Figure S1

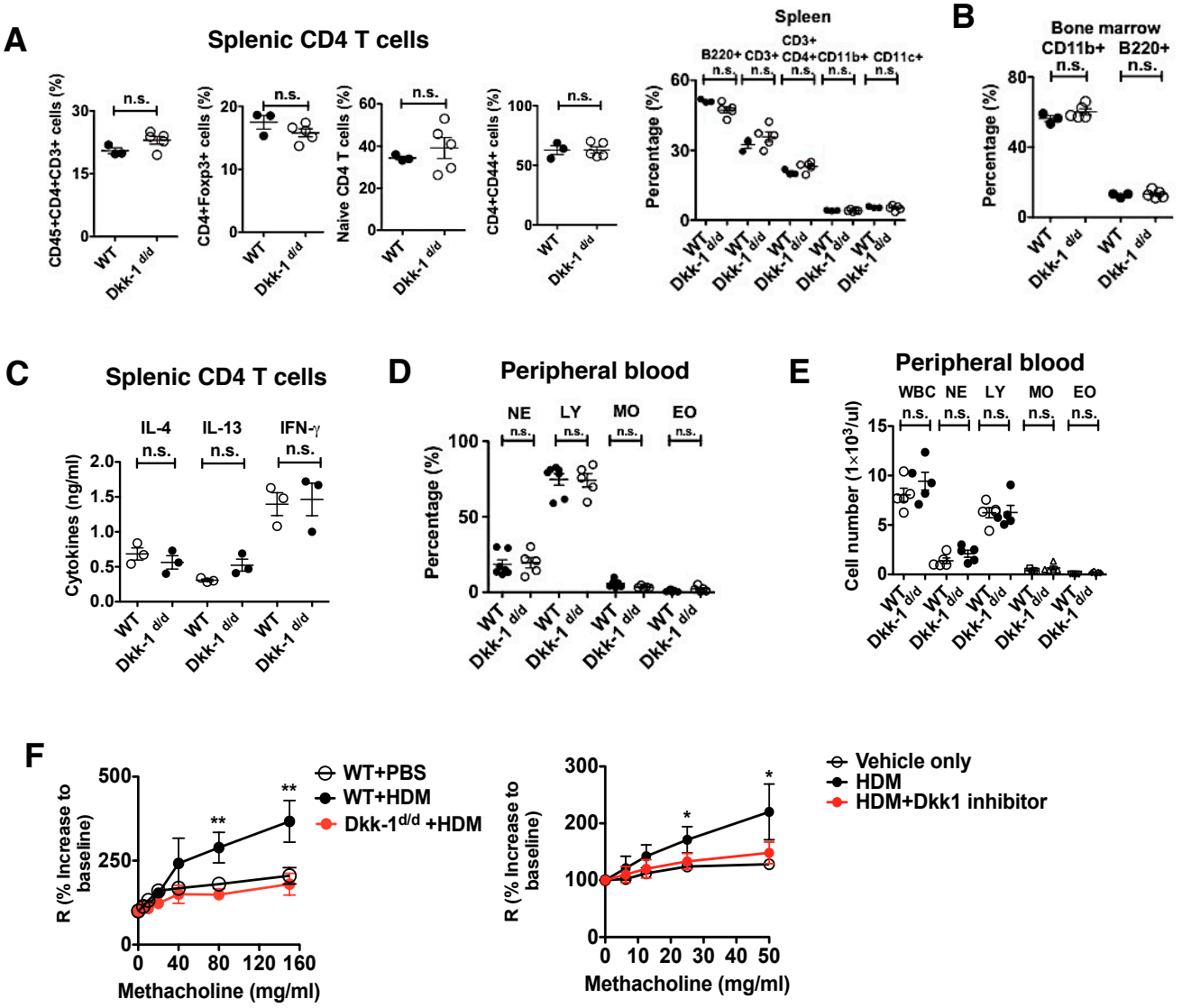

Figure S1, related to Figure 1. Characterization of Dkk-1$^{d/d}$ mice (**A, B**) Six to ten week-old Dkk-1$^{d/d}$ mice (n=5) and their wildtype littermate controls (n=3) were used. Single cell suspensions from spleen and bone marrow (**B**) were prepared and analyzed by flow cytometry. (**C**) Splenic CD4 T cells were isolated by MACS and stimulated with αCD3 and αCD28 mAb (2 μg/ml) for 4 days, and analyzed by ELISA. (**D, E**) Ten to twelve week-old Dkk-1$^{d/d}$ (n=5) and wildtype littermate control (n=5) mice were sacrificed and peripheral blood samples were measured by HEMAVAT analyzer. WBC (White blood cells), NE (Neutrophils), LY (Lymphocytes), MO (Monocytes), EO (Eosinophils) were counted. (**F**) (**Upper panel**) Seven week-old C57Bl/6 mice (n=5 each group) were challenged with HDM allergen as described in Figure 1B. Dkk-1 inhibitor (10 mg/kg) was injected intraperitoneally at day -1, +1, +6, +8, +10, and +12. Airway resistance was measured at day 14. (**Lower panel**) Seven week-old Dkk-1$^{d/d}$ (n=3) and their littermate control mice (n=4) were challenged with 100 μg HDM extract/mouse on day 0, day 7 and day 14. Control group mice (n=3) were challenged with PBS. Airway resistance was measured at day 16. A representative of two independent experiments are shown. Student's t-test was used. n.s, not significant. Small horizontal lines indicate the mean (± s.e.m.). *, p< 0.05

