first examined after infection of mice with a sublethal or
196 a 50% lethal dose (LD₅₀) of IAV A/PR/8/34. Immunohistochemistry of the lungs, using
197 monoclonal antibodies for IAV nucleoprotein (NP) and CD41, was used to detect virus-
198 infected cells and platelets, respectively (Figure 1A). At both doses, many IAV-infected cells
199 and marked platelet infiltrates were detected in the lungs of infected mice compared to
200 uninfected mice. To confirm these results, platelet counts in the BAL of infected versus
201 uninfected mice (sublethal dose or LD₅₀) were assessed using a blood cell counter (Figure
202 1B). In the BAL of infected mice, the platelet levels increased in a dose-dependent manner
203 and were significantly higher than in those of uninfected mice, reaching 50×10^9 cells/L on
204 day 6 post-inoculation (LD₅₀). Differences were not significant upon infection with IAV at the
205 sublethal dose.

206

207 **Viral proteins are present within platelets**

208 The presence of viral proteins was next determined in platelets from the BAL of infected
209 mice. Platelets were identified by immunofluorescence as CD41 positive, DAPI-negative
210 elements and IAV particles were detected using the viral anti-hemagglutinin (HA) antibody.
211 In contrast to uninfected mice (NI), upon infection (LD₅₀), CD41-positive DAPI-negative
212 platelets stained positively for viral HA, demonstrating that platelets engulfed IAV particles,
213 fragments of IAV or viral proteins *in vivo* (Figure 1C). CD41-negative/DAPI-positive cells
214 were used as controls for antibody specificity. To confirm these results, immunogold labeling
215 of ultrathin cryosections of lungs from uninfected or infected mice was performed using a
216 specific anti-HA antibody. Examination of platelets clearly showed a positive and specific

217 staining of viral HA proteins, which were located predominantly within platelet granule-like
218 structures (Figure 1D, middle and upper right panels). The sparse staining could have been
219 due to the procedure. Indeed, as a control, we used immunogold labelling of HA on highly
220 purified A/PR/8/34 virus particles (17). Although virions of IAV contain approximately 500
221 molecules of HA per virion, few gold particles were observed (Figure 1D, lower right panel).

222

223 **Platelet activation and aggregation**

224 Upon activation, platelets become immobilized, secrete their granule content, and aggregate.
225 Serotonin is released from platelet dense granules, and P-selectin is rapidly translocated from
226 the alpha granules to the plasma membrane and shed. Thus, we next analyzed these responses
227 in the lungs of infected mice (sublethal or LD₅₀). Serotonin and soluble P-selectin (sP-
228 selectin) were measured in BAL and plasma, respectively, by ELISA (Figure 2A). Serotonin
229 and sP-selectin were significantly higher in the fluid of infected mice compared to uninfected
230 mice. Significant differences were only observed upon infection with IAV at LD₅₀.
231 Furthermore, exposure of P-selectin on the surface of platelets isolated from IAV-infected
232 mice was increased compared to uninfected mice (Figure 2B, left panel). The average
233 percentage of P-selectin-positive platelets reached 23% upon infection, versus 5% in
234 uninfected mice (Figure 2B, right panel). Moreover, transmission electron microscopy
235 showed that platelets in the lungs of influenza virus-infected mice were tightly packed,
236 forming large extravascular aggregates with signs of shape change and some platelets were
237 devoid of granules (Figure 2C). In contrast, in the lungs of uninfected mice, only a few
238 isolated platelets were detected.

239

240 **Platelets contribute to influenza pathogenesis**

241 Platelet GPIIIa^{+/-} mice were intercrossed to generate wild-type (WT) and platelet GPIIIa^{-/-}
242 mice, which were then infected with IAV A/PR/8/34, and the survival rates were monitored.
243 As shown in Figure 2D, compared to WT mice, GPIIIa^{-/-} mice were significantly more
244 resistant to IAV-induced death.

245

246 **Time course of platelet activation, IL1- β release and platelet binding to leukocytes**

247 Platelets were counted in the BAL of infected mice (LD₅₀) at various times post-inoculation.
248 Upon infection, platelet counts increased in a time-dependent manner (Figure 3A), peaked on
249 day 3 and stayed elevated until day 8. Plasmatic sP-selectin significantly increased during the
250 course of infection and plateaued on days 3-8 (Figure 3B). Increased IL1- β was also detected
251 in the BAL and blood of infected mice but with different lags (Figure 3C-D). IL1- β was
252 released in the BAL paralleled platelet activation, whereas IL1- β peaked in the blood on day 2
253 post-inoculation and then rapidly decreased. Ultrastructural analysis of the lungs of
254 A/PR/8/34-infected mice showed that platelet-leukocyte complexes formed *in vivo*.
255 Neutrophils and monocytes were associated with platelet aggregates, although not all platelets
256 adhered to leukocytes (Figure 3E).

257

258 **PAR4 promotes pathogenesis of IAV infection in a platelet-dependent pathway**

259 Mice were inoculated with a sublethal dose of IAV A/PR/8/34 and stimulated with 100
260 μ g/mouse of the PAR4 agonist peptide AYPGKF-NH₂ (PAR4-AP) or the inactive control
261 peptide YAPGKF-NH₂ (Control-P). As expected, the content of serotonin and sP-selectin was
262 increased in the BAL of infected mice treated with PAR4-AP compared to Control-P,
263 indicating an increased level of platelet activation (Figure 4A). More interestingly, upon
264 infection, mice treated with PAR4-AP displayed significantly higher mortality rates compared
265 with mice treated with Control-P (Figure 4B). In contrast, treatment with PAR4-AP did not

266 affect the survival of uninfected mice. The effect was platelet dependent, as treatment of mice
267 with eptifibatide, an antagonist of the GPIIb/IIIa platelet receptor, abrogated the deleterious
268 effect of PAR4-AP (Figure 4C), as did the platelet GPIIIa deficiency (Figure 4D). This
269 indicated that PAR4-AP-induced platelet aggregation increased the severity of the IAV
270 symptoms. No significant differences in lung virus titer were observed 3 or 6 days post-
271 inoculation between mice treated with PAR4-AP and those treated with Control-P (Figure
272 4E). However, on day 6, treatment with PAR4-AP significantly increased total proteins in the
273 BAL (Figure 4F). The response levels of IL-6, IL-1 β and MIP-2 were also enhanced, while
274 those of interferon (IFN)- γ , RANTES and KC were unaffected (Figure 5A). On day 3, no
275 difference was observed. Thus, PAR4 activation promoted IAV-induced inflammation of the
276 lungs at later time points post-infection. Similarly, staining of lung sections on day 6 revealed
277 marked cellular infiltrates of leukocytes (HE) and neutrophils (Ly6G) in the lungs of PAR4-
278 AP-treated mice compared to controls (Figure 5B). Similar numbers of IAV-infected cells
279 were detected by immunohistochemistry using an anti-NP antibody. No staining was observed
280 in the lungs of uninfected control mice.

281

282 **PAR4 antagonism protects against influenza virus pathogenicity**

283 When mice were infected with IAV A/PR/8/34 (LD₅₀), treatment with pepducin p4pal-10
284 protected them from death (Figure 6A). Substantial protection was also observed against
285 infection with an H3N2 virus, A/HK/1/68. The protection conferred by PAR4 antagonism
286 correlated with the degree of inhibition of platelet activation. In the BAL of pepducin p4pal-
287 10-treated mice, decreased thromboxane B2 (TXB2), a specific marker of platelet activation,
288 was observed (Figure 6B). In contrast, no difference in the mean lung virus titers was detected
289 on day 3 or 6 after inoculation with IAV A/PR/8/34 (Figure 6C). However, treatment with
290 pepducin p4pal-10 significantly reduced the recruitment of leukocytes (Figure 6D), including

291 neutrophils, in BAL on day 6. Total proteins (Figure 6E) and IL-6, IL-1 β and MIP-2 (Figure
292 6F) were also decreased. Consistent with those results, histopathology revealed that treatment
293 with pepducin p4pal-10 reduced infiltration of inflammatory cells (HE), including neutrophils
294 (Ly6G), in the lungs of infected mice (Figure 6G), while similar numbers of IAV-infected
295 cells (NP) were detected by immunohistochemistry.

296

297 **The anti-platelet drug eptifibatide protects mice from lethal influenza infection**

298 Mice were inoculated with IAV A/PR/8/34 (LD₅₀) and were treated or not with 500 μ g/kg of
299 eptifibatide every 3 days. This dosage is comparable to the lowest doses used clinically in
300 humans (20-22). Eptifibatide treatment had a dramatic effect on lung infiltration by platelets:
301 platelet aggregation was totally prevented, and only isolated platelets were observed (Figure
302 7A). Furthermore, this effect was accompanied by decreases in TXB2 and sP-selectin in the
303 fluid of infected mice compared to controls (Figure 7B), showing that inhibition of platelet
304 aggregation also limited the extent of platelet activation. More importantly, treatment with
305 eptifibatide improved the outcome of infection with A/PR/8/34 virus and prevented mortality
306 of the mice (Figure 7C). Protection was also observed with other influenza strains. No effect
307 of eptifibatide was observed in GPIIIa^{-/-} mice (Figure 8A), showing the specificity of the drug.

308

309 The protective effect of eptifibatide was independent of virus replication in lungs (Figure 8B)
310 and IFN- α release in the BAL (Figure 8C). In contrast, it was correlated with decreased total
311 proteins and levels of certain cytokines in the BAL of eptifibatide-treated mice (Figure 8D-E).
312 Immunohistochemistry confirmed that treatment by eptifibatide prevented IAV-induced lung
313 alveolar damage (HE) and neutrophil infiltration (Ly6G) but not viral replication (NP) on day
314 6 post-infection (Figure 8F). This effect was not observed on day 2 (data not shown).

315 Treatment of infected mice with MRS 2179 and clopidogrel, which inhibits the ADP
316 receptors P2Y1 and P2Y12, improved the outcome of IAV infection (Figure 8G).

317

318 **Eptifibatide treatment protects mice from lung injury induced by influenza**

319 Histopathological analyses of lung tissues were performed to evaluate the extent of
320 hemorrhage after eptifibatide treatment. Mice were infected with A/PR/8/34 virus and treated
321 with eptifibatide or vehicle, and lungs were then harvested 6 days post-inoculation for
322 histopathology. In the infected group, lungs presented signs of congestion with infiltration of
323 neutrophils and monocytes, interstitial and alveolar hemorrhages, as well as thrombosis
324 (Figure 9A). Fibrin and erythrocyte-rich thrombi were observed in small vessels. Figure 9B
325 summarizes the blinded semi-quantitative scoring of the different parameters. Eptifibatide
326 markedly reduced the severity of pulmonary injury induced by influenza virus infections, and
327 a marked reduction in neutrophil infiltration was observed. (Figure 9A-B). More importantly,
328 almost no hemorrhage was detected in the lungs of infected mice treated with eptifibatide.

