The Phosphatidic Acid Phosphatase Lipin-1 Facilitates Inflammation-Driven Colon Carcinogenesis

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Extended Methods

T cell isolation

Single-T lymphocyte populations were isolated using nylon wool columns (1). Briefly, a cell suspension was obtained by grinding spleens between the ground ends of two microscope slides. Sample was depleted of red blood cell using ammonium chloride–mediated lysis. Cells were then resuspended in RPMI 1640 with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated for 2 h at 37°C in a 5% CO_2 incubator. The non-adherent lymphocytic population was further subjected to nylon wool column (Polysciences) purification by letting the cells to adhere to the column for 1h at 37°C. T cells were then eluted using warm RPMI 1640.

In vitro T cells activation

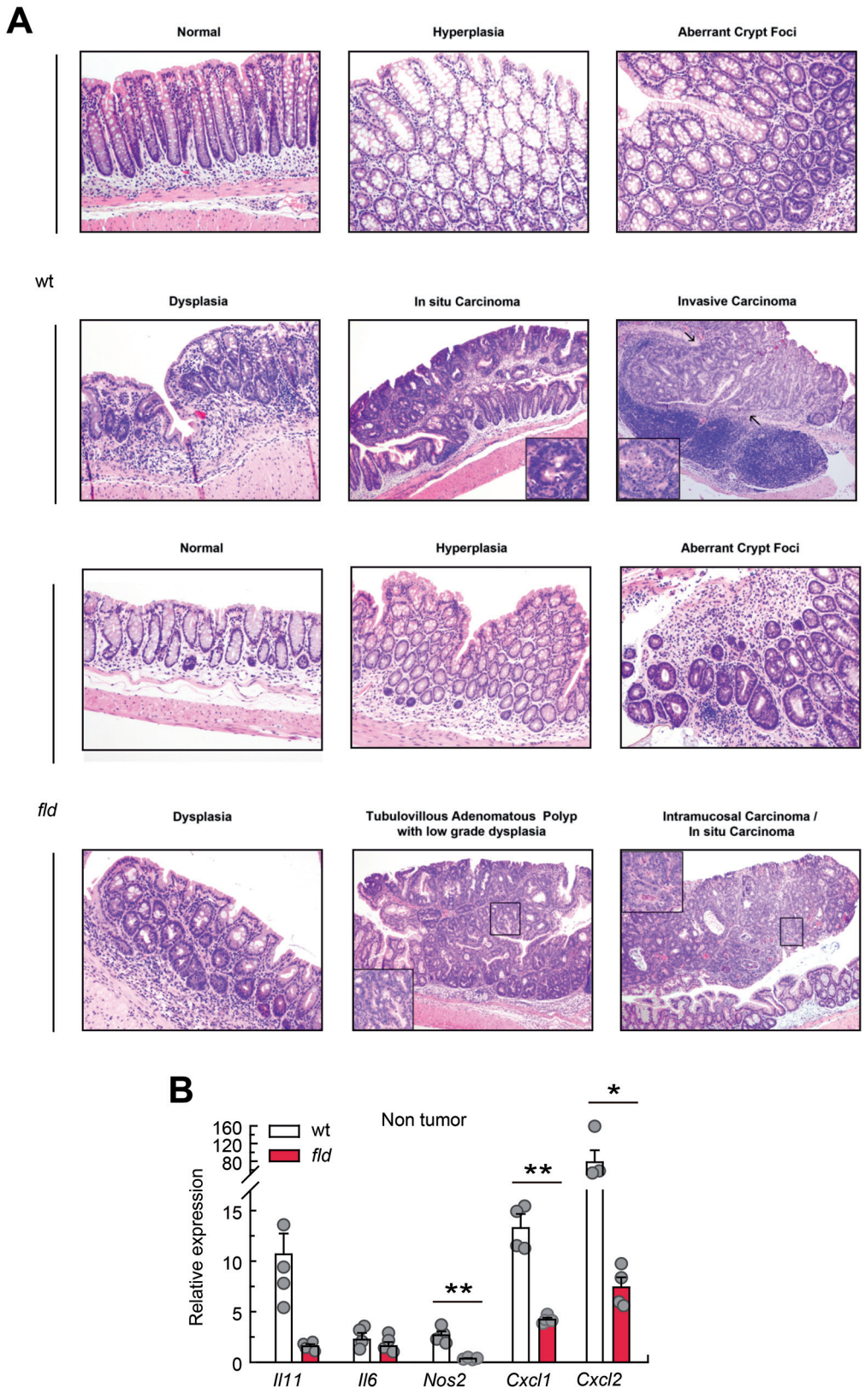
T lymphocyte activation was analyzed by CD69 surface expression (2). For that purpose, T lymphocytes were resuspended at 2.5 x 10^6 cells/ml in RPMI 1640 with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were activated with antibodies against CD3e (0.5 µg/ml, Biolegend,100303) and CD28 (1 µg/ml, Biolegend, 102101). After 24 h of activation, cells were stained with PE-conjugated anti-mouse CD69 (BD Pharmingen, 0150513) or the isotype control antibody. Data collection was performed in a Beckman Coulter Gallios flow cytometer in FL2, and analyses were done using Kaluza software (Beckman Coulter).

