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Original Research

Colonic Lesions, Cytokine Profiles, and Gut Microbiota in Plasminogen-Deficient Mice

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Plasminogen-deficient (FVB/NPan-*plg*^{tm1Jld}, *plg*^{tm1Jld}) mice, which are widely used as a wound-healing model, are prone to spontaneous rectal prolapses. The aims of this study were 1) to evaluate the fecal microbiome of *plg*^{tm1Jld} mice for features that might contribute to the development of rectal prolapses and colonic inflammation and 2) to assess the relevance of the inflammatory phenotype to the variability in wound healing in this model. The *plg*^{tm1Jld} mice exhibited delayed wound healing, and they could be divided into 3 distinct groups that differed according to the time until wound closure. Colonic lesions in *plg*^{tm1Jld} mice, which were characterized by necrotizing ulcerations and cystically dilated glands, were restricted to the intermediate and distal parts of the colon. The cytokine profile was indicative of chronic tissue damage, but the genetic modification did not change the composition of the gut microbiota, and none of the clinical or biochemical parameters correlated with the gut microbiota composition.

Several studies using plasminogen-deficient (*plg*^{tmJld}) mice have demonstrated that plasminogen, the proenzyme of plasmin, can degrade fibrin and other extracellular matrix proteins.[44] Plasminogen is essential for wound healing in skin,[40] which begins with inflammation, followed by epithelial proliferation, and thereafter tissue remodeling. Because the migrating keratinocytes of *plg*^{tm1Jld} mice have a decreased ability to dissect the platelet-rich fibrin matrix, they exhibit severely impaired wound healing.[15,40] In addition, plasmin mediates various pathologic processes, such as tumor growth and cancer metastasis,[8] and therapeutic intervention related to plasminogen has shown encouraging results in experimental tumors.[31] Therefore, one important application of these mice is the induction of wound healing to study basic mechanistic functions of plasmin, such as the clearance of the extracellular matrix and activation of tumor growth factors.[31]

Spontaneous rectal prolapse and colonic ulceration in *plg*^{tm1Jld} mice compromise studies using these mice by leading to loss of body weight (wasting disease)[6] and wellbeing-related, early study termination.[6] Like other inflammatory conditions, rectal prolapse and chronic colonic inflammation might affect wound healing and contribute to the wide interindividual variation in the wound-healing processes of *plg*^{tm1Jld} mice.[28,40]

The development of rectal prolapses and colonic ulcerations in *plg*^{tm1Jld} mice reportedly is due to vascular occlusion.[6] This pathologic condition is alleviated by superimposing fibrinogen deficiency on plasminogen deficiency, suggesting that fibrin is the primary substrate for plasmin.[7,15] The wide variation in effective tissue remodeling during the wound healing of plasminogen-deficient mice remains unexplained.

Wound healing depends to a large extent on cells and factors of the immune system.[3,53] We previously have shown that disease development in mouse models for various inflammatory conditions, including type 1 diabetes,[17-19,35] type 2 diabetes,[4,13,42] atopic dermatitis[30] and inflammatory bowel disease,[20] is influenced by the composition of gut microbiota. Therefore, gut inflammation can be presumed to interfere with wound healing and thus may increase the uncontrolled interindividual variation in these models. In addition, gut inflammatory conditions in humans, such as inflammatory bowel disease[43] and irritable bowel syndrome,[23] are linked to dysbiosis in the intestine. In mice deficient in IL10 or IL2 and in rats carrying HLA-B27,[52] inflammatory bowel disease can be alleviated by germ-free status[10,49,52] or ampicillin.[20] However, the possible role of the gut microbiome in rectal prolapse, colonic lesions, and wound healing in plasminogen-deficient mice has not previously been assessed.

The aims of the current study were 1) to evaluate the fecal microbiome of *plg*^{tm1Jld} mice and their unaffected WT littermates for features that might contribute to their rectal prolapse and colonic inflammation phenotypes and 2) to assess the relevance of the inflammatory phenotype to the variability in wound healing in this model.

Materials and Methods

In vivo procedures. All procedures were done in accordance with The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123 of 1986) and The Danish Animal Experimentation Act (LBK no 1306 of 23/11/2007) and were approved by the Animal Experiments Inspectorate, Ministry of Food, Fisheries, and Agriculture, Denmark (permit no. 2010-561-1778). Health monitoring of viruses, bacteria, mycoplasma, and fungi was done in accordance with FELASA

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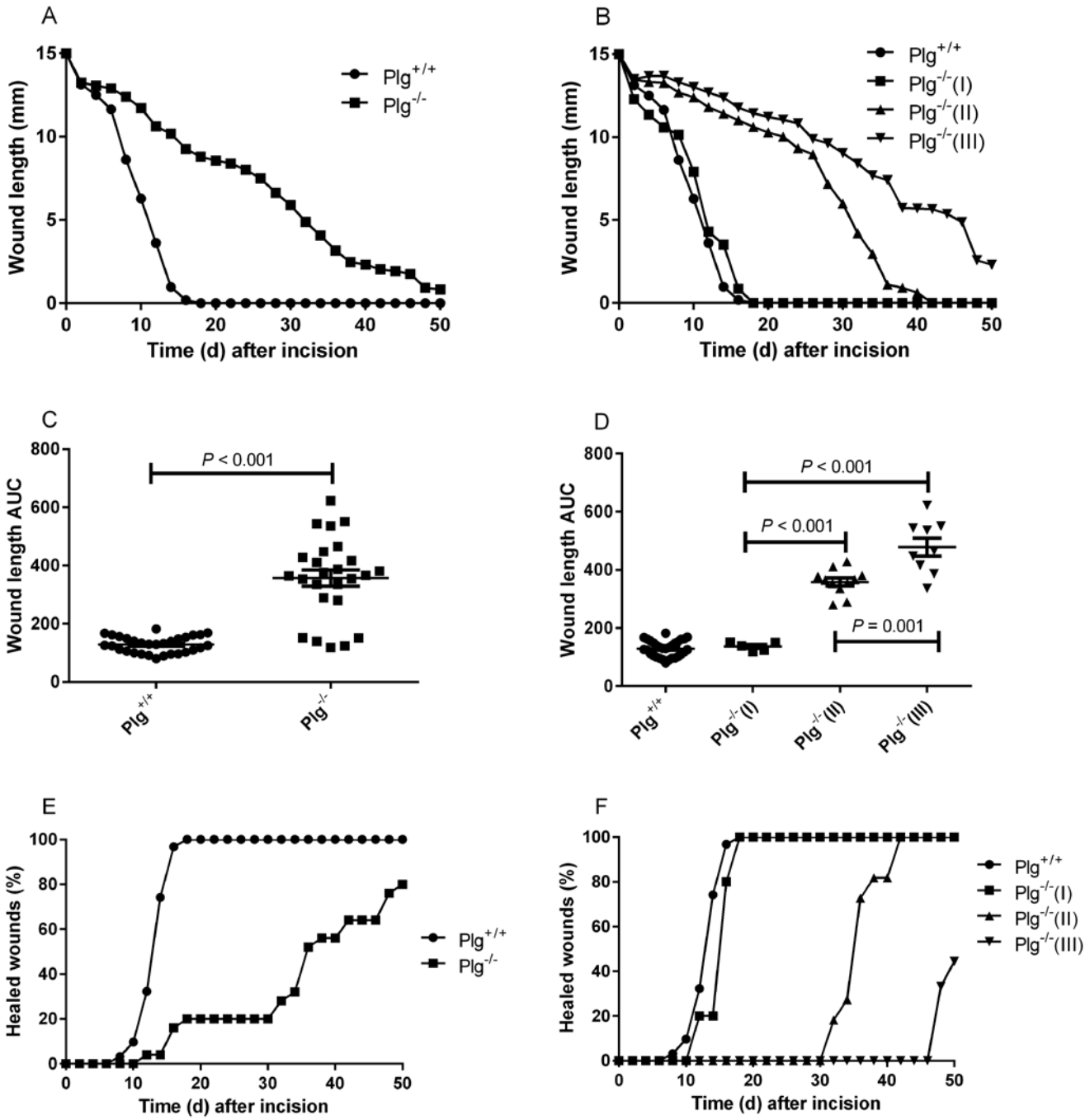

Figure 1. (A) Average skin wound length of WT ($plg^{+/+}$) and plg^{tm1Jld} mice during the course of the study. (B) Average wound length over time in the WT controls and the 3 groups of plg^{tm1Jld} mice. (C) Wound-length AUC of WT and plg^{tm1Jld} mice. (D) Wound-length AUC of WT, $plg^{+/+}$, plg^{tm1Jld} (I), plg^{tm1Jld} (II), and plg^{tm1Jld} (III) mice, for which WT mice differed from plg^{tm1Jld} (II) ($P < 0.001$) and plg^{tm1Jld} (III) ($P < 0.001$) mice. (E) Percentage of fully healed wounds in WT and plg^{tm1Jld} mice. (F) Percentage of fully healed wounds in WT and the 3 groups of plg^{tm1Jld} mice.