329

330 **DISCUSSION**

331

332 The present study shows that platelets play an active role in fueling the dysregulation of
333 inflammation and promoting pathogenesis of influenza virus infections. Histological analysis
334 of lungs provided evidence that platelets massively infiltrate the lungs of infected mice.
335 **Additionally**, infiltrated platelets stained positive for viral HA, **based on** immunofluorescence
336 staining of BAL and immunogold labeling of ultrathin cryosections of lungs. The technical
337 limitation of the staining did not allow **us to determine** whether platelets engulfed the entire
338 virions, only **IAV** fragments or antigens. **However, because** platelets incorporate influenza
339 viruses *in vitro* (23), our results suggest that platelets recruited to the lungs most likely take up
340 IAV particles *in vivo* as well. This could consist of a passive passage of particles through the
341 open canalicular system, the tortuous invaginations of platelet surface membranes tunneling
342 through the cytoplasm, in a manner similar to bacterial ingestion (24). Alternatively, uptake of
343 IAVs may be compared to phagocytosis by macrophages and neutrophils, as previously
344 observed for human immunodeficiency viruses (25).

345

346 **Ultrastructural** analysis showed that features of platelets in the lungs of infected mice are
347 those of aggregates of activated platelets: platelets were tightly stacked without interplatelet
348 spaces, and some platelets were devoid of granules, suggesting that they had degranulated.
349 Consistent **with those observations**, markers of platelet activation were detected in the **BAL**
350 **and plasma** of infected mice. **Thus, upon lethal IAV infection, platelets are activated in the**
351 **lung and in the peripheral circulation.** Our observations are **consistent** with the recent **findings**
352 that influenza virus activates platelets through FcγRIIA signaling or thrombin generation (26).
353 **Also**, thrombin triggers the release of serotonin and TXA2 from platelets, promotes P-selectin
354 translocation to the platelet plasma membrane and activates the GPIIb/IIIa **complex** (27).

355

356 Platelets contribute to the host defense against bacterial infectious agents by limiting vascular
357 lesions and inducing injury repair (28, 29). However, unbalanced platelet activation may have
358 pathological consequences. In the IAV infection model used in this study, platelet activation
359 and aggregation proved to be deleterious. PAR4 and GPIIIa are both key molecules in platelet
360 function. PAR4 is strictly required for platelet activation in mice, while GPIIIa is required for
361 platelet aggregation. First, mice deficient in GPIIIa were protected from lung injury and
362 death. Furthermore, stimulation of PAR4 increased lung inflammation and the severity of
363 IAV infection. In contrast, PAR4 antagonists protected mice from death. Our results indicate
364 that PAR4 acts through platelet activation because the effect of PAR4-AP was abrogated
365 when infected mice were treated with the platelet specific inhibitor eptifibatide (30), or when
366 mice were deficient in platelet GPIIIa protein. Altogether, the data indicate that platelets
367 regulate IAV pathogenesis.

368

369 Interestingly, the observation by others that influenza virus activates platelets through
370 thrombin generation (26) suggests that thrombin may also act in a deleterious manner against
371 IAV infection. Thrombin mediates signal transduction mainly by activating PAR4 and PAR1
372 (31, 32). Because mouse platelets do not express PAR1, thrombin-mediated platelet activation
373 most likely occurs through PAR4 activation, but thrombin activation of PAR1 may also be
374 involved in the pathogenesis of IAV infection. Indeed, we recently found that PAR1 signaling
375 contributes to IAV pathogenicity in mice (33). In this context, PAR1 cooperates with
376 plasminogen, which controls pathogenesis, via fibrinolysis (34). Thus, investigations into the
377 role of hemostasis dysregulation may help better understand IAV pathogenesis (35-37).

378

379 In several models of injury, **uncontrolled** platelet activation drives deleterious inflammation
380 (38). Activated platelets release an arsenal of potent pro-inflammatory molecules (39), which
381 exacerbate neutrophil rolling, adhesion and recruitment (40-42). In addition, the physical
382 interaction between platelets and neutrophils further contributes to neutrophil retention and
383 activation (42). **Because** dysregulation of inflammation is a hallmark of severe influenza virus
384 infections, it **is** likely that platelets have a pro-inflammatory effect with a key role in IAV
385 pathogenesis. In our study, electron microscopy demonstrated the presence of neutrophil-
386 platelet complexes upon IAV infection. **The** anti-platelet molecule eptifibatide inhibited
387 neutrophil recruitment into inflamed lungs (Figure 9). Thus, platelet interaction with
388 neutrophils is likely to play a role during severe inflammation induced by influenza.

389

390 Interestingly, **the** exacerbation of cytokine production induced by platelet activation was only
391 observed at later time points after infection. Upon infection, the virus is recognized as foreign
392 by highly conserved receptors known as **pattern recognition receptors**. Activation of these
393 receptors results in the secretion of cytokines and chemokines, which corresponds to the early
394 inflammatory response against IAV infection (35). Thus, the amplification and intensity of
395 inflammation **depends** on the replicative capacity of the virus. When the response is tightly
396 controlled, a resolution phase of inflammation is engaged at later time points post-infection,
397 and this partly determines the duration of inflammation. Resolution of inflammation is largely
398 influenced by the vascular endothelium (43). Upon injury of the latter, platelets are activated.
399 Our data show coordinated platelet activation/aggregation and inflammatory responses at late
400 time points post-infection, **indicating** that platelets may affect the recovery phase **after**
401 infection and wound healing. In this scenario, extravasation of large **numbers** of platelets and
402 **leukocytes** would be the basis of the defect in the resolution phase of the inflammation. Most

403 likely, this further promotes hemostasis dysregulation, such as fibrinolysis (18, 44) or PAR1
404 activation (33, 45), fueling the vicious circle of inflammation (34, 35).

405

406 Recurrent outbreaks of IAV that cause severe infections in humans have raised serious
407 concerns about therapeutic strategies available for these pathogens. Current treatments that
408 target viral proteins have a number of disadvantages, including the rapid development of
409 resistant virus variants as a result of selective pressure (46, 47). Because targeting the host
410 rather than the virus would not easily lead to resistance, drugs regulating inflammation are
411 appealing as potential new therapeutics for IAV symptoms (13, 33, 34, 48). Here, we found
412 that available anti-platelet drugs efficiently protected mice from IAV pathogenesis induced by
413 several influenza strains. These results are consistent with other studies showing that aspirin,
414 known to inhibit platelet activation, blocks IAV propagation via NF-kB inhibition (49).
415 Altogether, these results suggest that anti-platelet drugs might be explored as new anti-
416 inflammatory treatments against severe influenza.

417

418

419 **ACKNOWLEDGMENTS**

420 Authors are grateful to Dr. P. Clézardin (Inserm UMR S1033, France) and Dr. C. Dumontet
421 (Cancer Center of Lyon, France) for help with the immunohistochemistry and
422 immunofluorescence. Our study received only governmental funding. No pharmaceutical
423 industries have been involved in this study.

424

425

426 **REFERENCES**

427

428 1. Kuiken T, Riteau B, Fouchier RA, Rimmelzwaan GF. Pathogenesis of influenza virus
429 infections: The good, the bad and the ugly. *Curr Opin Virol* 2012;2:276-286.

430 2. Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: The
431 contributions of virus and host factors. *Current opinion in immunology* 2011;23:481-486.

432 3. Foucault ML, Moules V, Rosa-Calatrava M, Riteau B. Role for proteases and hla-g in
433 the pathogenicity of influenza a viruses. *J Clin Virol* 2011;51:155-159.

434 4. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y,
435 Peiris JS. Induction of proinflammatory cytokines in human macrophages by influenza a
436 (h5n1) viruses: A mechanism for the unusual severity of human disease? *Lancet*
437 2002;360:1831-1837.

438 5. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM,
439 Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT,
440 Hien TT, Farrar J. Fatal outcome of human influenza a (h5n1) is associated with high viral
441 load and hypercytokinemia. *Nature medicine* 2006;12:1203-1207.

442 6. Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH,
443 Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H,
444 Kawaoka Y. Aberrant innate immune response in lethal infection of macaques with the 1918
445 influenza virus. *Nature* 2007;445:319-323.

446 7. Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, Martinborough
447 E, Peach R, Oldstone MB, Rosen H. Endothelial cells are central orchestrators of cytokine
448 amplification during influenza virus infection. *Cell* 2011;146:980-991.

- 449 8. Rumbaut RE, Thiagarajan P. Platelet-vessel wall interactions in hemostasis and
450 thrombosis. San Rafael (CA); 2010.
- 451 9. Duerschmied D, Suidan GL, Demers M, Herr N, Carbo C, Brill A, Cifuni SM, Mauler
452 M, Cicko S, Bader M, Idzko M, Bode C, Wagner DD. Platelet serotonin promotes the
453 recruitment of neutrophils to sites of acute inflammation in mice. *Blood* 2013;121:1008-1015.
- 454 10. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;420:885-891.
- 455 11. Degen JL, Bugge TH, Goguen JD. Fibrin and fibrinolysis in infection and host
456 defense. *J Thromb Haemost* 2007;5 Suppl 1:24-31.
- 457 12. Riteau B, Moreau P, Menier C, Khalil-Daher I, Khosrotehrani K, Bras-Goncalves R,
458 Paul P, Dausset J, Rouas-Freiss N, Carosella ED. Characterization of hla-g1, -g2, -g3, and -g4
459 isoforms transfected in a human melanoma cell line. *Transplant Proc* 2001;33:2360-2364.
- 460 13. Khoufache K, LeBouder F, Morello E, Laurent F, Riffault S, Andrade-Gordon P,
461 Boullier S, Rousset P, Vergnolle N, Riteau B. Protective role for protease-activated receptor-2
462 against influenza virus pathogenesis via an ifn-gamma-dependent pathway. *J Immunol*
463 2009;182:7795-7802.
- 464 14. Riteau B, de Vaureix C, Lefevre F. Trypsin increases pseudorabies virus production
465 through activation of the erk signalling pathway. *J Gen Virol* 2006;87:1109-1112.
- 466 15. Riteau B, Faure F, Menier C, Viel S, Carosella ED, Amigorena S, Rouas-Freiss N.
467 Exosomes bearing hla-g are released by melanoma cells. *Hum Immunol* 2003;64:1064-1072.
- 468 16. LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Pechoux C, Delmas B, Riteau B.
469 Annexin ii incorporated into influenza virus particles supports virus replication by converting
470 plasminogen into plasmin. *J Virol* 2008;82:6820-6828.
- 471 17. Berri F, Haffar G, Le VB, Sadewasser A, Paki K, Lina B, Wolff T, Riteau B. Annexin
472 v incorporated into influenza virus particles inhibits gamma interferon signaling and promotes
473 viral replication. *J Virol* 2014;88:11215-11228.