T cell proliferation

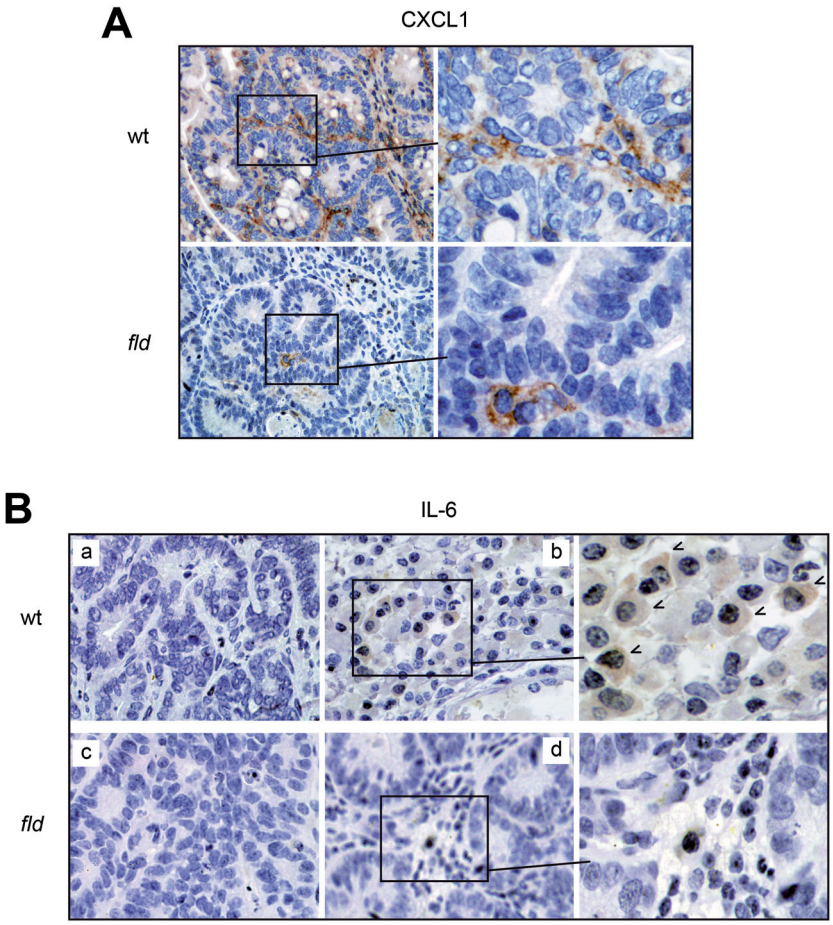
T cell proliferation was measured using the CellTracker™ Deep red dye (Molecular Probes) following the manufacturer instructions. Briefly, Isolated T lymphocytes were incubated in 0.1 % BSA-PBS with 2 µM of the probe at 37 ^oC for 30 min in the dark. Cells were then placed in RPMI 1640 with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and stimulated with antibodies against CD3e (0.5 µg/ml, Biolegend,100303) and CD28 (1 µg/ml, Biolegend, 102101) for 6 days. Fluoresce intensity was measured using the Beckman Coulter Gallios flow cytometer in FL6. Analysis were performed with Kaluza software (Beckman Coulter) and ModFit LT software.