recommendations,[34] and the housing room was negative for all viruses, bacteria, mycoplasma, and fungi tested, except that 4 of 8 mice tested during the most-recent screening were positive for *Helicobacter bilis*. Male FVB/NPan-plg^{tm1Jld} (plg^{tm1Jld}; $n = 25$) and WT littermate controls ($plg^{+/+}$; 31) obtained through a heterozygous breeding scheme were used in a parallel group design. *Plg* gene-targeted 129/Black Swiss mice[6] were backcrossed into FVB/N mice, and the mice were fully congenic after 40 generations of backcrossing (Faculty of Health and Medical Sciences, Copenhagen, Denmark). All mice were housed in Eurostandard type III transparent cages (Techniplast, Varese, Italy) with aspen bedding (4HV, Tapvei, Kortteinen, Finland), a Des Res Mouse House (Lillico Biotechnology, Hookwood, United Kingdom), Enviro-Dri bedding (Brogaarden, Lynge, Denmark), Fun Tunnels (mini size, Lillico), and aspen bricks ($1 \times 1 \times 5$ cm^3, Tapvei) changed once weekly for group-housed mice and every 2 wk for single-housed mice. The mice were fed extruded complete feed (Altromin 1314, Brogaarden) and bottled citric-acidified tap water (approximately pH 3; changed weekly)

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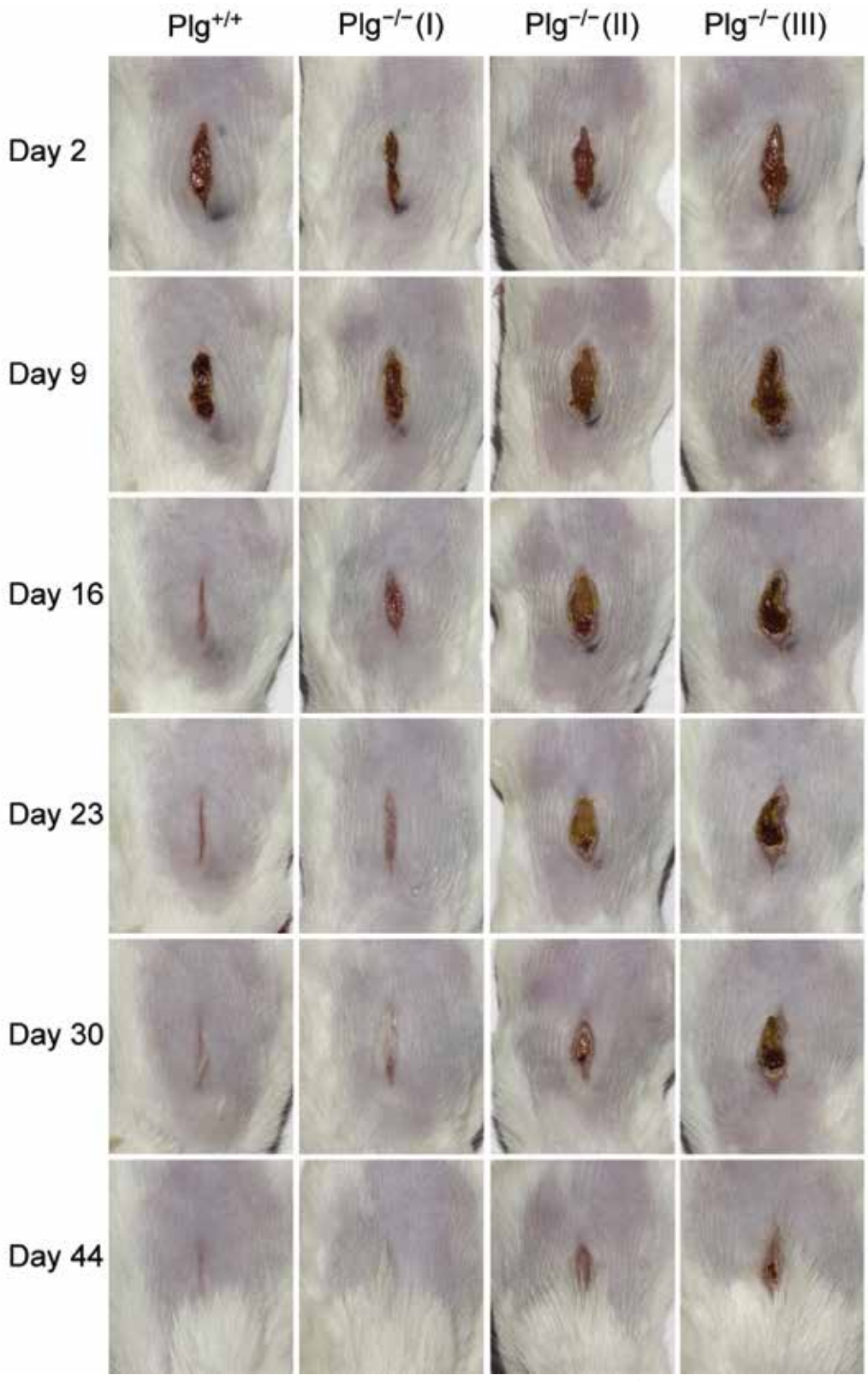

Figure 2. Selected macroscopic images of wounds illustrate the wound-healing progress of WT (*plg*$^{+/+}$) and the 3 groups of *plg*^{tm1Jld} mice.

free choice. The room had a temperature of 21 to 22 °C, a relative humidity of 55% (± 10%), 12 to 14 air exchanges per hour, and a 12:12-h light:dark cycle (lights on, 0600). For anesthesia, a mixture of fentanyl–fluanison (Hypnorm, VetaPharma, Leeds, UK) and midazolam (Dormicum 5 mg/mL, Roche, Hvidovre, Denmark) was given subcutaneously as previously described.[14]

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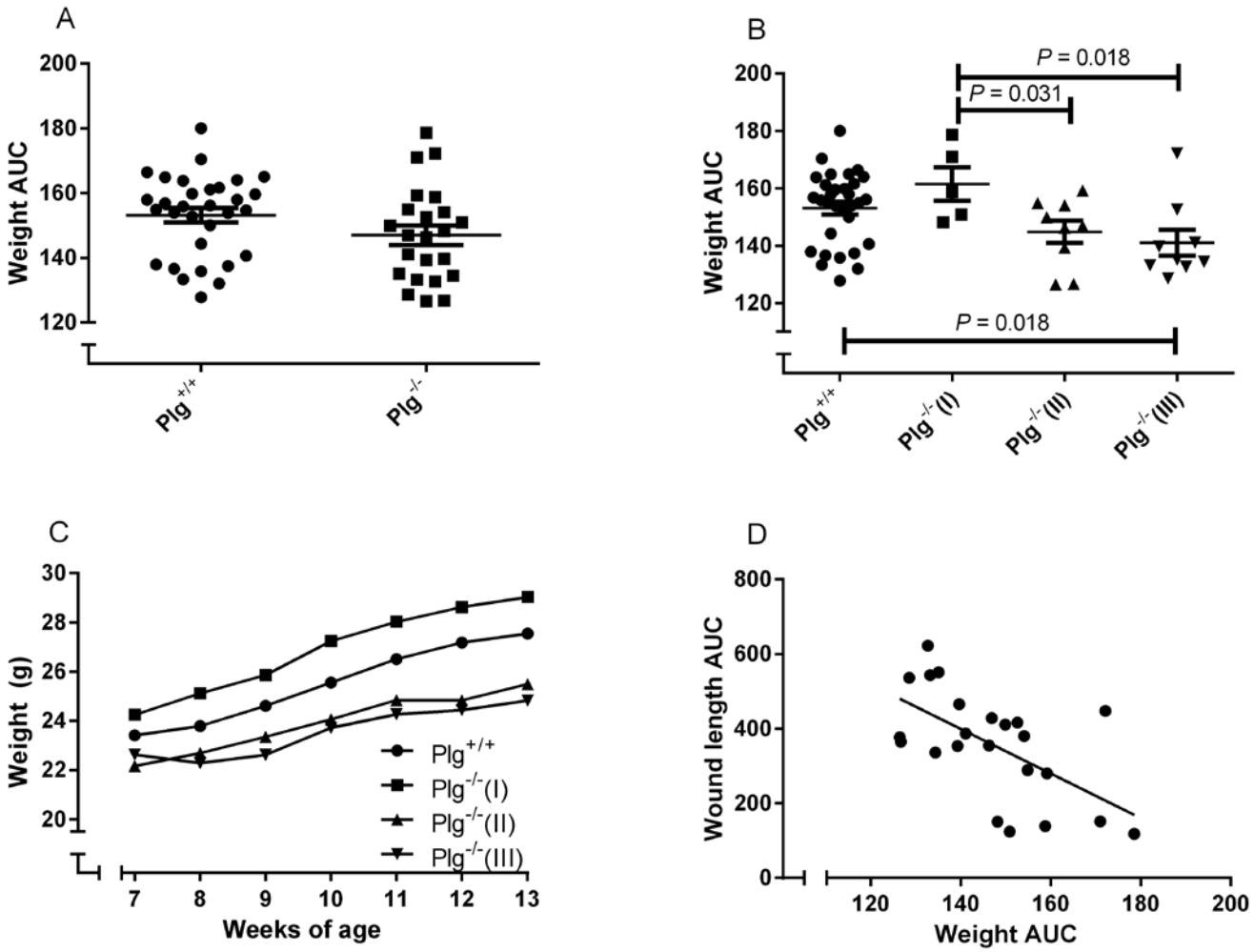