- 474 18. LeBouder F, Lina B, Rimmelzwaan GF, Riteau B. Plasminogen promotes influenza a
475 virus replication through an annexin 2-dependent pathway in the absence of neuraminidase. *J*
476 *Gen Virol* 2010;91:2753-2761.
- 477 19. LeBouder F, Khoufache K, Menier C, Mandouri Y, Keffous M, Lejal N, Krawice-
478 Radanne I, Carosella ED, Rouas-Freiss N, Riteau B. Immunosuppressive hla-g molecule is
479 upregulated in alveolar epithelial cells after influenza a virus infection. *Hum Immunol*
480 2009;70:1016-1019.
- 481 20. Hassan W, Al-Sergani H, Al Buraiki J, Dunn B, Al Turki F, Akhras N, Elshaer F,
482 Nawaz M, Kharabsheh S, ElKum N. Immediate and intermediate results of intracoronary
483 stand-alone bolus administration of eptifibatide during coronary intervention (ice) study.
484 *American heart journal* 2007;154:345-351.
- 485 21. Gilchrist IC, O'Shea JC, Kosoglou T, Jennings LK, Lorenz TJ, Kitt MM, Kleiman NS,
486 Talley D, Aguirre F, Davidson C, Runyon J, Tcheng JE. Pharmacodynamics and
487 pharmacokinetics of higher-dose, double-bolus eptifibatide in percutaneous coronary
488 intervention. *Circulation* 2001;104:406-411.
- 489 22. Tcheng JE, Talley JD, O'Shea JC, Gilchrist IC, Kleiman NS, Grines CL, Davidson CJ,
490 Lincoff AM, Califf RM, Jennings LK, Kitt MM, Lorenz TJ. Clinical pharmacology of higher
491 dose eptifibatide in percutaneous coronary intervention (the pride study). *The American*
492 *journal of cardiology* 2001;88:1097-1102.
- 493 23. Danon D, Jerushalmy Z, De Vries A. Incorporation of influenza virus in human blood
494 platelets in vitro. Electron microscopical observation. *Virology* 1959;9:719-722.
- 495 24. White JG. Platelets are coverocytes, not phagocytes: Uptake of bacteria involves
496 channels of the open canalicular system. *Platelets* 2005;16:121-131.

- 497 25. Youssefian T, Drouin A, Masse JM, Guichard J, Cramer EM. Host defense role of
498 platelets: Engulfment of hiv and staphylococcus aureus occurs in a specific subcellular
499 compartment and is enhanced by platelet activation. *Blood* 2002;99:4021-4029.
- 500 26. Boilard E, Pare G, Rousseau M, Cloutier N, Dubuc I, Levesque T, Borgeat P, Flamand
501 L. Influenza virus h1n1 activates platelets through fcgammariia signaling and thrombin
502 generation. *Blood* 2014;123:2854-2863.
- 503 27. Polley MJ, Leung LL, Clark FY, Nachman RL. Thrombin-induced platelet membrane
504 glycoprotein iib and iiiia complex formation. An electron microscope study. *J Exp Med*
505 1981;154:1058-1068.
- 506 28. Petaja J. Inflammation and coagulation. An overview. *Thromb Res* 2011;127 Suppl
507 2:S34-37.
- 508 29. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate
509 immunity. *Nat Rev Immunol* 2013;13:34-45.
- 510 30. Tardiff BE, Jennings LK, Harrington RA, Gretler D, Potthoff RF, Vorchheimer DA,
511 Eisenberg PR, Lincoff AM, Labinaz M, Joseph DM, McDougal MF, Kleiman NS,
512 Investigators P. Pharmacodynamics and pharmacokinetics of eptifibatide in patients with
513 acute coronary syndromes: Prospective analysis from pursuit. *Circulation* 2001;104:399-405.
- 514 31. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-
515 activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*
516 1999;103:879-887.
- 517 32. Kataoka H, Hamilton JR, McKemy DD, Camerer E, Zheng YW, Cheng A, Griffin C,
518 Coughlin SR. Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial
519 cells. *Blood* 2003;102:3224-3231.

- 520 33. Khoufache K, Berri F, Nacken W, Vogel AB, Delenne M, Camerer E, Coughlin SR,
521 Carmeliet P, Lina B, Rimmelzwaan GF, Planz O, Ludwig S, Riteau B. Par1 contributes to
522 influenza a virus pathogenicity in mice. *J Clin Invest* 2013;123:206-214.
- 523 34. Berri F, Rimmelzwaan GF, Hanss M, Albina E, Foucault-Grunenwald ML, Le VB,
524 Vogelzang-van Trierum SE, Gil P, Camerer E, Martinez D, Lina B, Lijnen R, Carmeliet P,
525 Riteau B. Plasminogen controls inflammation and pathogenesis of influenza virus infections
526 via fibrinolysis. *PLoS Pathog* 2013;9:e1003229.
- 527 35. Berri F, Le VB, Jandrot-Perrus M, Lina B, Riteau B. Switch from protective to
528 adverse inflammation during influenza: Viral determinants and hemostasis are caught as
529 culprits. *Cellular and molecular life sciences : CMLS* 2014;71:885-898.
- 530 36. Antoniak S, Mackman N. Multiple roles of the coagulation protease cascade during
531 virus infection. *Blood* 2014;123:2605-2613.
- 532 37. Pryzdial EL, Sutherland MR, Ruf W. The procoagulant envelope virus surface:
533 Contribution to enhanced infection. *Thromb Res* 2014;133 Suppl 1:S15-17.
- 534 38. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G,
535 Kroczek RA. Cd40 ligand on activated platelets triggers an inflammatory reaction of
536 endothelial cells. *Nature* 1998;391:591-594.
- 537 39. von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, Weber C.
538 Rantes deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic
539 endothelium. *Circulation* 2001;103:1772-1777.
- 540 40. Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling,
541 arrest, and transmigration across activated, surface-adherent platelets via sequential action of
542 p-selectin and the beta 2-integrin cd11b/cd18. *Blood* 1996;88:146-157.

- 543 41. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling
544 and extravasation are severely compromised in p selectin-deficient mice. *Cell* 1993;74:541-
545 554.
- 546 42. Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions:
547 Linking hemostasis and inflammation. *Blood reviews* 2007;21:99-111.
- 548 43. Kadl A, Leitinger N. The role of endothelial cells in the resolution of acute
549 inflammation. *Antioxidants & redox signaling* 2005;7:1744-1754.
- 550 44. Berri F, Le VB, Jandrot-Perrus M, Lina B, Riteau B. Switch from protective to
551 adverse inflammation during influenza: Viral determinants and hemostasis are caught as
552 culprits. *Cellular and molecular life sciences : CMLS* 2013.
- 553 45. Aerts L HM, Rhéaume C, Lavigne S, Couture C, Kim W, Susan-Resiga D, Prat A,
554 Seidah NG, Vergnolle N, Riteau B, Boivin G. Modulation of protease activated receptor 1
555 influences human metapneumovirus disease severity in a mouse model. *Plos One*
556 2013;28;8(8):e72529.
- 557 46. Song MS, Hee Baek Y, Kim EH, Park SJ, Kim S, Lim GJ, Kwon HI, Pascua PN,
558 Decano AG, Lee BJ, Kim YI, Webby RJ, Choi YK. Increased virulence of neuraminidase
559 inhibitor-resistant pandemic h1n1 virus in mice: Potential emergence of drug-resistant and
560 virulent variants. *Virulence* 2013;4:489-493.
- 561 47. Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L, Guarnaccia T, Baas C,
562 Xue L, Vitesnik S, Leang SK, McVernon J, Kelso A, Barr IG, McCaw JM, Bloom JD, Hurt
563 AC. Estimating the fitness advantage conferred by permissive neuraminidase mutations in
564 recent oseltamivir-resistant a(h1n1)pdm09 influenza viruses. *PLoS Pathog*
565 2014;10:e1004065.
- 566 48. Walsh KB, Teijaro JR, Wilker PR, Jatzek A, Fremgen DM, Das SC, Watanabe T,
567 Hatta M, Shinya K, Suresh M, Kawaoka Y, Rosen H, Oldstone MB. Suppression of cytokine

568 storm with a sphingosine analog provides protection against pathogenic influenza virus. *Proc*
569 *Natl Acad Sci U S A* 2011;108:12018-12023.

570 49. Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, Puthavathana P, Silberzahn T, Wolff T,
571 Planz O, Ludwig S. Acetylsalicylic acid (asa) blocks influenza virus propagation via its nf-
572 kappaB-inhibiting activity. *Cell Microbiol* 2007;9:1683-1694.

573

574

575

576

577 **FIGURE LEGENDS**

578

579 **Figure 1: Upon IAV infection, platelets infiltrate the lungs, and IAV particles are**
580 **observed in platelets.** (A) Immunohistochemistry analysis of lungs from uninfected (NI) or
581 infected mice inoculated with A/PR/8/34 virus, at a sublethal dose (75 pfu/mouse) or LD₅₀
582 (250 pfu/mouse; day 6 post-infection). Antibodies against the IAV nucleoprotein (NP) and
583 CD41 were used to detect virus infected cells and platelets, respectively. The results are
584 representative of three mice per group. (B) Platelet numbers in BAL were assessed using a
585 Vet ABC™ Hematology Analyzer on day 6 post-inoculation of mock or IAV-infected mice.
586 Data are presented as the means ± SEM of 4 mice per group, ** p<0.01 for LD₅₀ vs. NI. (C)
587 Immunofluorescence staining of viral particles in platelets from BAL was performed with
588 anti-influenza HA antibody. Platelets were detected with anti-CD41 antibody, and nuclei were
589 counterstained with DAPI. The merged images are shown on the right panel. CD41-negative
590 cells from BAL were used as a negative control. (D) Immunogold labeling of ultrathin
591 cryosections of lungs of uninfected (NI) or A/PR/8/34 virus-infected mice (LD₅₀, 250
592 pfu/mouse, day 6 post-infection) was performed using a specific anti-HA antibody. Black
593 arrows indicate viral particles. Staining of a platelet-granule like structure is shown on the
594 upper right panel. As a control for HA staining, electron microscopic immunogold labeling
595 was performed on purified A/PR/8/34 viruses using the anti-HA antibody (lower right panel).