References

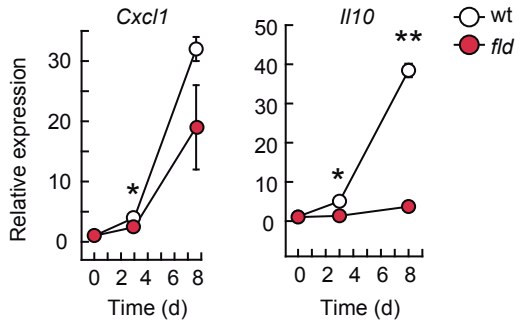
1. Meana C, et al. Lipin-1 integrates lipid synthesis with proinflammatory responses during TLR activation in macrophages. *J Immunol*. 2014;193(9):4614-4622.
2. Balboa MA, Izquierdo M, Sánchez-Madrid F, Fernández-Rañada JM, López-Botet M. Analysis of different protein kinase C-dependent events in T cells from allogeneic bone marrow transplantation recipients. *Clin Exp Immunol*. 1992;87(3):478-484.



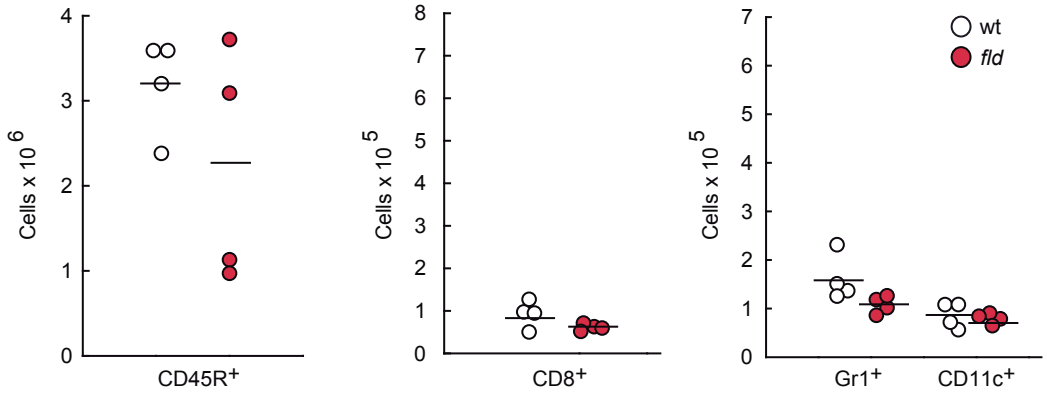
Supplemental Figure S1. Types of tumors generated in animals after AOM+DSS treatment (related with Fig. 2). Wt and lipin-1-deficient (*fld*) animals were treated with AOM+DSS, and tumors were analyzed at day 80. A, individual lesions were histologically analyzed and classified after fixation and H&E staining. Examples of microscopic lesions and tumorigenesis in wt and lipin-deficient animals are shown with magnified inserts (10x). Original magnification was 10x, except for carcinomas (4x). B, mRNA was extracted from adjacent areas to tumors and relative mRNA abundance for the indicted genes was analyzed by qPCR and normalized to Gapdh (n=4). Data represents mean ± SEM, *p<0.05, **p<0.01. *p* values were obtained by Student's t test.