Figure 3. (A) Weight AUC of WT ($plg^{+/+}$) and plasminogen-deficient (plg^{tm1Jld}) mice. The mean body weight ($P = 0.032$) and its interindividual variation ($P = 0.007$) differ between groups. (B) Weight AUC of the 3 groups of plg^{tm1Jld} mice and the WT controls. The AUC differs between plg^{tm1Jld} (III) and plg^{tm1Jld} (I) ($P = 0.018$) and WT ($P = 0.018$) mice. (C) Average weight of the 3 groups of plg^{tm1Jld} mice and the WT controls during the course of the study. (D) Wound-length AUC compared with weight AUC in plg^{tm1Jld} mice shows significant correlation ($P = 0.002$ and $r^2 = 0.3611$).

Skin wounding of anesthetized mice (age, 6 to 7 wk) was performed by using a scalpel to create a 15-mm craniocaudal incision along the midline of the shaved and disinfected back, as previously described.[40] The wounds were inspected visually, and a digital caliper (ToolMate, www.silvan.dk) was used to measure the length and width of the crusts every second day until fully healed, that is, until the wound crust was lost and 2 consecutive measurements of 0 mm from the cranial wound angle to the caudal wound angle were obtained. The mice were weighed and the wounds photographed once weekly. At every handling, the mice were inspected for rectal prolapses. Fecal samples were collected by temporarily transferring individual mice to clean cages without bedding on the day before euthanasia. Fecal samples were stored in autoclaved 1.5-mL test tubes (Eppendorf, Hamburg, Germany) at –80 °C within 1 h after collection.

Termination. Except for 2 mice euthanized at 37 and 39 d after wounding because of rectal prolapses, all mice were euthanized 50 to 52 d after wounding regardless of whether the wounds were fully or only partially healed. Mice were anesthetized, the thoracic cavity was entered after 70% ethanol skin disinfection, and a 23-gauge needle was used to collect blood through the left ventricle of the heart and into a 1-mL syringe containing 0.05 mL 47 mM K_3-EDTA; a total of 0.5 mL blood was drawn, which was transferred to an autoclaved 1.5-mL Eppendorf test tube and stored on ice for 1 to 2 h before being centrifuged (30 min, 2000 × *g*, 4 °C). Plasma (approximately 300 µL per mouse) was transferred to autoclaved 1.5-mL Eppendorf test tubes and stored at –80 °C. The mice were euthanized by bleeding under anesthesia, a small hole was cut in the right auricle, and 10 mL PBS and then 10 mL of 4% phosphate-buffered formaldehyde (VWR–Bie and Berntsen, Rødovre, Denmark) was slowly administered directly into the left ventricle. The anus, rectum, and colon were removed piece en bloc. The colon was filled with the same fixative as used for perfusion, and the gut was rolled into a 'Swiss-roll'[32] without opening the colon. All samples were placed in 4% formaldehyde, which was replaced with 70% ethanol after 24 h.

Postmortem examinations. When a rectal prolapse was present, it was removed, bisected longitudinally, and embedded in paraffin together with the colon. Longitudinal sections were stained with hematoxylin and eosin and examined to quantify the lesions in the colon and confirm rectal prolapse. Furthermore, the distribution of colonic lesions was recorded, with lesions being located

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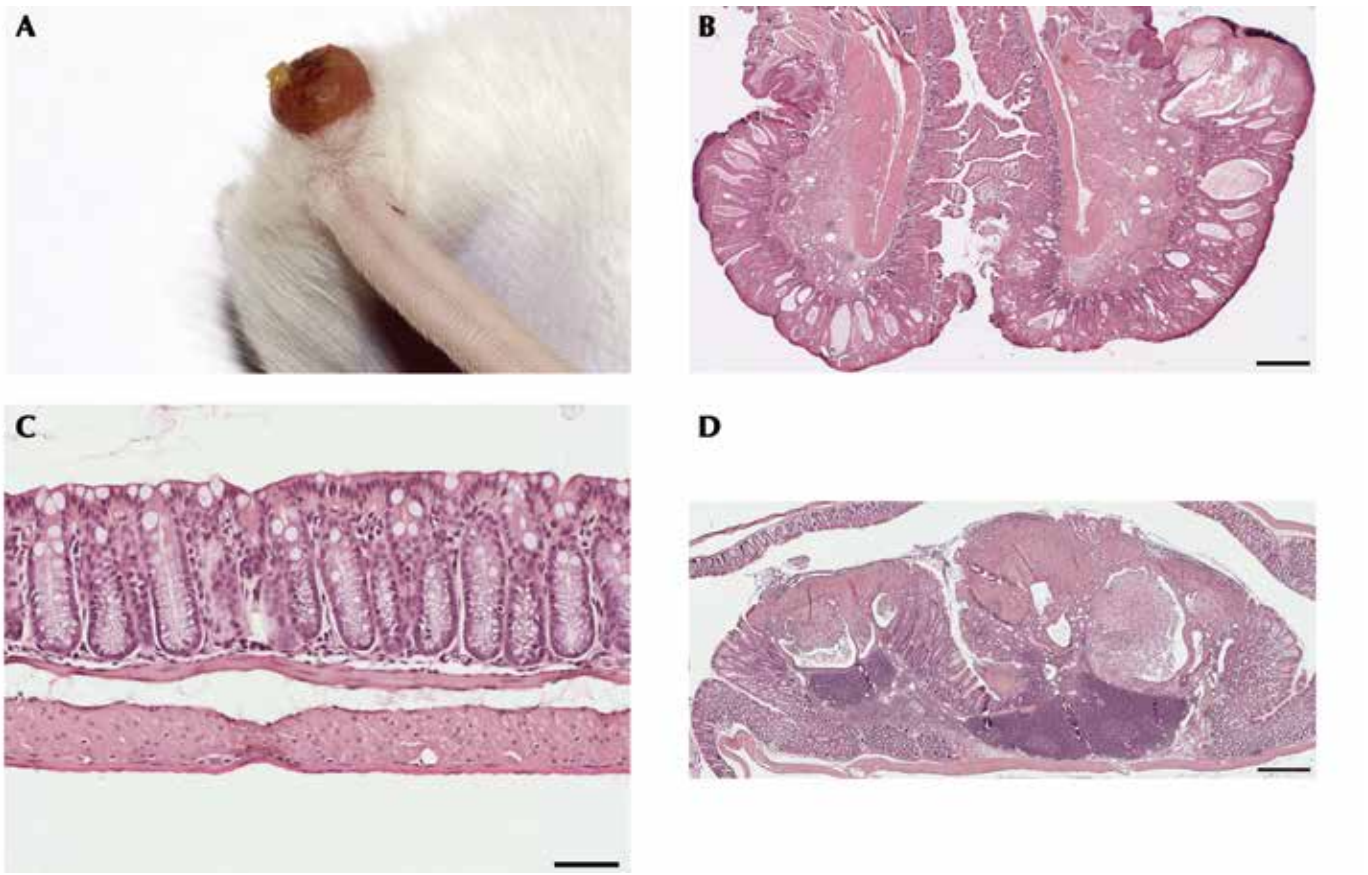

Figure 4. (A) Gross pathology of rectal prolapse in a *plg*^{tm1Jld} mouse. (B) Histology of a rectal prolapse in which cystically dilated glands are seen (bar, 300 µm). (C) Normal colonic histology in a WT mouse (bar, 50 µm). (D) Typical necrotizing ulceration in the colon of a plasminogen-deficient mouse with cystically dilated glands and enlarged lymphoid follicles containing distinct germinal centers (bar, 225 µm). Hematoxylin and eosin stain.