596

597 **Figure 2: Upon IAV infection, platelets are stimulated and contribute to influenza**

598 **pathogenesis.** (A) Serotonin and sP-selectin were measured by ELISA in the BAL and
599 plasma of Mock (NI) or A/PR/8/34 virus-infected mice, respectively, on day 6 post-
600 inoculation (75 pfu/mouse, sublethal dose or 250 pfu/mouse, LD₅₀). Data represent the means
601 ± SEM of 4 mice per group, * p<0.05 for LD₅₀ vs. NI; ** p<0.01 for LD₅₀ vs. NI. (B) Blood

602 samples from uninfected (NI) or infected mice were double-stained with anti-P-selectin and
603 anti-CD41 antibody as a platelet identifier. The mean percentage \pm SEM of activated platelets
604 (CD41 and P-selectin-positive) from **five** mice per group is shown **in** the right panel, * $p < 0.05$
605 for LD₅₀ vs. NI. (C) Ultrastructure analysis of platelets in the lungs of uninfected and infected
606 mice (A/PR/8/34, 250 pfu/mouse, LD₅₀). Note the aggregation of platelets in the lungs of
607 infected mice along with their morphological changes (arrows) and the absence of granules in
608 some of them, which reflects their degranulation (asterisks). Sections show platelet aggregates
609 with an interstitial localization. (D) Survival of platelet GPIIIa^{-/-} mice and WT littermates after
610 infection with A/PR/8/34 virus at a LD₅₀ (250 pfu/mouse, n=9-10 mice per group) or lethal
611 dose (350 pfu/mouse n=6 mice per group); * $p < 0.05$ and ** $p < 0.01$, respectively.

612

613 **Figure 3: Platelet activation and inflammation.** (A) Platelet numbers in BAL of A/PR/8/34
614 virus infected mice (250 pfu/mouse, LD₅₀) were assessed using a Vet ABC™ Hematology
615 Analyzer, at the indicated time post-inoculation. **The results** are represented **as the means** \pm
616 SEM of 4 mice per group. **On** days 0 and 6, additional results from Figure 1B **are** included.
617 (B-D) sP-selectin in the plasma (B), IL1- β in the BAL (C) and IL1- β in the plasma (D) of
618 infected mice (A/PR/8/34, 250 pfu/mouse, LD₅₀) were determined by ELISA at the indicated
619 times. **The results** from panels B-D represent **the means** \pm SEM of 4 mice per group. From A-
620 D: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the indicated time vs. 0. (E) Ultrastructural analysis
621 of platelets associated with leukocytes in the lungs of infected mice (A/PR/8/34, 250
622 pfu/mouse, LD₅₀). **The star shows platelet aggregates that are not adherent to leukocytes.**

623

624 **Figure 4: Effects of PAR4 activation on IAV pathogenicity, virus replication and**
625 **inflammation.** (A) Serotonin and sP-selectin were **measured** by ELISA in the BAL and
626 plasma, **respectively**, of infected mice (A/PR/8/34, 75 pfu/mouse, sublethal dose) after

627 treatment with PAR4-AP or control peptide (Control-P) on day 6 post-inoculation. Columns
628 represent the means \pm SEM (n=4-5). ** p<0.01; * p<0.05. (B) Time course of IAV-induced
629 death in mice in response to PAR4 stimulation. Mice were mock-infected or inoculated with
630 A/PR/8/34virus (75 pfu/mouse, sublethal dose, n=18-19 mice per group; or 250 pfu/mouse,
631 LD₅₀, n=6-12 mice per group) and treated with either control peptide or PAR4-AP. * p<0.05;
632 ** p<0.01. (C) Time course of IAV-induced death in mice (A/PR/8/34virus) in response to
633 PAR4 stimulation and after treatment or no treatment with eptifibatide (n=6-18 mice per
634 group). * p<0.05 for PAR4-AP vs. control-P. A significant difference (p<0.01) was also found
635 between groups treated with PAR-AP \pm eptifibatide (not shown) (D) Time course of IAV-
636 induced death in WT (n=10 mice per group) and GPIIIa^{-/-} mice (n=7-9 mice per group) in
637 response to PAR4 stimulation (A/PR/8/34virus). The same mice were used in Figure 2D (250
638 pfu/mouse, dose LD₅₀). *** p<0.001 for PAR4-AP vs. control-P in WT mice. (E) Lung virus
639 titers after infection of mice with A/PR/8/34 virus (sublethal dose) stimulated or not with
640 PAR4-AP. (F) Total protein quantification in BAL of infected mice in response to PAR4
641 stimulation. For E and F, the results represent the means \pm SEM (n=3-5). ** p<0.01 for
642 PAR4-AP vs. control-P.

643

644 **Figure 5: PAR4-AP increases lung inflammation upon A/PR/8/34 virus infection.** (A)
645 Cytokines in the BAL of infected mice (75 pfu/mouse, sublethal dose), treated with PAR4-AP
646 or control peptide, were measured by ELISA 3 and 6 days after inoculation. Uninfected mice
647 (NI) were used as control. The results represent the means \pm SEM (n=3-5). * p<0.05, **
648 p<0.01 for PAR4-AP vs. control-P. (B) Histopathological analysis of lungs from uninfected
649 mice or mice infected with a sublethal dose (75 pfu/mouse) of A/PR/8/34 virus after treatment
650 with PAR4-AP or control peptide, on day 6 post-infection. Thin sections of lungs were
651 stained with hematoxylin and eosin (HE). Note the marked infiltration of cells in the lungs of

652 infected mice stimulated with PAR4-AP. Immunohistochemistry used antibodies against
653 Ly6G. Viral NP was used to detect neutrophils and virus-infected cells. Data are
654 representative of three mice per group.

655

656 **Figure 6: PAR4 antagonist protects mice against IAV infection and deleterious lung**
657 **inflammation.** (A) IAV-induced pathogenesis in mice treated or not with the PAR4
658 antagonist pepducin p4pal-10 (pepducin). Mice were inoculated with A/PR/8/34 virus (250
659 pfu/mouse, LD₅₀, n=13 mice per group) or A/HK/1/68 (100 pfu/mouse, LD₅₀, n=12 mice per
660 group) and treated with pepducin or saline. Survival was then monitored for 2 weeks. *
661 p<0.05. (B) Thromboxane B2 (TXB2) was measured by ELISA in the BAL of infected mice
662 (A/PR/8/34, 250 pfu/mouse, LD₅₀) after treatment with pepducin or vehicle, on day 6 post-
663 inoculation. Data represent the mean ± SEM of 4-6 mice per group. (C) Lung virus titers after
664 infection of mice with A/PR/8/34 virus (250 pfu/mouse, LD₅₀) treated with pepducin or
665 vehicle. The results represent the means ± SEM from 3 individual animals per group. (D)
666 Relative leukocyte and neutrophil numbers in BAL from mice treated with pepducin or
667 vehicle, determined by May-Grünwald-Giemsa staining 6 days after inoculation. Data
668 represent the means ± SEM from 6 individual mice per group. (E, F) Total proteins and levels
669 of cytokines were determined by ELISA in the BAL of infected mice (A/PR/8/34, 250
670 pfu/mouse, LD₅₀) after treatment with pepducin or vehicle, on day 6 post-inoculation. The
671 results represent the means ± SEM of 6 mice per group. (G) Histopathological analysis of
672 lungs from mice infected with A/PR/8/34 virus (250 pfu/mouse, LD₅₀) after treatment with
673 pepducin or vehicle, on day 6 post-infection. Lung sections were stained with hematoxylin
674 and eosin (HE). Immunohistochemistry using antibodies against Ly6G, viral NP was used to
675 detect neutrophils and virus-infected cells. Data are representative of three mice per group.
676 (B-F). * p<0.05, ** p<0.01 for pepducin vs. saline.

677

678 **Figure 7: Eptifibatide protects mice against IAV infection, independently of the strain.**

679 (A) Ultrastructural analysis of platelets in the lungs of infected mice (A/PR/8/34, 250
680 pfu/mouse, LD₅₀), treated or not with eptifibatide, was performed by transmission electron
681 microscopy. Note the aggregation of platelets in the lungs of infected mice, and their
682 disaggregation after treatment of mice with eptifibatide. Sections show platelet aggregates
683 with an interstitial localization. (B) Thromboxane B2 (TXB2) was measured by ELISA in the
684 BAL of infected mice (A/NL/602/09, 30,000 pfu/mouse LD₅₀) after treatment with
685 eptifibatide or vehicle. The results represent the means ± SEM of 3-5 mice per group. sP-
686 selectin was measured by ELISA in the plasma of A/PR/8/34 virus-infected mice (250
687 pfu/mouse, LD₅₀) that were treated or not with eptifibatide, on day 6 post-inoculation. Data
688 represent the means ± SEM of 4 mice per group. * p<0.05 for pepducin vs. saline. (C)
689 Survival of mice treated with eptifibatide or vehicle after infection with IAV A/PR/8/34
690 (n=13 mice per group, 250 pfu/mouse), A/NL/602/09 (n=9-12 mice per group, 30,000
691 pfu/mouse) or A/HK/1/68 (n=12 mice per group, 100 pfu/mouse) at their respective LD₅₀
692 values. A/FPV/Bratislava/79 was used at 5 Pfu/mouse (n=6-7 mice per group). * p<0.05, **
693 p<0.01 for pepducin vs. saline.

694

695 **Figure 8: Eptifibatide treatment prevents severe inflammation during influenza virus**

696 **infections.** (A) Survival of GPIIIa^{-/-} (n=5 mice/group) and WT mice (n=12 mice/group) after
697 infection with IAV A/PR/8/34 (300 pfu/mouse) and treatment or no treatment with
698 eptifibatide. * p<0.05 for eptifibatide vs. saline. (B) Lung virus titers after infection of mice
699 with the A/NL/602/09 virus (30,000 pfu/mouse, LD₅₀) treated with eptifibatide or vehicle.
700 Data represent the means ± SEM from 3 individual animals per group. (C) IFN-α was
701 measured by ELISA in the BAL of infected mice (A/PR/8/34, 250 pfu/mice) after treatment

702 with eptifibatide or vehicle. The results represent the means \pm SEM of 4 mice per group. (D,
703 E) Total proteins and levels of cytokines were determined by ELISA in the BAL of infected
704 mice (30 000 pfu/mouse, A/NL/602/09, LD₅₀) after treatment with eptifibatide or vehicle. The
705 results represent the means \pm SEM of 3-5 mice per group. * p<0.05, ** p<0.01 for eptifibatide
706 vs. saline. (F) Histopathological analysis of lungs from mice infected with A/NL/602/09 virus
707 (30 000 pfu/mouse, LD₅₀) after treatment with eptifibatide or vehicle, on day 6 post-infection.
708 Lung sections were stained with hematoxylin and eosin (HE). Immunohistochemistry using
709 antibodies against Ly6G and viral NP was used to detect neutrophils and virus-infected cells.
710 Data are representative of three mice per group. (G) Survival of mice treated with MRS 2179,
711 clopidogrel or vehicle after infection with IAV A/PR/8/34 (250 pfu/mouse; n=12 mice per
712 group). ** p<0.01 for MRS 2179 vs. saline; * p<0.05 for clopidogrel vs. saline.