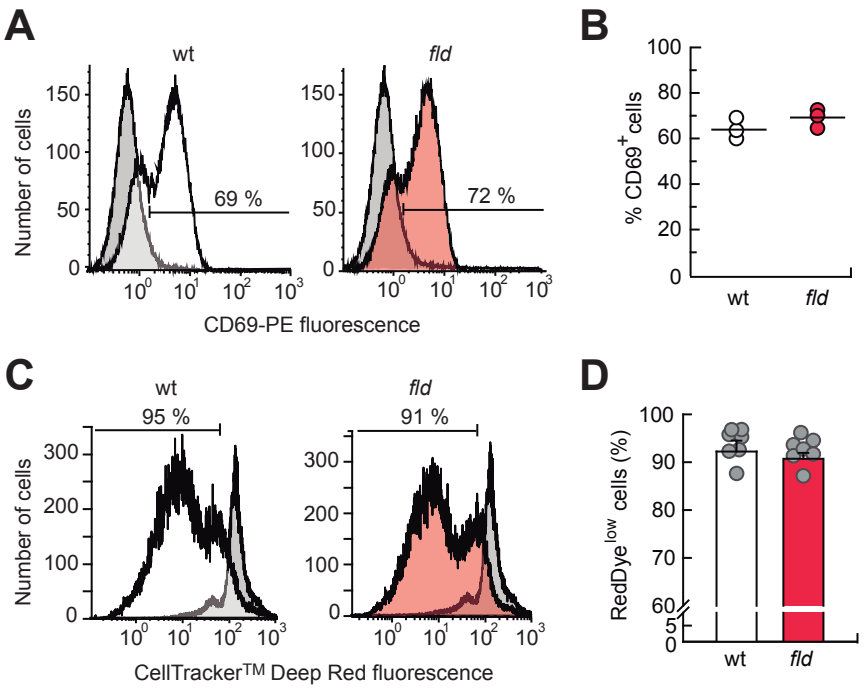
Supplemental Figure S2. Lipin-1 deficiency decreases the expression of CXCL1 and IL-6 in colon tumors (related to Figure 2). A-B, representative pictures of colon tumors sections from wt and lipin-1-deficient (*fld*) animals with the same grade of dysplasia immunostained with antibodies against CXCL1 (A) or IL-6 (B). Original magnification was 60x. Inserts are amplified to the right (120x). In B, a and c show epithelial tumoral cells, and b and d show stromal cells. Arrows in B point to macrophage-like cells expressing IL-6.



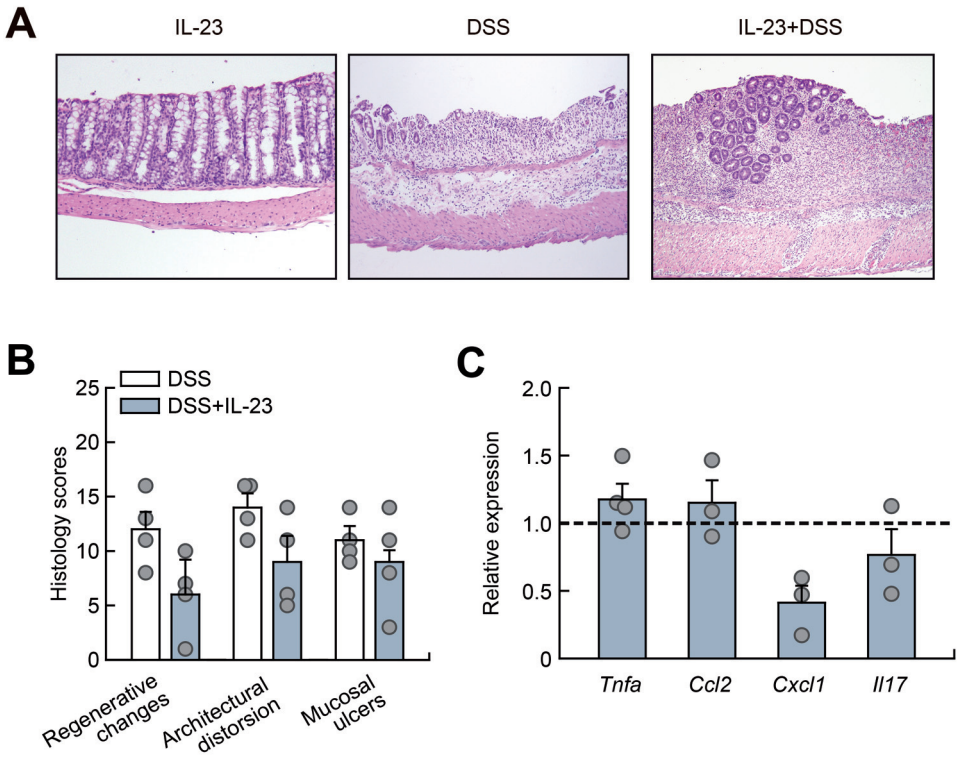
Supplemental Figure S3. Lipin-1 deficiency reduces DSS-induced expression of *Cxcl1* and *Il10* mRNA levels in the colon (related to Fig. 5). Wt and lipin-1-deficient (*fld*) animals were treated with DSS as indicated in M&M. Colons were collected and the content of Cxcl1 and Il10 mRNAs was analyzed by qPCR. The amount of each mRNA in colons of untreated mice was given an arbitrary value of 1. Data represents mean± SEM, *p<0.05, **p<0.01. *p* values were obtained by Student's *t* test