Table 1. Number of rectal prolapses and colonic lesions in *plg*^{tm1Jld} and WT mice

Group	*n*	No. of rectal prolapses	No. of colonic lesions			
			0	1	2	3 to 7
WT	31	*0*	31	0	0	0
plg^{tm1Jld} (I)	5	2	0	0	2	3
plg^{tm1Jld} (II)	11	6	1	1	5	4
plg^{tm1Jld} (III)	9	4	0	4	2	3
plg^{tm1Jld} total	25	12	1	5	9	10
All mice total	56	12	32	5	9	10

WT and *plg*^{tm1Jld} mice differed significantly in regard to the numbers of mice with rectal prolapses ($P < 0.001$) and colonic lesions ($P < 0.001$), but the number of colonic lesions did not differ among the 3 groups of *plg*^{tm1Jld} mice.

Table 2. Lesions in the intermediate and aboral colons of WT and *plg*^{tm1Jld} mice

	Intermediate colon (no. of lesions)			Distal colon (no. of lesions)		
	0	1	2 to 4	0	1	2 to 4
WT	31	0	0	31	0	0
plg^{tm1Jld} (I)	2	2	1	1	0	4
plg^{tm1Jld} (II)	3	6	2	1	6	4
plg^{tm1Jld} (III)	4	3	2	1	4	4
plg^{tm1Jld} total	9	11	5	3	10	12

Colonic lesions did not differ among the 3 groups of *plg*^{tm1Jld} mice but were borderline more numerous ($P = 0.052$) in the distal compared with the intermediate colon of *plg*^{tm1Jld} mice overall.

in the proximal, intermediate, or distal third of the colon. Rectal prolapse was scored as being present or absent.

Cytokine levels in stored plasma samples were measured by using a Mouse Th1/Th2 10plex FlowCytomix kit (eBioscience, Vienna, Austria). Briefly, standards and samples were mixed with the antibody-coated beads and biotin-conjugated secondary antibodies in a 96-well filter plate and incubated at room temperature on a plate shaker for 2 h. Subsequently, the plate was washed twice on a vacuum manifold (Enzo, AH Diagnostics, Aarhus, Denmark) followed by a 1-h incubation on the plate shaker with assay buffer and streptavidin–phycoerythrin followed by 2 more washes. Beads were suspended in assay buffer, and the fluorescence was measured on a flow cytometer (FACSCanto II, BD Biosciences, Albertslund, Denmark). Cytokine levels were calculated by using Flowcytomix Pro 2.4 software (eBioscience).

Genotyping of the mice at weaning and euthanasia was performed on tail-tip DNA as previously described.[16]

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Table 3. Bacterial sequences from fecal samples

						plg^{tm1Jld} (%)		*plg*$^{+/+}$ (%)	
Phylum	Class	Order	Family	Genus	Species	Mean	1 SD	Mean	1 SD
Other	Other	Other	Other	Other	Other	1.618	0.566	1.993	0.913
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Salinibacterium*	Other	0.000	0.000	0.009	0.033
Bacteroidetes	Other	Other	Other	Other	Other	0.070	0.061	0.052	0.054
Bacteroidetes	Bacteroidia	Bacteroidales	Other	Other	Other	1.788	0.879	2.003	1.440
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Other	Other	0.084	0.106	0.067	0.100
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Unclassified	Unclassified	0.002	0.010	0.000	0.000
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	Other	0.406	0.260	0.383	0.483
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	Unclassified	1.278	1.701	0.855	1.498
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*acidifaciens*	1.186	1.066	0.921	0.933
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*helcogenes*	0.002	0.010	0.005	0.020
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*uniformis*	0.002	0.010	0.000	0.000
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Other	Other	0.002	0.010	0.000	0.000
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	Other	0.018	0.032	0.019	0.039
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	Unclassified	0.114	0.116	0.081	0.082
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	*distasonis*	0.244	0.262	0.179	0.137
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	*gordonii*	0.010	0.020	0.017	0.036
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Other	Other	0.006	0.017	0.005	0.020
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	Other	0.006	0.017	0.003	0.013
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	Unclassified	2.884	2.680	2.119	1.999
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Other	Other	0.158	0.122	0.209	0.204
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Unclassified	Unclassified	18.244	6.252	17.586	5.749
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	Unclassified	Unclassified	17.640	8.561	18.379	9.680
Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	*Odoribacter*	Unclassified	3.390	1.368	4.045	2.252
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	*[Prevotella]*	Unclassified	0.080	0.256	0.212	1.057
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*	Unclassified	0.000	0.000	0.002	0.009
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Flexibacteraceae	*Hymenobacter*	Unclassified	0.002	0.010	0.002	0.009
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Unclassified	Unclassified	0.000	0.000	0.002	0.009
Cyanobacteria	Other	Other	Other	Other	Other	0.002	0.010	0.000	0.000
Cyanobacteria	4C0d-2	YS2	Unclassified	Unclassified	Unclassified	0.044	0.109	0.228	0.375
Cyanobacteria	Chloroplast	Streptophyta	Unclassified	Unclassified	Unclassified	0.000	0.000	0.002	0.009
Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Other	Other	0.002	0.010	0.000	0.000
Cyanobacteria	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae	Unclassified	Unclassified	0.002	0.010	0.000	0.000
Cyanobacteria	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae	*Pseudanabaena*	Unclassified	0.002	0.010	0.000	0.000
Firmicutes	Other	Other	Other	Other	Other	0.048	0.055	0.040	0.045
Firmicutes	Bacilli	Other	Other	Other	Other	0.000	0.000	0.002	0.009
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	Other	0.004	0.014	0.000	0.000
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	Unclassified	0.000	0.000	0.002	0.009
Firmicutes	Bacilli	Lactobacillales	Other	Other	Other	0.002	0.010	0.002	0.009
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Other	Other	0.000	0.000	0.002	0.009
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Other	Other	0.008	0.024	0.003	0.013
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Unclassified	Unclassified	0.012	0.026	0.003	0.013
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	Other	0.014	0.034	0.021	0.037
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	Unclassified	0.166	0.330	0.069	0.097
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.048	0.065	0.060	0.110
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Weissella*	Unclassified	0.000	0.000	0.005	0.015

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Table 3. Continued

						plg^{tm1Jld} (%)		*plg*$^{+/+}$ (%)	
Phylum	Class	Order	Family	Genus	Species	Mean	1 SD	Mean	1 SD
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	Unclassified	0.008	0.019	0.000	0.000
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	Other	0.002	0.010	0.000	0.000
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	Unclassified	0.002	0.010	0.000	0.000
Firmicutes	Clostridia	Other	Other	Other	Other	0.878	0.417	1.060	0.632
Firmicutes	Clostridia	Unclassified	Unclassified	Unclassified	Unclassified	0.318	0.186	0.341	0.241
Firmicutes	Clostridia	Clostridiales	Other	Other	Other	0.404	0.206	0.483	0.301
Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	Unclassified	1.378	0.784	1.572	1.025
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Other	Other	0.008	0.024	0.002	0.009
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Unclassified	Unclassified	0.170	0.091	0.169	0.110
Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	*Dehalobacterium*	Unclassified	0.268	0.188	0.266	0.238
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Other	Other	1.420	0.920	1.378	0.691
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified	Unclassified	30.212	11.383	29.209	9.309
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Anaerostipes*	Unclassified	0.034	0.047	0.029	0.043
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Coprococcus*	Unclassified	0.124	0.262	0.202	0.334
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Dorea*	Unclassified	0.046	0.068	0.098	0.201
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Roseburia*	Unclassified	0.080	0.089	0.072	0.104
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*[Ruminococcus]*	Unclassified	0.002	0.010	0.003	0.013
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*[Ruminococcus]*	*gnavus*	0.236	0.217	0.266	0.270
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Unclassified	Unclassified	0.008	0.019	0.007	0.018
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*rc4-4*	Unclassified	0.098	0.133	0.067	0.109
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Other	Other	0.434	0.211	0.397	0.143
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unclassified	Unclassified	3.320	2.936	2.819	2.160
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Anaerotruncus*	Unclassified	0.252	0.249	0.183	0.159
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	Other	0.056	0.067	0.059	0.063
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	Unclassified	5.354	3.216	4.964	2.347
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	*guilliermondii*	0.002	0.010	0.007	0.018
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	Other	0.304	0.459	0.243	0.226
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	Unclassified	1.014	0.505	1.395	0.803
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	*flavefaciens*	0.182	0.479	0.152	0.417
Firmicutes	Clostridia	Coriobacteriales	Unclassified	Unclassified	Unclassified	0.000	0.000	0.003	0.013
Firmicutes	Clostridia	Coriobacteriales	Coriobacteriaceae	Other	Other	0.002	0.010	0.000	0.000
Firmicutes	Clostridia	Coriobacteriales	Coriobacteriaceae	*Adlercreutzia*	Unclassified	0.004	0.014	0.005	0.015
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Other	Other	0.004	0.020	0.002	0.009
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Unclassified	Unclassified	0.022	0.033	0.016	0.027
Firmicutes	Erysipelotrichi	Erysipelotrichales	[Coprobacillaceae]	Unclassified	Unclassified	0.000	0.000	0.007	0.029
Proteobacteria	Other	Other	Other	Other	Other	0.000	0.000	0.002	0.009
Proteobacteria	Alphaproteobacteria	Other	Other	Other	Other	0.006	0.017	0.002	0.009
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Unclassified	Unclassified	0.002	0.010	0.000	0.000
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium*	Unclassified	0.000	0.000	0.002	0.009
Proteobacteria	Alphaproteobacteria	RF32	Unclassified	Unclassified	Unclassified	0.108	0.174	0.114	0.247
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Other	Other	0.000	0.000	0.002	0.009
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other	Other	0.000	0.000	0.002	0.009
Proteobacteria	Betaproteobacteria	Other	Other	Other	Other	0.080	0.099	0.074	0.085
Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other	Other	0.002	0.010	0.002	0.009
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*	Unclassified	0.850	0.618	0.883	0.682
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other	Other	0.000	0.000	0.002	0.009
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Delftia*	Unclassified	0.002	0.010	0.005	0.028
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Polaromonas*	Unclassified	0.004	0.020	0.005	0.028
Proteobacteria	Betaproteobacteria	Ellin6067	Unclassified	Unclassified	Unclassified	0.000	0.000	0.002	0.009
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Bilophila*	Unclassified	0.006	0.030	0.000	0.000
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*	*C21_c20*	0.008	0.031	0.007	0.029