713

714 **Figure 9: Histopathological analysis of lungs from infected mice after treatment with**
715 **eptifibatide.** (A) Histopathological analysis of lungs obtained from mice inoculated with
716 A/PR/8/34 virus (250 PFU/mouse) and treated or not with eptifibatide. In the infected group,
717 note the extended areas with interstitial and peribronchial inflammation and interstitial and
718 alveolar hemorrhage. In the infected group treated with eptifibatide, note the limited areas
719 with slight peribronchial inflammation but no major hemorrhage. (B) Blinded
720 semiquantitative scoring of inflammatory infiltration, vascular congestion, hemorrhage, fibrin
721 deposits and epithelial cell apoptosis in the lungs of infected mice treated or not with
722 eptifibatide. All lung fields were examined (50x) for each sample. The scoring was performed
723 as follows: 0=no lesion, x=mild, xx=moderate, xxx=severe.

Figure 1

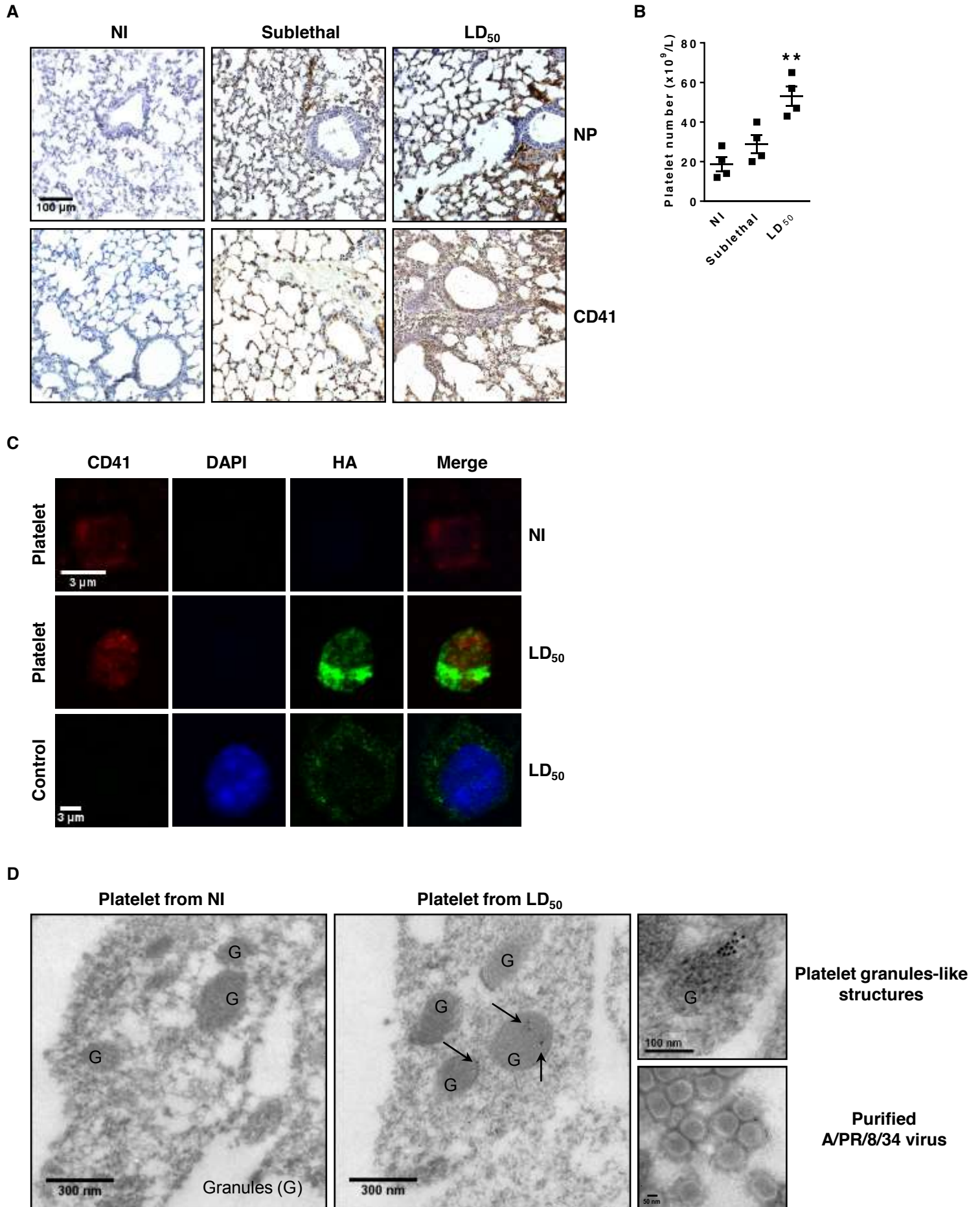


Figure 2

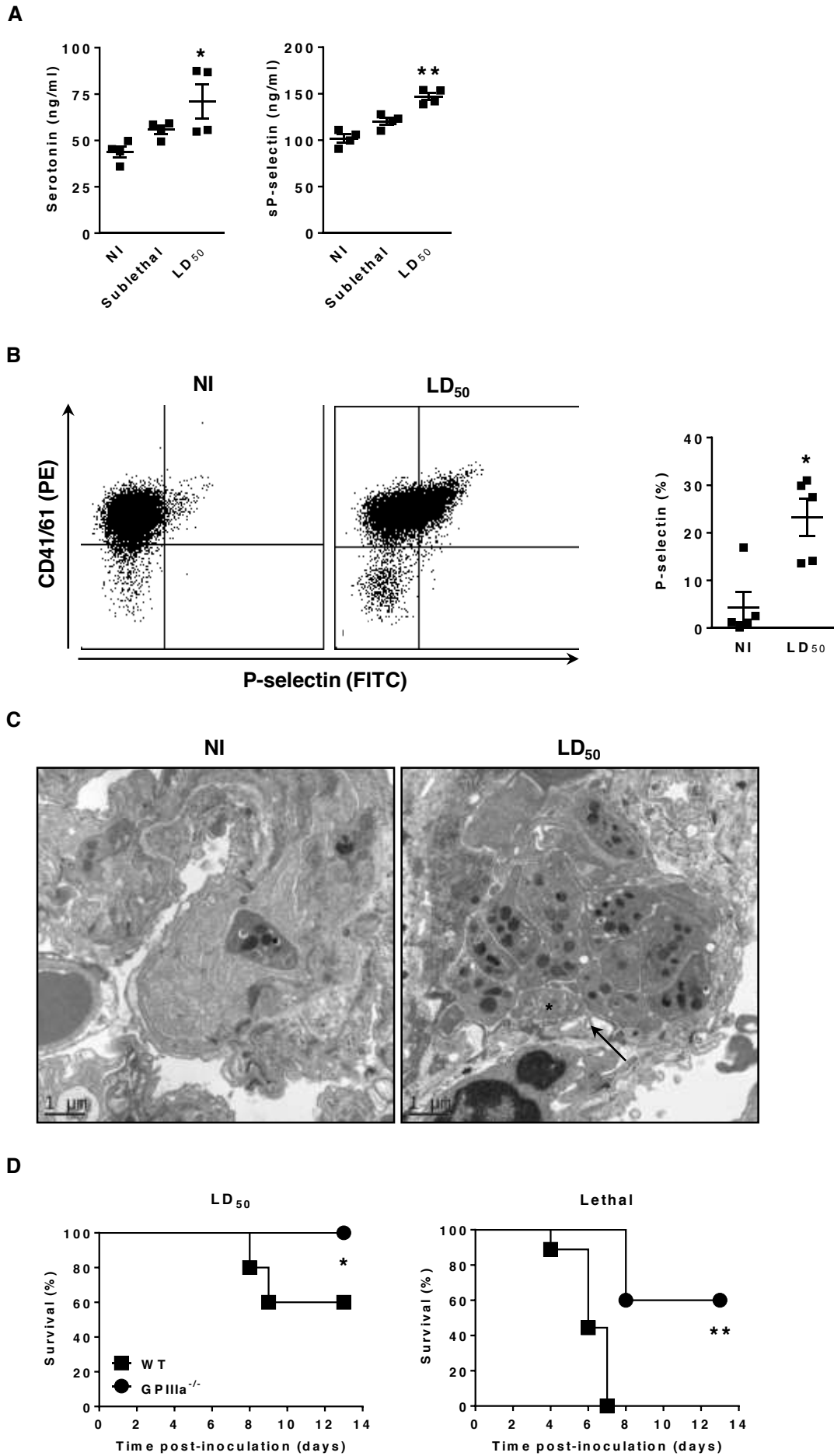
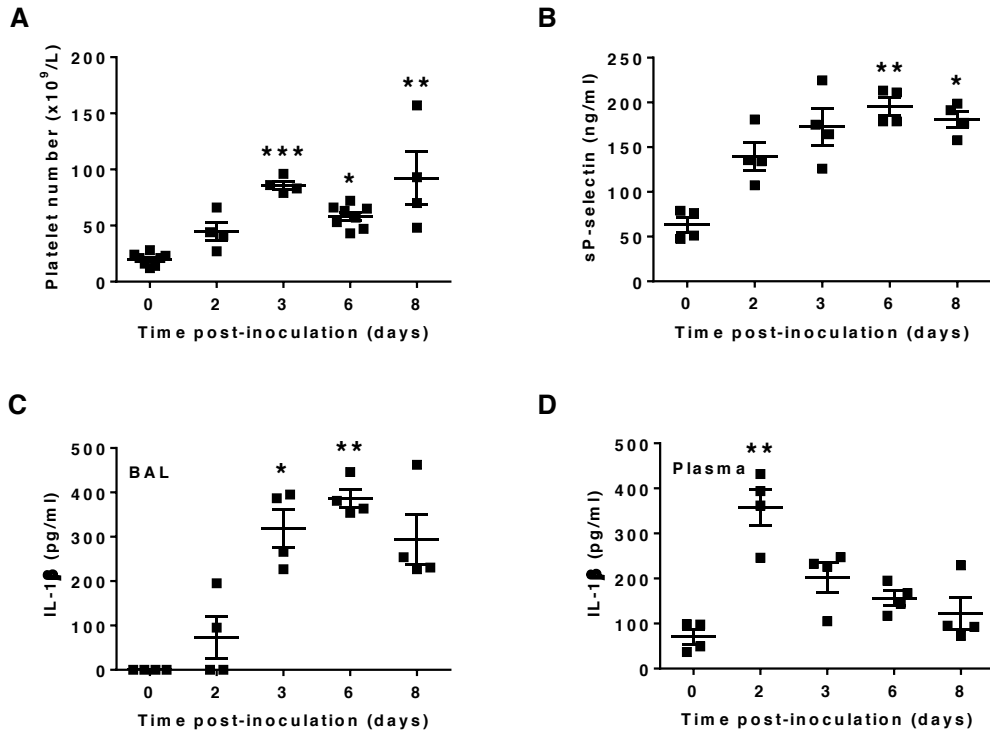