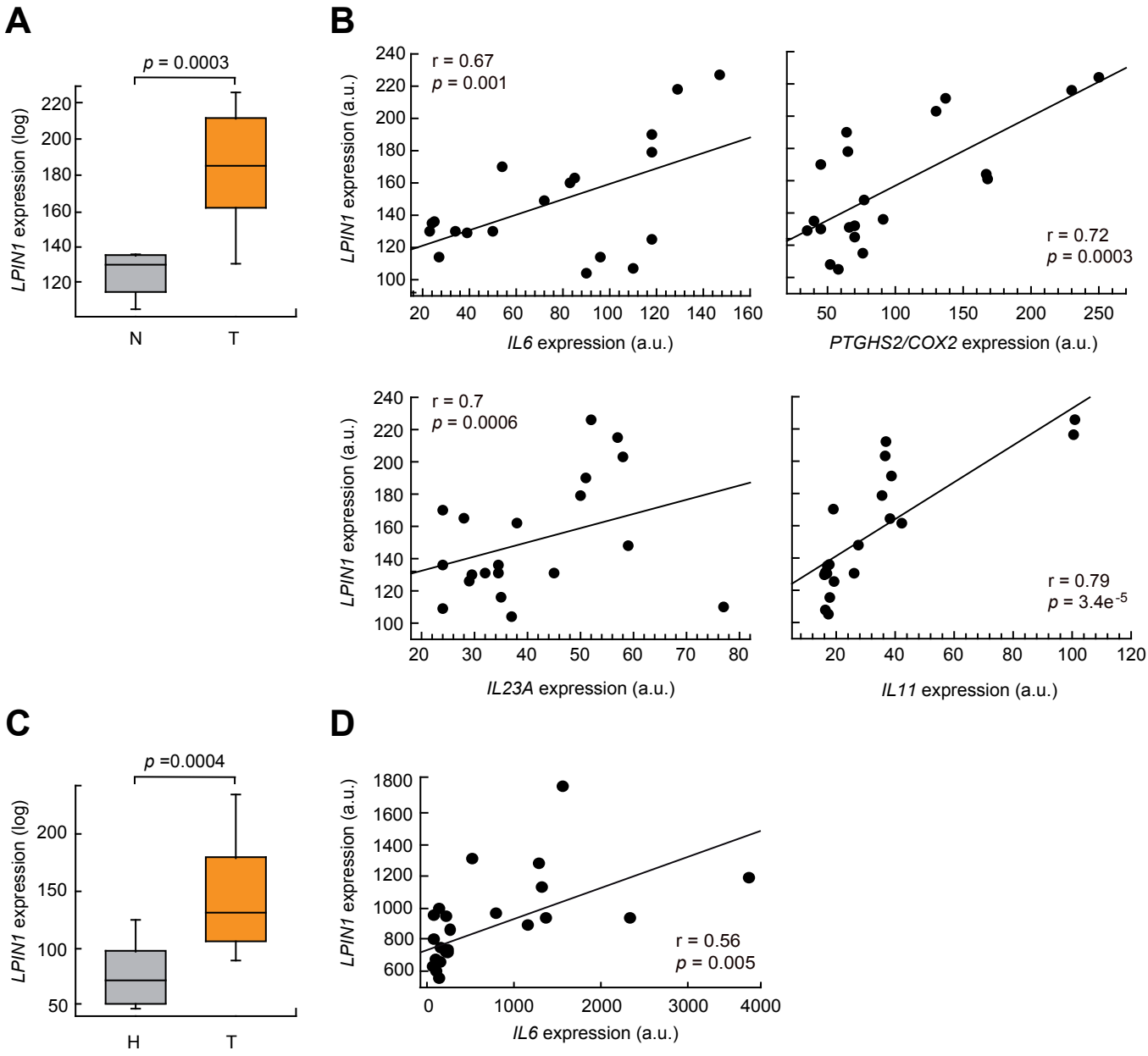
Supplemental Figure S4. Subtypes of immune populations found in lamina propria after DSS treatment (related to Fig. 4). Lamina propria cells from wt and lipin-1-deficient (*fld*) animals were isolated 8 days after DSS treatment as mentioned in M&M, counted, stained with specific antibodies and analyzed by flow cytometry. $CD45^+$ cells were gated and total $CD45R^+$, $CD8^+$, $Gr1^+$ or $CD11c^+$ cells found in colons are shown (n=4). *p* values were obtained by Student´s *t* test.