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Table 3. Continued

						plg^{tm1Jld} (%)		$plg^{+/+}$ (%)	
Phylum	Class	Order	Family	Genus	Species	Mean	1 SD	Mean	1 SD
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter*	Other	0.292	0.350	0.609	0.640
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter*	*cinaedi*	0.042	0.069	0.072	0.131
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Other	Other	0.002	0.010	0.000	0.000
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	Unclassified	0.000	0.000	0.002	0.009
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	Unclassified	0.002	0.010	0.000	0.000
TM7	TM7-3	Other	Other	Other	Other	0.002	0.010	0.002	0.009
TM7	TM7-3	CW040	Other	Other	Other	0.004	0.014	0.005	0.015
TM7	TM7-3	CW040	F16	Unclassified	Unclassified	1.806	1.456	2.197	1.403
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	*Anaeroplasma*	Unclassified	0.016	0.031	0.012	0.029
Tenericutes	Mollicutes	RF39	Unclassified	Unclassified	Unclassified	0.032	0.043	0.026	0.037
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	*muciniphila*	0.500	2.386	0.912	3.084
WPS2	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	0.000	0.000	0.002	0.009

Taxa denoted as 'unclassified' means that the reference database does not have an official taxonomy for this cluster. Taxa denoted as 'other' indicates ambiguity in that more bacteria could be assigned to this cluster. Square brackets indicate a proposed taxonomy.

All fecal samples were homogenized, DNA was extracted (QIAamp DNA Stool Mini Kit, Qiagen, Germany) and stored at –20 °C, and PCR amplification of the V3–V4 region of the 16S rRNA gene, purification, and 454–FLX-based pyrosequencing of amplified PCR products were done as previously described.[19] The open-source software package Quantitative Insight Into Microbial Ecology (QIIME version 1.7.0) was used to analyze the pyrosequencing data (NCBI accession no. SRP031769) as previously described,[25] including slight modification such as reference database update (Greengenes version 12.10).

Data management and statistical analysis. For weight and wound-length data, AUC were calculated by using Prism version 5.02 (GraphPad Software, La Jolla). Weight AUC was calculated for mice from 7 to 13 wk of age, excluding the 2 mice that were euthanized preterm. The Anderson–Darling test for normality was applied by using Minitab 16.1.1 (Minitab, State College, PA). The weight and wound-length AUC were compared by using linear regression; all other normally distributed data were tested by using ANOVA followed by testing of significant differences with unpaired Student *t* tests. For data not normally distributed, the Kruskal–Wallis test was used. Confidence interval was 95%. Fractions of mice with or without colon or rectal lesions and scorings of lesions in the 3 parts of the colon were compared between the 2 genotypes by χ^2 testing. Variances were compared by F tests for normally distributed data and the Levene test for nonnormally distributed data.

Bacterial consortia according to pyrosequencing data were examined as previously described.[19] Briefly, jack-knifed β diversity based on weighted and unweighted UniFrac distance metrics was calculated for subsampled operational taxonomic unit tables unified to the 2000 reads per sample and projected by using principal coordinate analysis. α diversity measures, such as estimated species number and Chao1 and Shannon indexes, were calculated for rarefied operational taxonomic unit tables (2000 reads per sample). Two samples were discarded due to the low number of reads (fewer than 2000). Differences in taxa abundance at all taxonomic levels ranging from phylum to genus between categories were verified by using Metastats (http://metastats.cbc.umd.edu). Correlation between values representing bacterial relative distribution and inflammatory markers was examined with MINE (http://www.exploredata.net). The nonparametric *t* test with 999 Monte Carlo permutations was used to discriminate differences in α diversity measures.

Results

In vivo examination. Wound healing was significantly ($P < 0.001$) faster in WT ($plg^{+/+}$) mice compared with plg^{tm1Jld} mice (AUC, 128.9 ± 27.7 compared with 357.2 ± 138.9, respectively) and showed less interindividual variation ($P < 0.001$). According to the wound-healing phenotype and genotype data, mice were classified into 4 significantly ($P < 0.001$) different groups (Figure 1). These groups were: WT ($plg^{+/+}$), the 31 mice with full healing between days 8 and 18 and no obvious interindividual differences; I, the 5 plg^{tm1Jld} mice with healing similar to that of $plg^{+/+}$; II, the 11 plg^{tm1Jld} mice with full healing between days 32 and 42; and III, the 9 plg^{tm1Jld} mice with full healing later than day 48 or no healing (Figures 1 and 2). At the ages of 12 wk ($P = 0.038$) and 13 wk ($P = 0.032$; Figure 3), WT mice weighed more than plg^{tm1Jld} mice did . In addition, the body weight AUC differed significantly ($P = 0.013$, Figure 3) among the 4 groups of mice, with lower AUC in groups with impaired wound healing, and the body-weight and wound-healing AUC correlated significantly in plg^{tm1Jld} mice ($P = 0.002$, Figure 3) but not in WT mice. Furthermore, interindividual variation differed significantly ($P = 0.007$) between the 2 genotypes.

Pathology. Rectal prolapse (Figure 4 A) occurred in 10 of the 25 plg^{tm1Jld} mice but in 0 of the 31 WT mice ($P < 0.001$). In addition, histologic examination revealed partial rectal prolapses in 2 additional plg^{tm1Jld} mice (Figure 4 B, Table 1). Colonic lesions were present in 24 of the 25 plg^{tm1Jld} mice and were characterized by necrotizing ulceration and cystically dilated glands with epithelial hyperplasia, lymphoid follicles with activated germinal centers, and subepithelial edema (Figure 4 D). In contrast, all of the WT mice were free of colonic lesions (Figure 4 C, $P < 0.001$ compared with plg^{tm1Jld} mice). For statistical calculations, the sole plg^{tm1Jld} mouse with no colonic lesions was grouped with the 5 mice that each had single lesions. Colonic lesions showed no correlation with either wound healing or body weight in plg^{tm1Jld} mice. No mouse had any lesions in the proximal part of the colon, thus differing from the lesion counts in the intermediate ($P < 0.001$) and distal ($P < 0.001$) parts, whereas lesions were borderline ($P = 0.052$) more frequent in the distal compared with the intermediate colon (Table 2). The 3 plg^{tm1Jld} wound-healing groups did not differ in colon lesion counts (Table 2). No significant correlations were found between rectal prolapse compared with colonic lesion count, wound healing, or body weight in plg^{tm1Jld} mice.