Figure 3



E

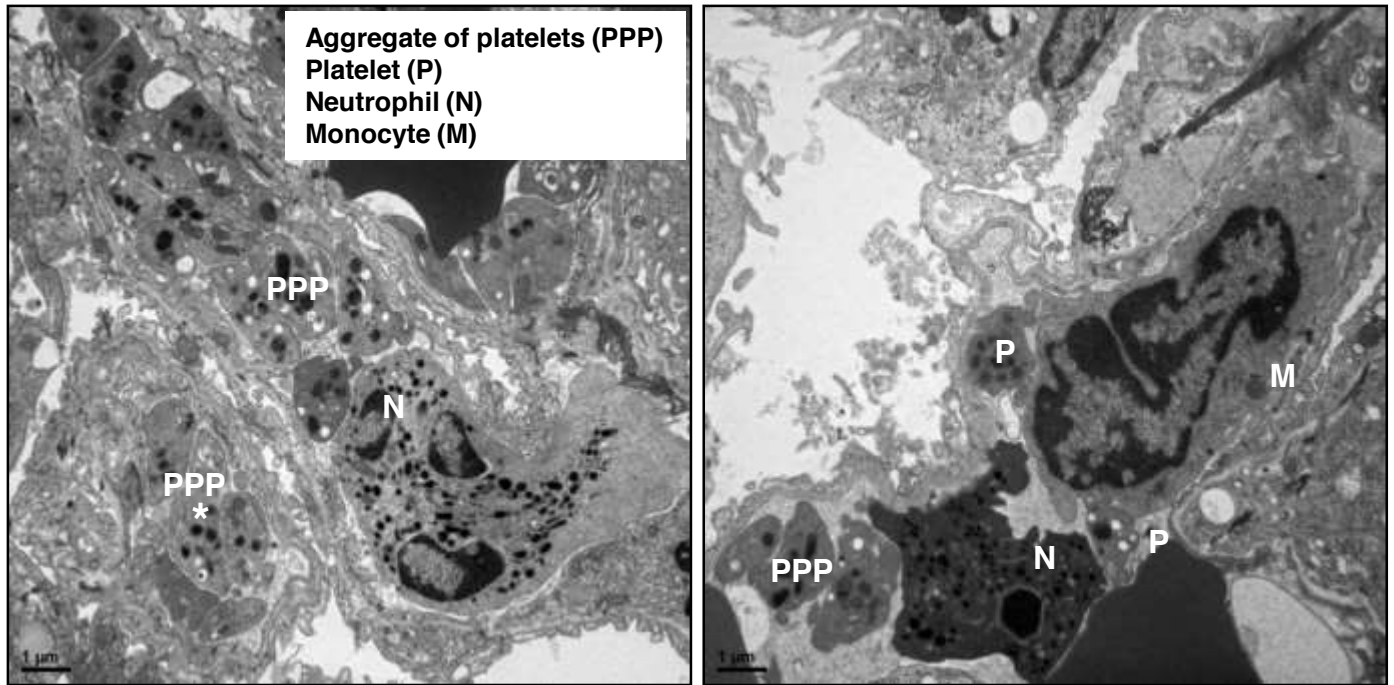


Figure 4

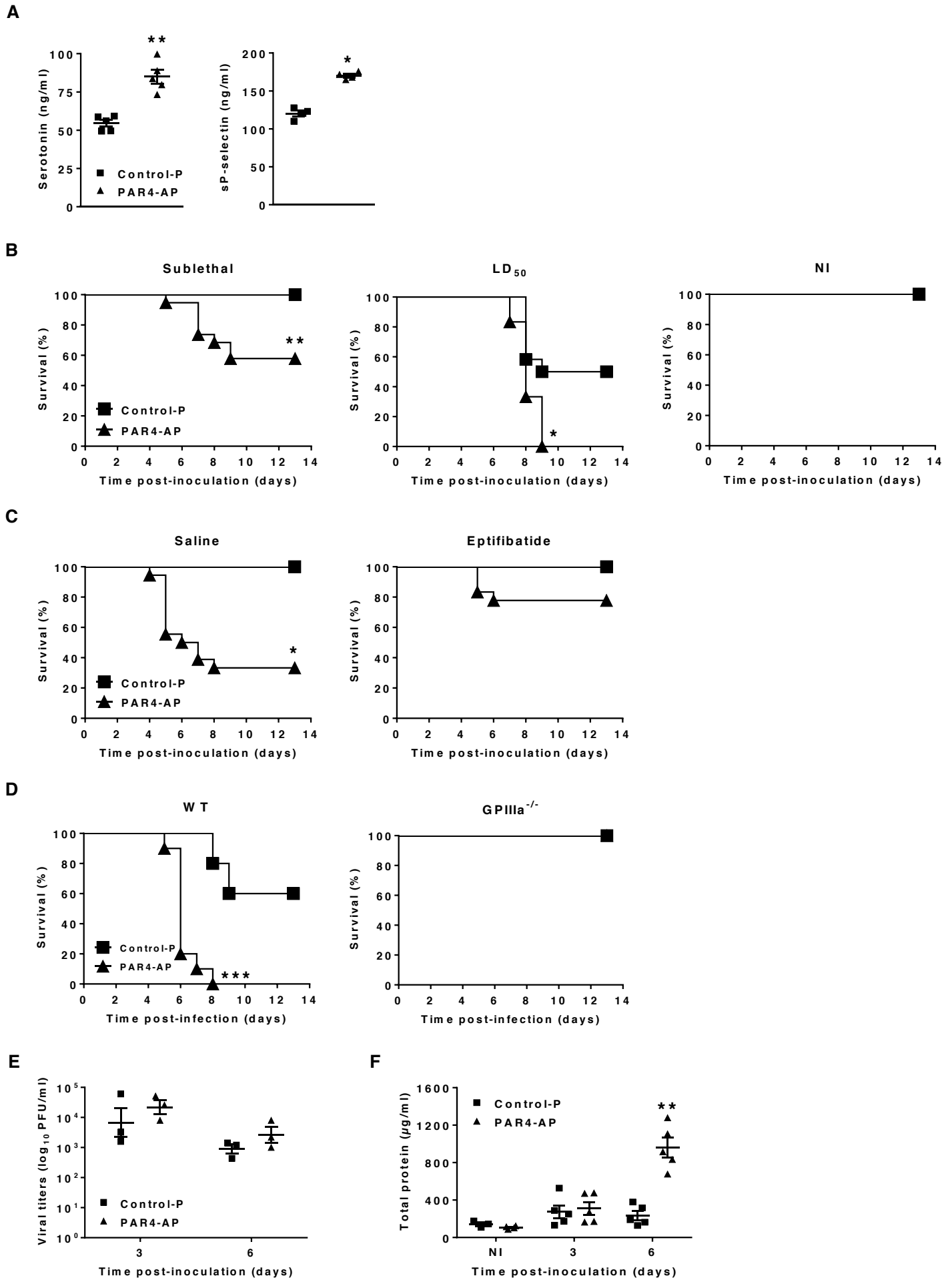


Figure 5

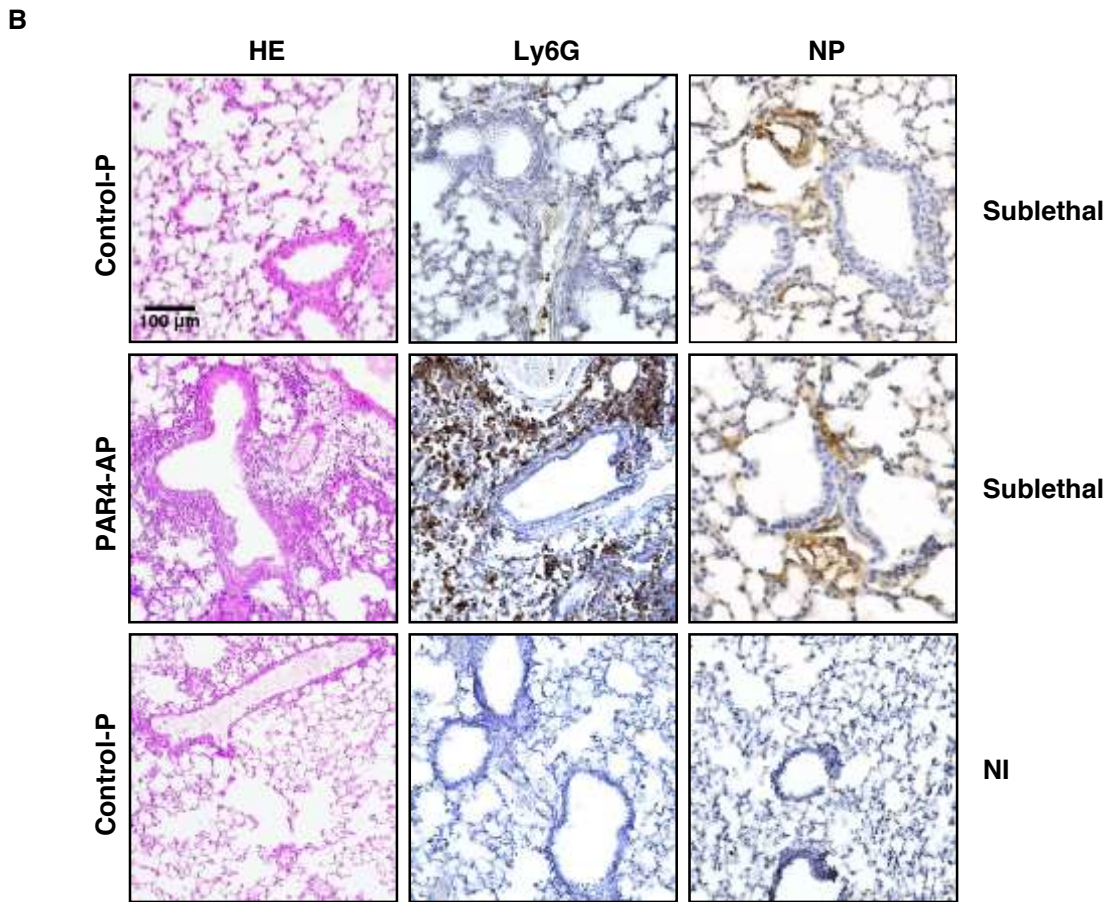