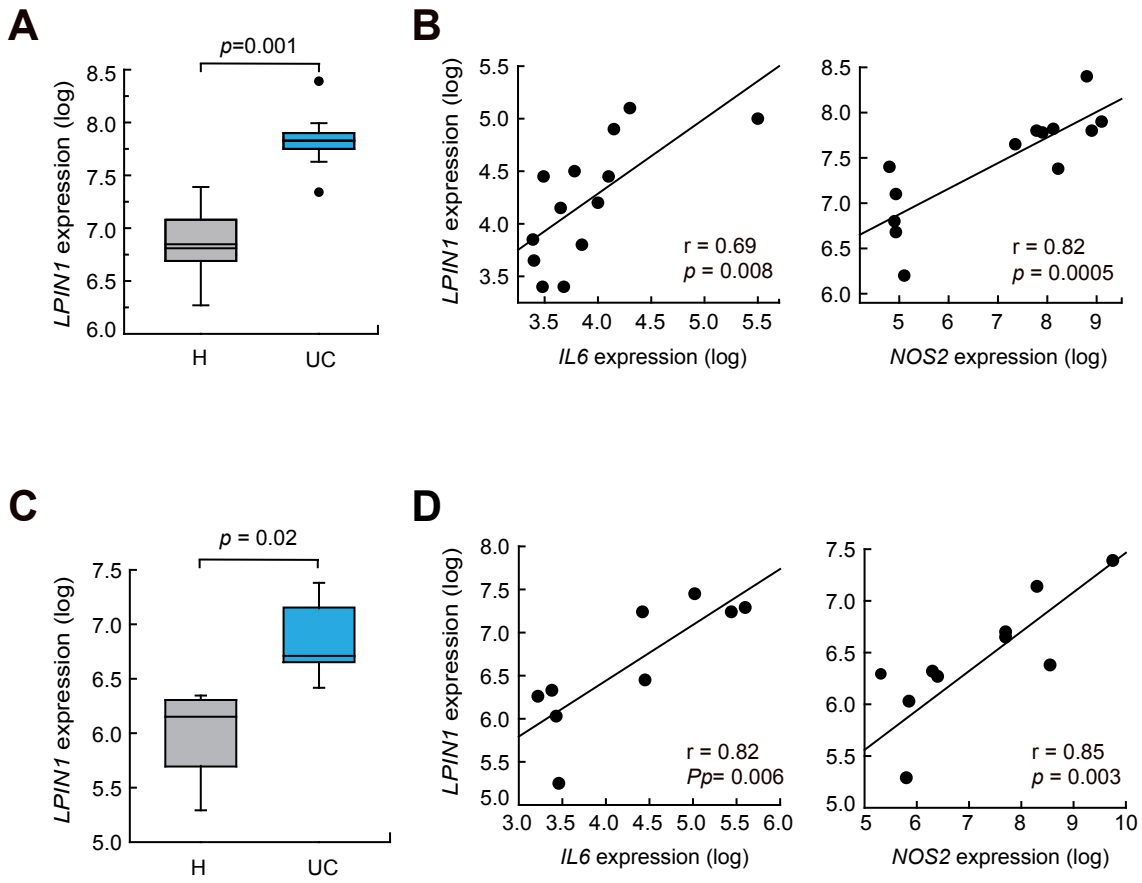
Supplemental Figure S5. Lipin-1 deficiency does not affect activation or proliferation of T cells. T cells isolated from spleens of wt and lipin-1-deficient (*fld*) animals were activated with antibodies against CD3 and CD28 as indicated in M&M. A-B, 24h later cells were stained with antibodies against CD69 and analyzed by flow cytometry. A, a representative analysis is shown. B, percentage of $CD69^+$ T cells from different animals (n=3). Differences between wt and *fld* activated T cells were not statistically significant by Student's *t* test. C-D, isolated T cells were stained with CellTracker™ Deep Red dye and stimulated as mentioned in M&M. Six days later fluorescence was analyzed by flow cytometry to study proliferation. C, a representative analysis is shown. D, percentage of CellTracker™ Deep Red^+ T cells from different animals (n=7). Differences between wt and fld proliferating T cells were not statistically significant by Student's *t* test.