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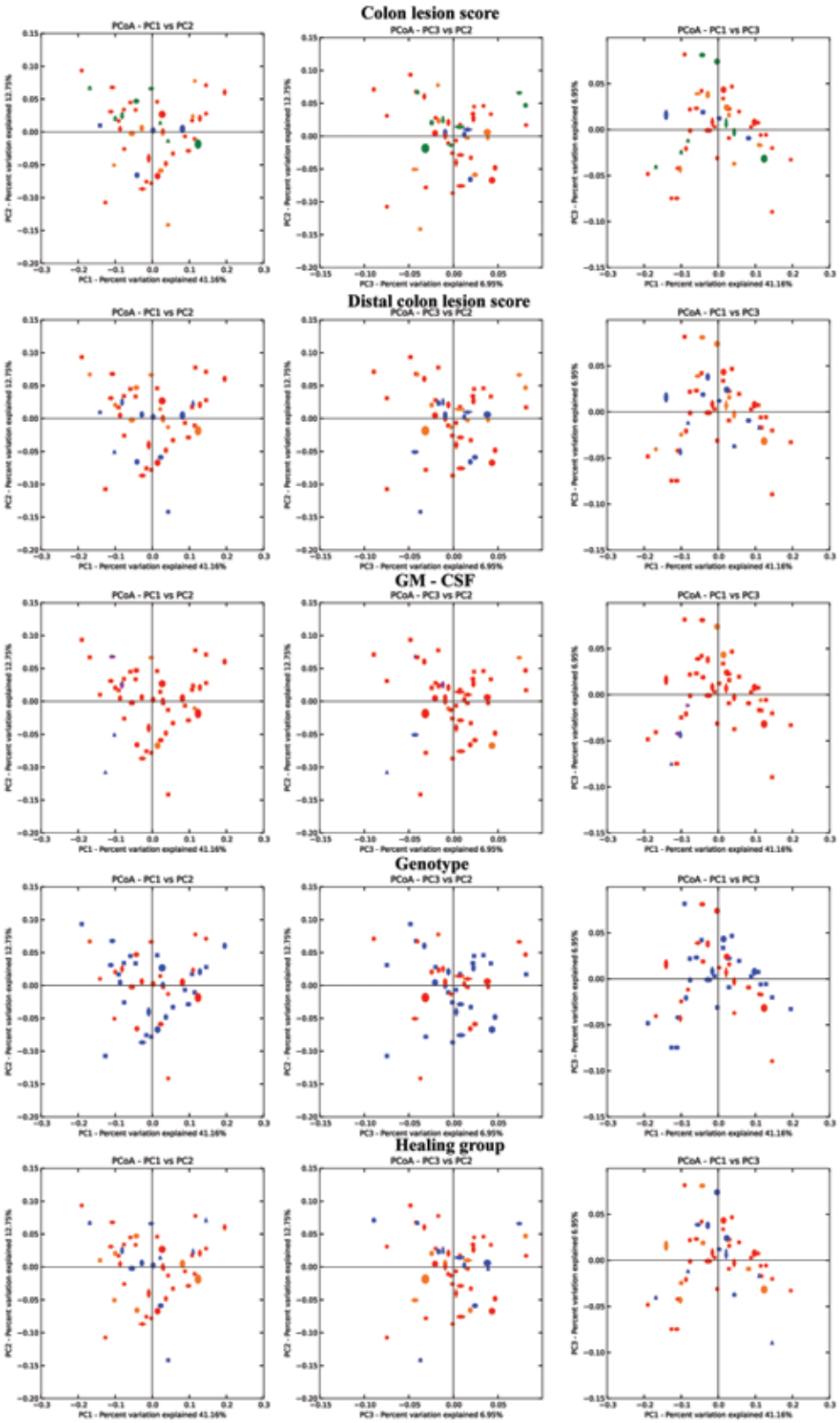

Figure 5. Principal coordinate analysis of the composition of the prokaryotic community of fecal samples representing *plg*$^{+/+}$ and *plg*^{tm1Jld} mice and determined by using tag-encoded 454/FLX-based pyrosequencing of the V3–V4 region of the 16S rRNA gene did not reveal clustering according to genotype; healing group (with or without WT); IL1α, IL2, IL4, IL5, Il6, IL10, IFNγ, IL17, or GM-CSF level; lesion count in proximal, intermediate, or distal colon; or presence of rectal prolapse.

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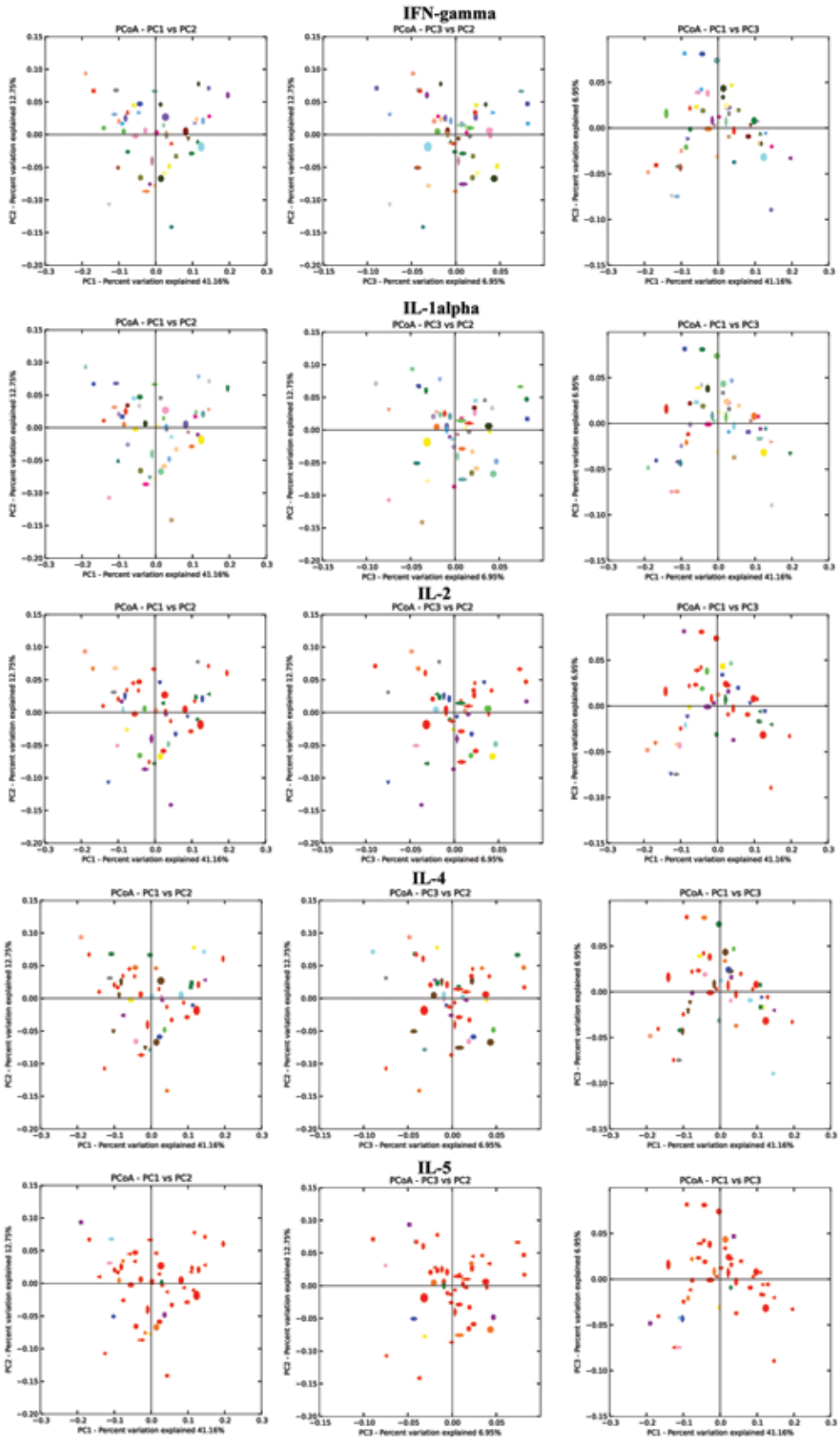