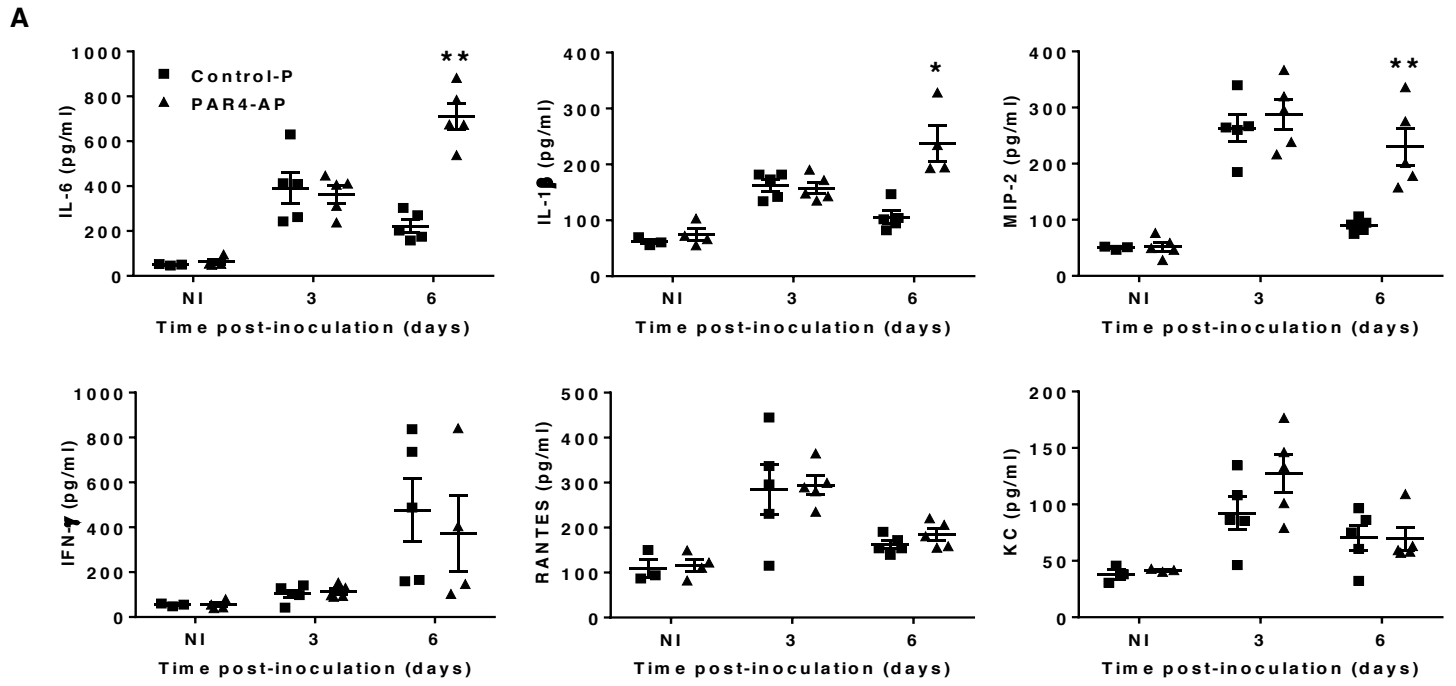


Figure 6

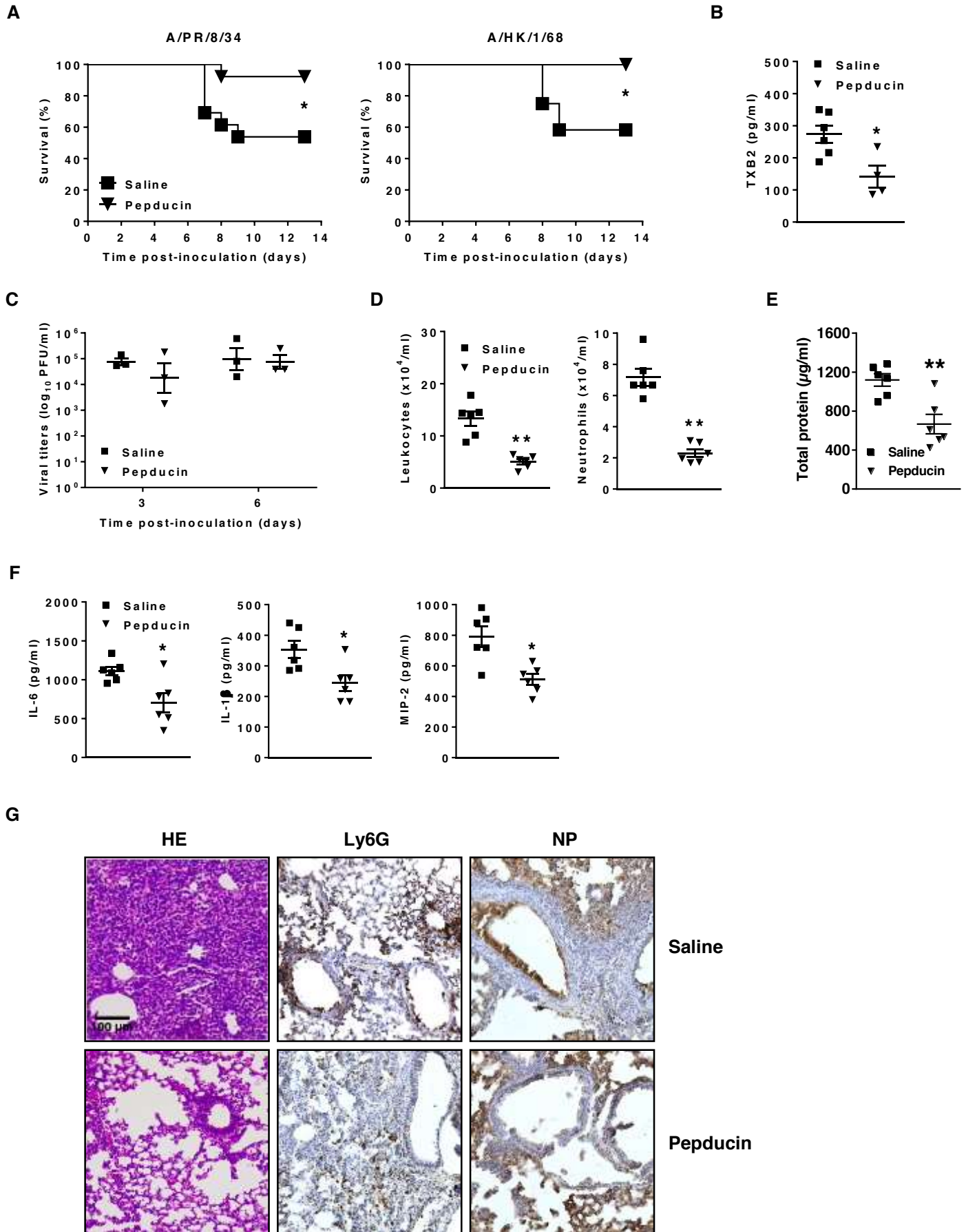
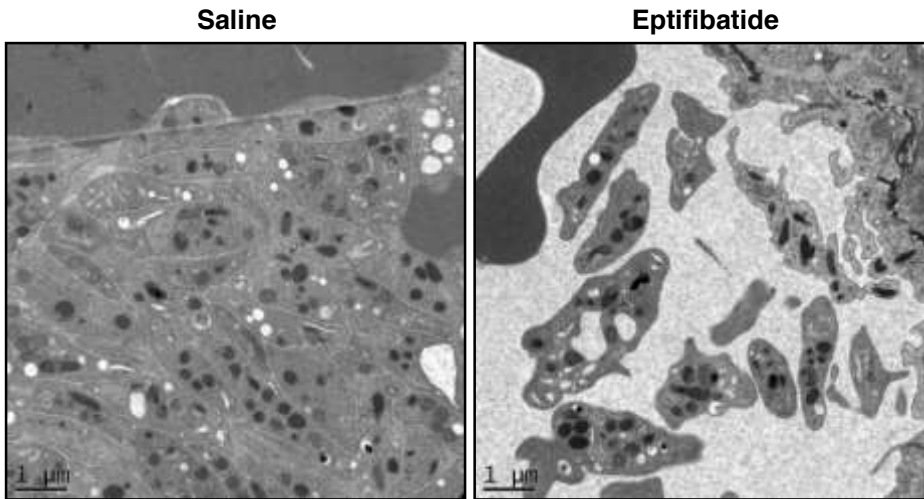
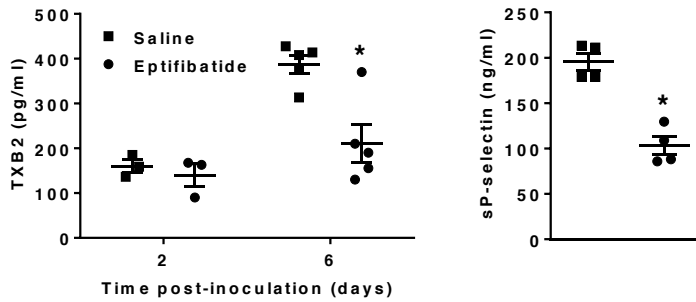