Supplemental Figure S6. Effects of exogenous IL-23 on DSS-induced inflammation in wt animals (related to Fig. 7). Wt animals were treated with DSS with or without a daily injection of 400 ng recombinant IL-23 for 6 days. A, representative photographs of H&E-stained colon sections where ulcers can be observed. Original magnification was 10x. Tissues were obtained 8 days after initiation of treatments. B, colonic histology scores for regenerative changes, architectural distortion and mucosal ulcers, calculated as indicated in M&M (n=4). C, abundance of mRNA for the indicated genes were analyzed by qPCR. *Gapdh* was used as the reference gene (n=3). The amount of each mRNA in just DSS-treated colons was given an arbitrary value of 1. Data in B-E represents mean ± SEM, *p<0.05, **p<0.01. Differences were not statistically significant by Student's *t* test.



Supplemental Figure S7. LPIN1 expression and correlation with inflammatory genes in colon cancer data sets. A-B, analysis performed in the GSE41328 data set (n=21). A, Box plots showing *LPIN1* expression analysis in paired normal mucosa (N) and tumor tissues (T) from colorectal cancer patients. B, scatter plot showing the correlation between *LPIN1* and *IL6*, *COX2*, *IL23*, and *IL11* expression levels in colon samples. Pearson coefficient tests were performed to assess statistical significance. C-D, analysis performed in the GSE4183 data set (n=23). C, Box plots showing LPIN1 expression analysis in healthy donors (H) and tumor tissues (T) from colorectal cancer patients. D, scatter plot showing the correlation between *LPIN1* and *IL6*, *COX2*, *IL23*, and *IL11* expression levels in colon samples. Pearson coefficient tests were performed to assess statistical significance.



Supplemental Figure S8. LPIN1 expression and correlation with inflammatory genes in ulcerative colitis data sets. A-B, analysis performed in the GSE9452 data set (n=36). A, Box plots showing *LPIN1* expression analysis in colon tissues from healthy donors (H) and colon tissues from ulcerative colitis patients (UC). B, scatter plot showing the correlation between *LPIN1* and *IL6*, and *NOS2* expression levels in colon samples. Pearson coefficient tests were performed to assess statistical significance. C-D, analysis performed in the GSE6731 data set (n=9). C, Box plots showing *LPIN1* expression analysis in colon tissues from healthy donors (H) and colon tissues from ulcerative colitis patients (UC). D, scatter plot showing the correlation between *LPIN1* and *IL6*, and *NOS2* expression levels in colon samples. Pearson coefficient tests were performed to assess statistical significance.