Figure 5b. Continued

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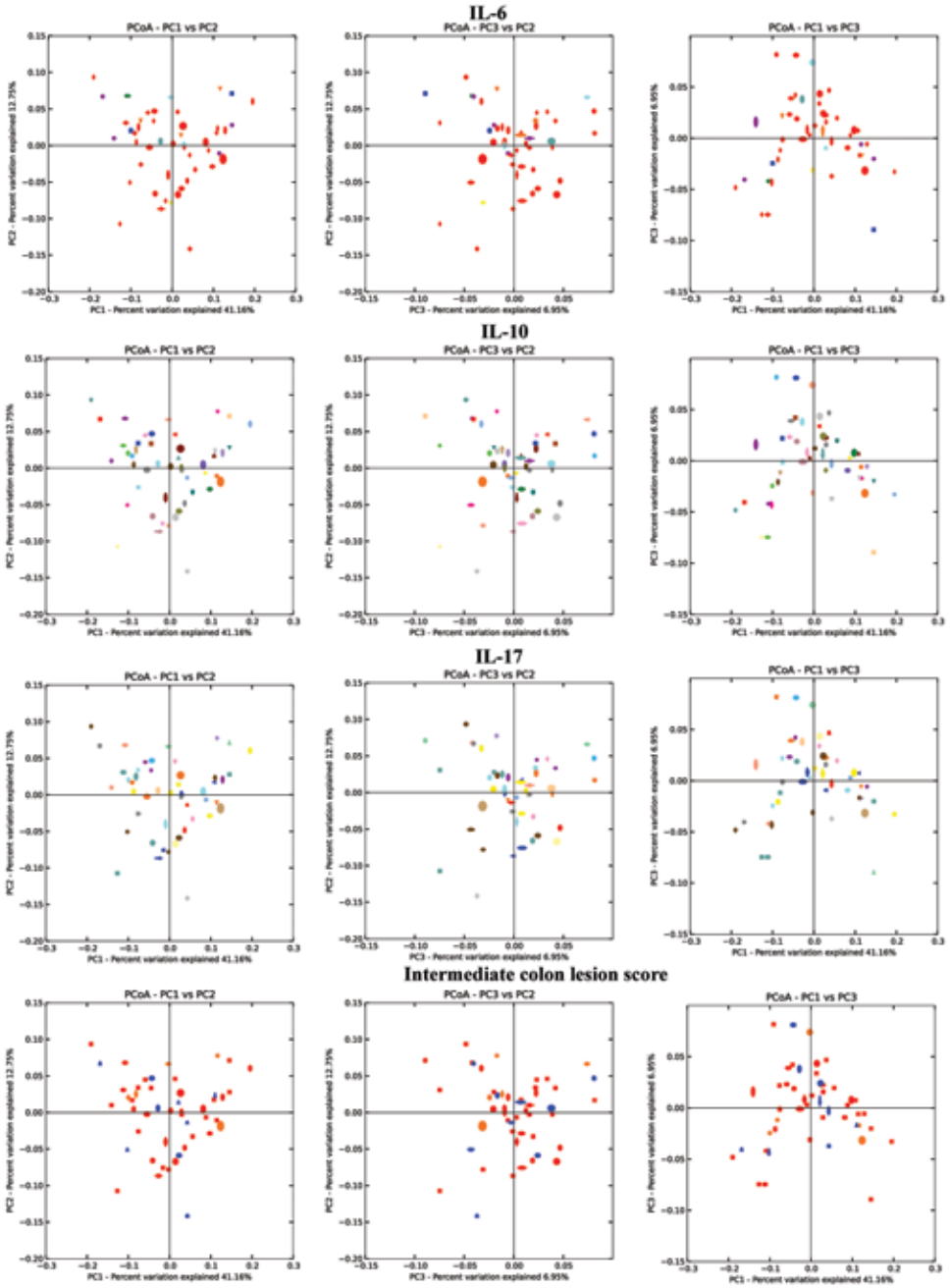

Figure 5c. Continued

Gut microbiota. All 454–FLX-based 16S rRNA sequences of fecal samples from WT and *plg*^{tm1Jld} mice were assigned to 9 phyla, namely, Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Firmicutes, Proteobacteria, Tenericutes, TM7, and Verrucomicrobia. The majority of the sequences (>90%) were distributed in practically equal proportions between Bacteroidetes and Firmicutes (Table 3). More than 70% of all reads were dispersed among 5 genera (listed beginning with the most abundant): an unclassified genus from the Lachnospiraceae family (approximately 31%, Firmicutes); an unclassified genera from the Rikenellaceae and S24-7 families (approximately 18% and 17%, respectively; Bacterioidetes); *Oscillospira* (approximately 5%; Firmicutes); and *Odoribacter* (approximately 4%; Bacterioidetes).

Principal coordinate analysis of fecal gut microbiota composition in WT and *plg*^{tm1Jld} mice did not reveal any clustering according to genotype; healing group (with or without WT); IL1α, IL2, IL4, IL5, IL6, IL10, IFNγ, IL17, or GM-CSF level; number of lesions in the proximal, intermediate, or distal colon lesion score; or the presence of a rectal prolapse (Figure 5). In addition, no intergroup differences were revealed in species diversity (Figure 6), phylum-level relative abundance, or genus-level relative abundance (Table 3). Furthermore, there was no correlation between any of the bacterial genera and either the presence of a rectal prolapse; number

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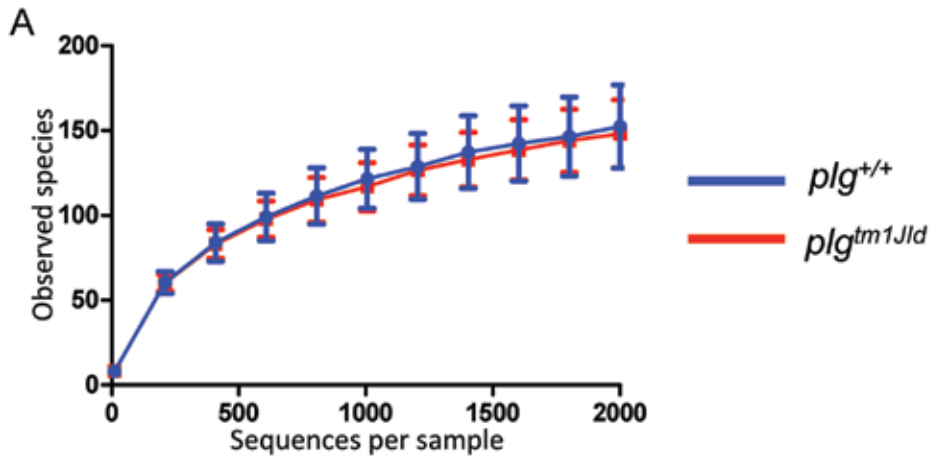
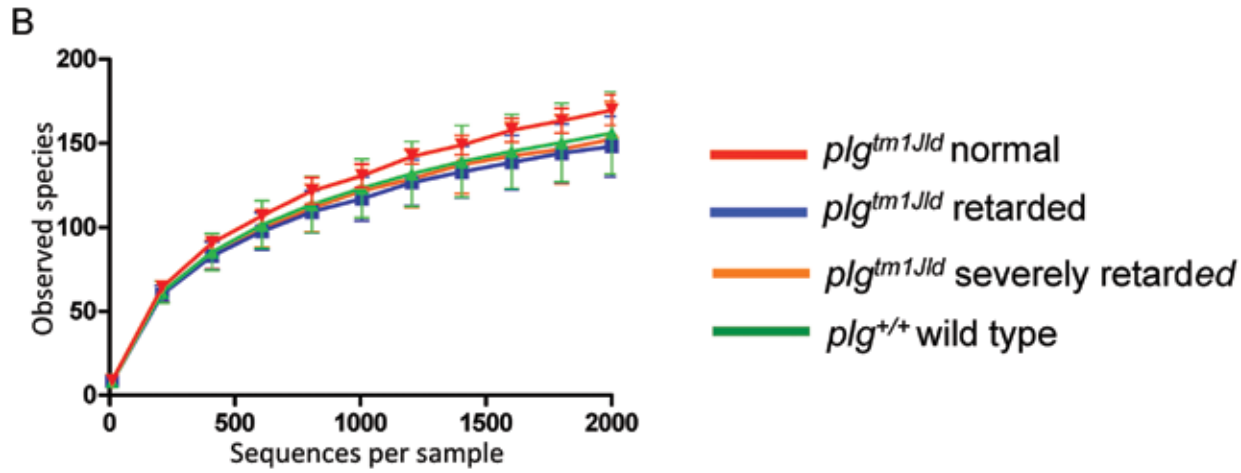


Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
plg$^{+/+}$	*plg*^{tm1Jld}	336.766	67.652	321.380	52.090	0.908	0.371



Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
plg$^{+/+}$ wild type	*plg*^{tm1Jld} retarded	336.766	67.652	309.309	38.010	1.240	1
plg^{tm1Jld} normal	*plg*^{tm1Jld} retarded	348.400	48.246	309.309	38.010	1.634	0.882
plg^{tm1Jld} severely retarded	*plg*^{tm1Jld} retarded	321.122	62.405	309.309	38.010	0.494	1
plg^{tm1Jld} severely retarded	*plg*$^{+/+}$ wild type	321.122	62.405	336.766	67.652	-0.601	1
plg^{tm1Jld} normal	*plg*^{tm1Jld} severely retarded	348.400	48.246	321.122	62.405	0.784	1
plg^{tm1Jld} normal	*plg*$^{+/+}$ wild type	348.400	48.246	336.766	67.652	0.358	1

Figure 6. Comparison of pyrosequenced fecal samples from WT and *plg*^{tm1Jld} mice did not reveal any difference in species diversity between the 2 genotypes.

of colonic lesions; wound AUC; or IL1α, IL2, IL10, IFNγ, IL4, or IL17 level (Table 4).