Figure 7

A



B



C

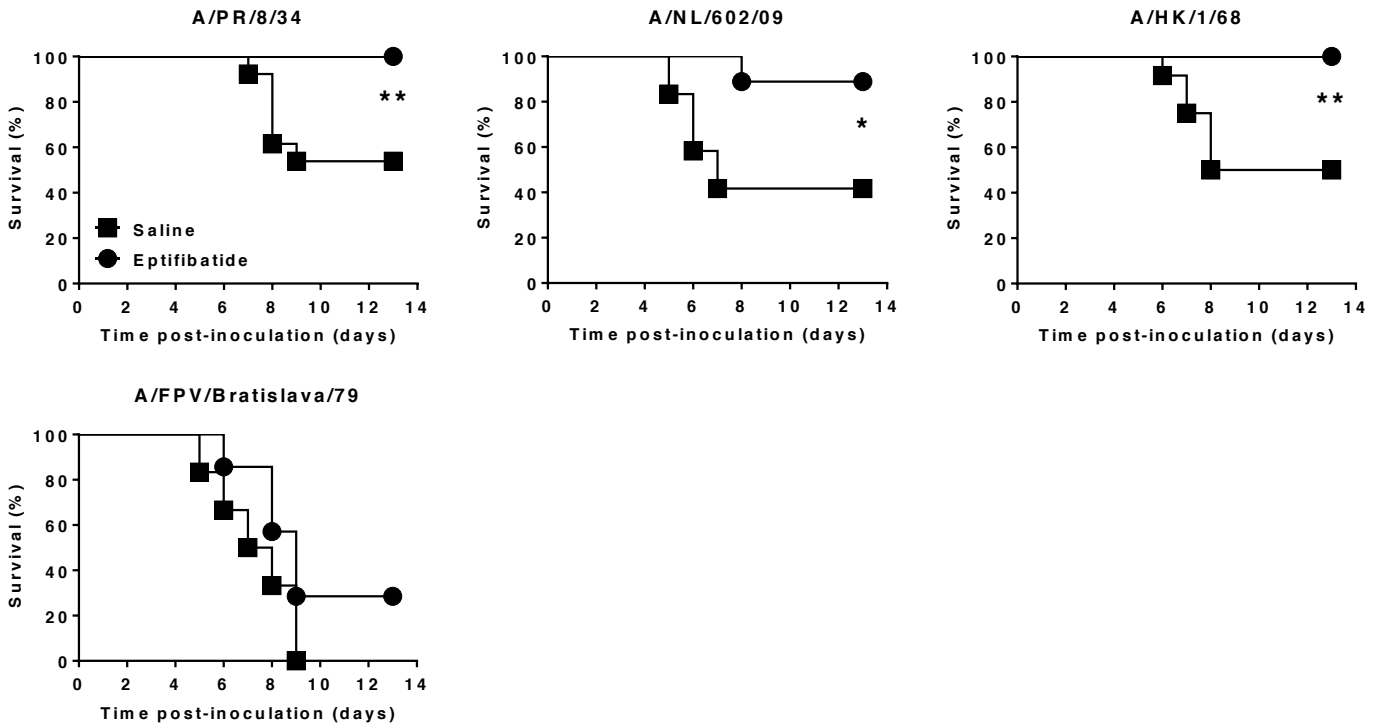


Figure 8

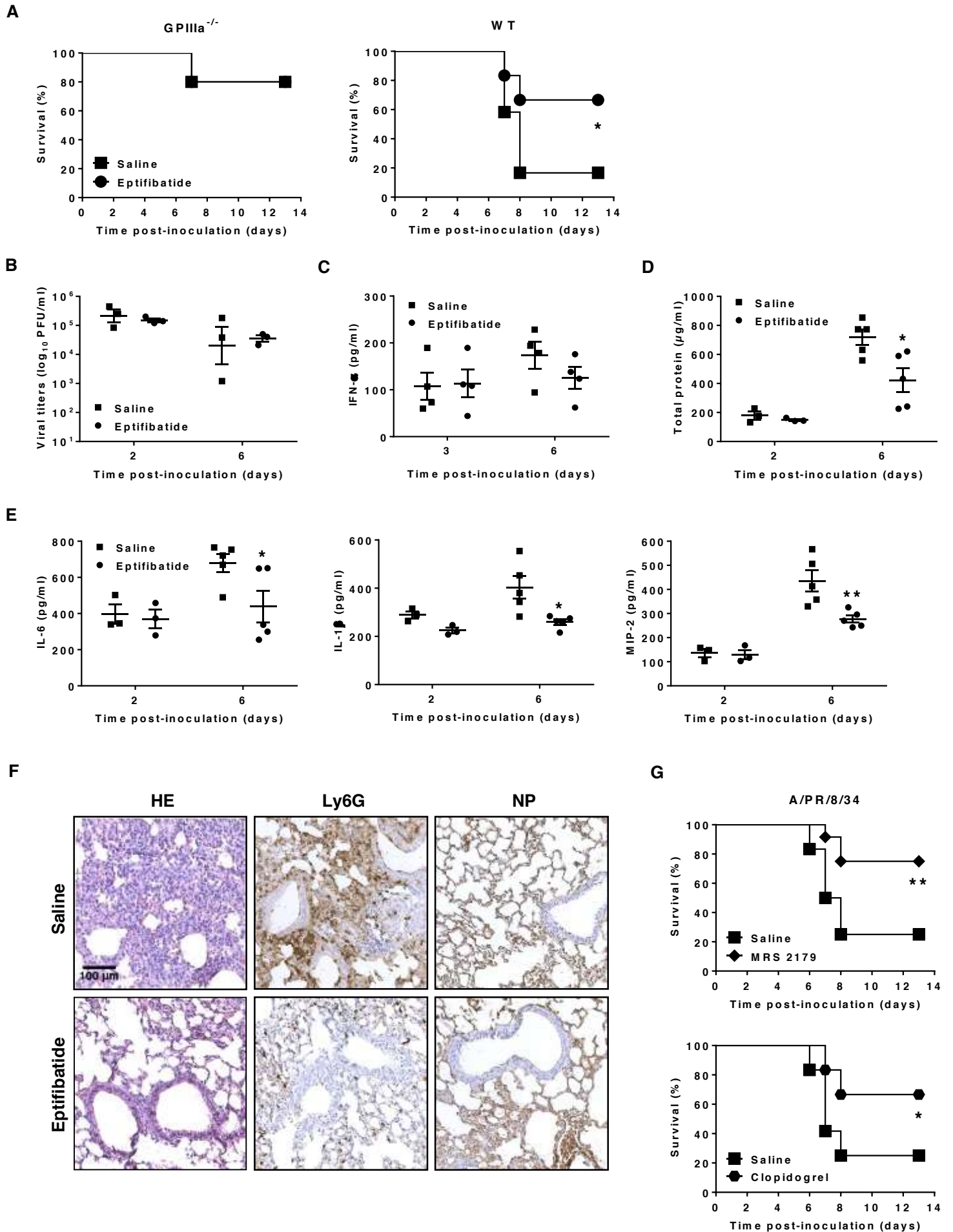
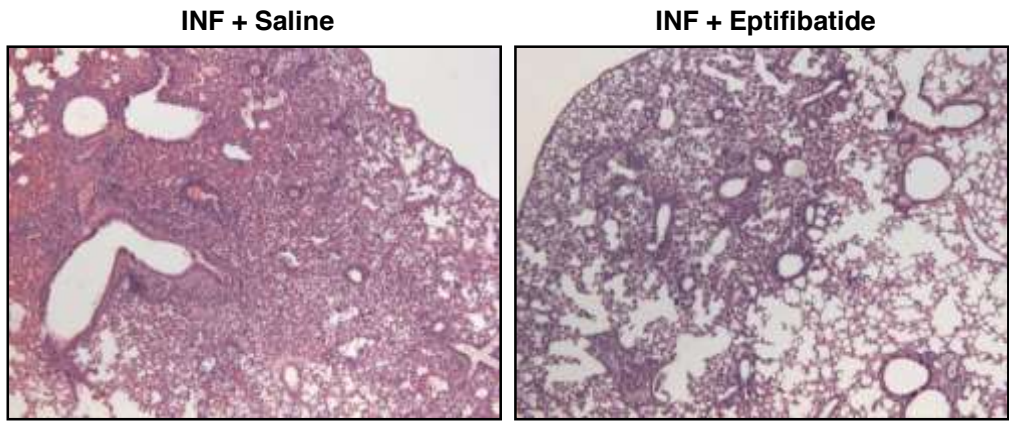


Figure 9

A



B

	Mouse	Saline			Eptifibatide		
		1	2	3	1	2	3
Neutrophil infiltration	Alveoli	++	+	+	+	+	+
	Bronchiole	++	+	+	+	+	+
	Bronchus	++	+	+	+	+	+
	Interalveolar septa	++	++	++	+	+	+
	Peribronchiolar parenchyma	++	++	++	+	+	+
	Big vessels	-	-	-	-	-	-
	Serosa	-	-	-	-	-	-
Mononuclear cell infiltration	Alveoli	+++	+++	+++	++	++	++
	Bronchiole	+++	+++	+++	++	++	++
	Bronchus	+++	+++	+++	++	++	++
	Interalveolar septa	+++	+++	+++	++	++	++
	Peribronchiolar parenchyma	+++	+++	+++	+++	+++	+++
	Big vessels	-	-	-	-	-	-
	Serosa	-	-	-	-	-	-
Vascular congestion	Alveoli	+++	++	++	-	-	+
	Bronchiole	+++	++	++	-	-	++
	Bronchus	+++	++	++	-	-	++
	Interalveolar septa	+++	++	++	-	-	++
	Peribronchiolar parenchyma	+++	++	++	-	-	++
	Big vessels	+++	++	++	+	+	++
	Serosa	+++	++	++	-	-	-
Hemorrhage	Alveoli	++	++	+	-	-	+
	Bronchiole	+	+	+	-	-	-
	Bronchus	-	-	-	-	-	-
	Interalveolar septa	-	-	-	-	-	-
	Peribronchiolar parenchyma	-	-	-	-	-	-
	Big vessels	-	-	-	-	-	-
	Serosa	-	-	-	-	-	-
Fibrin deposit	Alveoli	+++	++	++	-	-	-
	Bronchiole	-	-	-	-	-	-
	Bronchus	-	-	-	-	-	-
	Interalveolar septa	-	-	-	-	-	-
	Peribronchiolar parenchyma	-	-	-	-	-	-
	Big vessels	-	-	-	-	-	-
	Serosa	-	-	-	-	-	-
Epithelial cell apoptosis	Alveoli	++	++	++	-	-	+
	Bronchiole	++	++	++	-	-	-
	Bronchus	++	++	++	+	+	+
	Interalveolar septa	-	-	-	-	-	-
	Peribronchiolar parenchyma	-	-	-	-	-	-
	Big vessels	-	-	-	-	-	-
	Serosa	-	-	-	-	-	-