Cytokines. Cytokine profiles differed significantly between the 2 genotypes, in that *plg*^{tm1Jld} mice had less IL1α ($P = 0.036$), IL5 ($P < 0.000$), IFNγ ($P < 0.000$), and IL10 ($P < 0.000$) and more IL17 ($P = 0.017$) than did WT mice (Table 5). In the *plg*^{tm1Jld} mice, cytokine responses did not differ or correlate in regard to wound healing, number of colonic lesions, or GM-CSF profiles. However, *plg*^{tm1Jld} mice with rectal prolapses had significantly ($P = 0.044$) less IL5 (mean, 2.125 pg/mL) than did *plg*^{tm1Jld} mice without rectal prolapses (mean, 4.150 pg/mL).

Discussion

In the current study, *plg*^{tm1Jld} mice had delayed wound healing, frequent colonic lesions and altered cytokine profiles, thereby indicating a connection between plasminogen deficiency, gut immunology, and gut pathology. In particular, *plg*^{tm1Jld} mice had none

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Table 4. Correlations between taxa of gut bacteria and various parameters

Taxon						No. of significant observations per 1000 subsampled OUT tables	MIC
Phylum	Class	Order	Family	Genus	Parameter		
Bacteroidetes	Other	Other	Other	Other	IL1α	1	0.516
Bacteroidetes	Other	Other	Other	Other	Wound AUC	1	0.523
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	IL1α	2	0.512
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	IFNγ	1	0.509
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	IFNγ	2	0.517
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	IL10	1	0.512
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Other	IL1α	4	0.51
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	Unclassified	IL10	5	0.504
Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Odoribacter	Lesion score	1	0.501
Firmicutes	Other	Other	Other	Other	Wound AUC	1	0.529
Firmicutes	Clostridia	Unclassified	Unclassified	Unclassified	IL17	7	0.54
Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	IL17	1	0.541
Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	IL4	1	0.504
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified	IL10	1	0.502
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified	IL1α	4	0.503
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	IFNγ	3	0.505
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[Ruminococcus]	IFNγ	1	0.525
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[Ruminococcus]	IL10	1	0.513
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[Ruminococcus]	IL17	3	0.501
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unclassified	IL17	1	0.501
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unclassified	Wound AUC	1	0.506
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus	IL1α	1	0.508
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	IL4	1	0.523
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter	IL2	2	0.502
TM7	TM7-3	CW040	F16	Unclassified	Lesion score	1	0.519
Tenericutes	Mollicutes	RF39	Unclassified	Unclassified	Wound AUC	1	0.501

of the clinical signs observed in colitis models, including diarrhea, profound decline in body weight, profuse rectal bleeding, and progressive lethargy.[12] The wasting syndrome of *plg*^{tm1Jld} mice, which becomes more evident with age,[7] also is observed in IL10-deficient mice.[26,38] As previously noted,[40] *plg*^{tm1Jld} compared with *plg*$^{+/+}$ mice exhibited a larger interindividual variation in wound healing, thus segregating them into 3 groups according to the time needed for complete wound closure (Figures 1 and 2). This characteristic may reflect a genetic rather than an environmental difference between the mice, such as a gene linked to the targeted mutation in some but not all mice, for example.

The gut microbiota of the 2 genotypes presented neither quantitative nor qualitative differences. None of the clinical or biochemical scores correlated with the composition of gut microbiota, although some of the cytokine levels of the plasminogen-deficient mice were indicative of gut inflammation. This feature makes this condition highly different from other inflammatory colon diseases, such as inflammatory bowel disease and irritable bowel syndrome. In human patients as well as animal models, a decrease in microbial diversity is known to be an essential part of the dysbiosis related to inflammatory bowel disease.[27,41,47] To a large extent, Crohn disease is driven by Th1 and Th17 cells.[51] In the *plg*^{tm1Jld} mice, we noted increased levels of the proinflammatory cytokine IL17 (Table 3), which is produced by Th17 cells and is indicative of tissue damage.[50] However, the *Clostridium*-related Candidatus *Savagella* (perhaps better known as 'segmented filamentous bacteria'), which is a strong inducer of Th17 cells,[21,22] was not present in any of the mice, regardless of genotype. IFNγ levels, which reflect the abundance of Th1,[46] were low in the *plg*^{tm1Jld} mice (Table 3) and is a common observation in late-stage gut inflammation, when IL17 suppresses IFNγ-producing Th1 cells.[51] Amounts of the antiinflammatory IL10 were decreased in *plg*^{tm1Jld} mice (Table 3), and this situation, together with low levels of IL5 and IL10, perhaps suggests that the number of Th2 cells may be low in *plg*^{tm1Jld} mice.[39] However, IL10 is also produced by regulatory T cells. *Clostridium* spp. as well as other clostridia-related species are strong inducers of regulatory T cells, especially in the colonic mucosa of mice.[2] Indeed, the transfer of a mixture of human *Clostridium* spp. and related bacteria has been shown to expand the regulatory T-cell population of mice.[1] In humans, an important inducer of this phenomenon is the clostridia-related bacterium *Faecalibacterium prausnitzii*,[11] which occurs spontaneously in some[24] but not all[25] mouse colonies. When fed orally, *F. prausnitzii* reduces various forms of gut inflammation in mouse models,[9,36,48] but it was not present in any of our mice. *Prevotella* spp. were slightly increased in the *plg*^{tm1Jld} mice, which is of interest because these microbes are known to increase the severity of the dextran sodium sulfate–induced mouse model of colitis.[5,45] The common feature shared between that model and our *plg*^{tm1Jld} mice might be the disrupted mucosal barrier. *Bacteroides vulgatus*[37]

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Table 5. Plasma cytokine levels (pg*ml^{-1}) of *plg*$^{+/+}$ and *plg*^{tm1Jld} mice

	Genotype	*n*	Mean ± 1 SD	Median	Minimum	Maximum	*P*
IL1α[α]	WT	31	5.086 ± 2.022				
	plg^{tm1Jld}	25	4.012 ± 1.643				0.036
IL2	WT	31	0.1152	0.0000	0.9600		
	plg^{tm1Jld}	25	0.1016	0.0000	0.4400		
IL4	WT	31	0.1700	0.0000	1.6500		
	plg^{tm1Jld}	25	0.1700	0.0000	1.6500		
IL5[a]	WT	31	5.562 ± 2.110				
	plg^{tm1Jld}	25	3.262 ± 1.810				
IL6	WT	31		0.1000	0.0000	0.9000	
	plg^{tm1Jld}	25		0.1000	0.0000	1.5200	
IL10[a]	WT	31	14.953 ± 5.340				
	plg^{tm1Jld}	25	9.489 ± 4.374				0.000
IL17[a]	WT	31		0.2400	0.0000	0.8200	
	plg^{tm1Jld}	25		0.3400	0.0000	1.2200	0.017
IFNγ[α]	WT	31	12.628 ± 4.464				
	plg^{tm1Jld}	25	9.848 ± 3.402				0.000
TNFα	WT	31		0.0000	0.0000	1.3000	
	plg^{tm1Jld}	25		0.0000	0.0000	0.3100	
GM-CSF	WT	31	0.0000	0.0000	1.1800		
	plg^{tm1Jld}	25	0.0000	0.0000	1.1800		

Values are shown as mean ± 1 SD for normally distributed data, whereas the median, maximum, and minimum values are shown for nonnormally distributed data.
[a]Values differ significantly ($P < 0.05$) between the 2 genotypes.

and *B. fragilis*[33] are known to induce intestinal inflammation in rodent models. However, the various *Bacteroides* spp. did not differ among the experimental groups of our study, suggesting that this bacterial species does not contribute to the pathology present in plg^{tm1Jld} mice.

In conclusion, plasminogen-deficient *plg*^{tm1Jld} mice frequently develop colon lesions and rectal prolapses that can complicate the interpretation of data from studies involving this model. However, we were unable to demonstrate an altered fecal microbiome corresponding to the colon lesions and rectal prolapses in these mice, which are on an FVB/N background. Wide interindividual variability in the wound healing of *plg*^{tm1Jld} mice compared with their WT littermates further complicates the use of this model. We confirmed and characterized the interindividual variability in wound healing in *plg*^{tm1Jld} mice, but it did not correlate with the number of colonic lesions or with rectal prolapse.

Acknowledgments

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