Figure S2

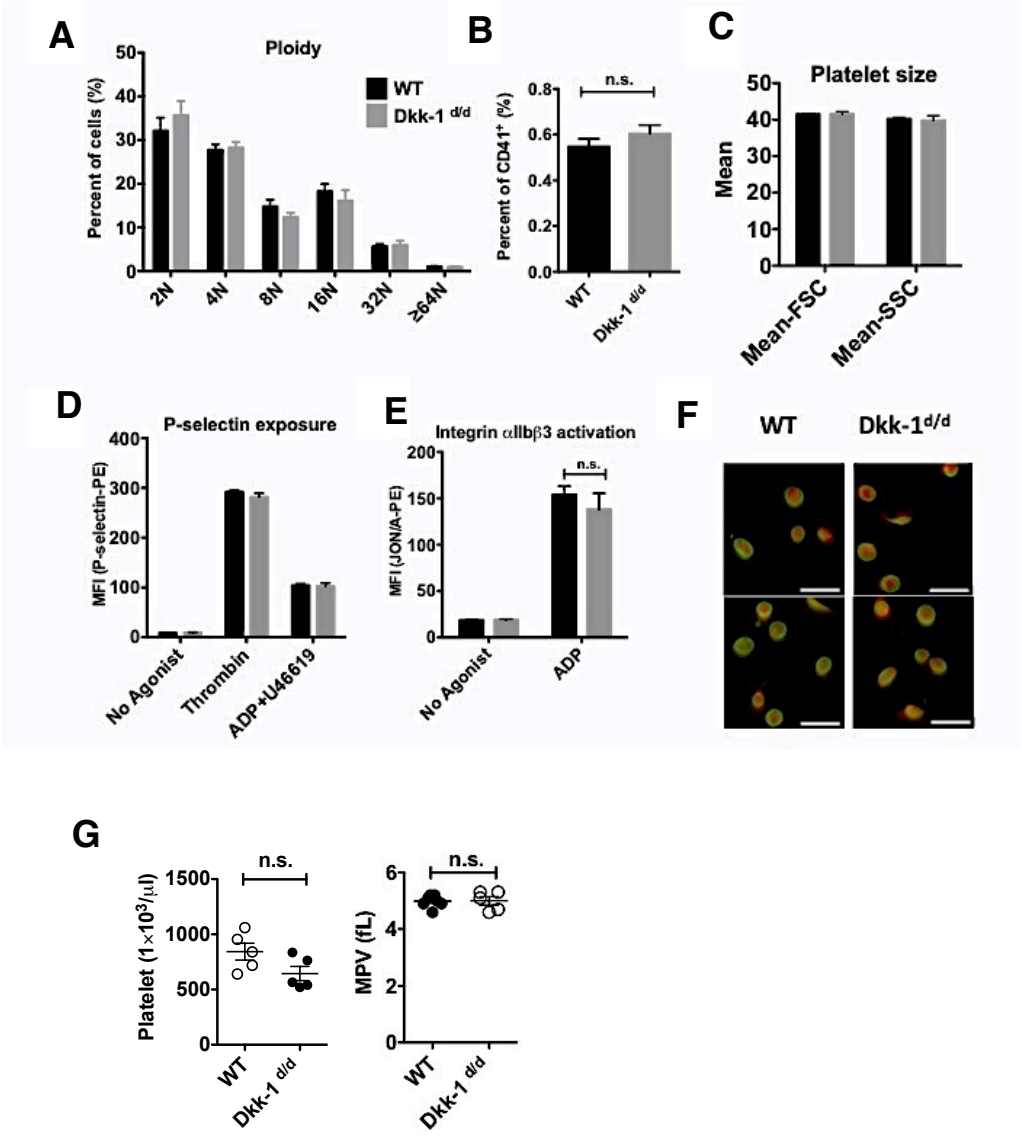

Figure S2, related to Figure 1. Dkk-1$^{d/d}$ mice have normal megakaryocyte ploidy, platelet size and platelet activation
Six to ten week-old Dkk-1$^{d/d}$ mice (n=3) and their wildtype littermate controls (n=3) were used. (**A**) Ploidy profile of CD41^{+} bone marrow cells assessed by propidium iodide staining (n=6 per genotype) shows no difference between WT and Dkk-1$^{d/d}$ mice. (**B**) Total CD41^{+} bone marrow cellular content is also normal (n=6 per genotype). Flow cytometry of peripheral blood indicates no difference in platelet size under resting conditions (**C**). Dkk-1$^{d/d}$ platelets can be activated and expose surface P-selectin in response to thrombin and ADP + U-46619 (**D**), and activate the αIIbβ3 receptor in response to ADP (**E**) at comparable levels to WT (n=3 per genotype). (**F**) Platelet cytoskeletal architecture assessed by b-tubulin and Phallodin (actin) is normal in Dkk-1$^{d/d}$ mice. (**G**) Platelet count and MPV (Mean platelet volume) were measured from Ten to twelve week- old Dkk-1$^{d/d}$ (n=5) and wildtype littermate control (n=5) mice. n.s., not significant. Student's t-test was performed. Small horizontal lines indicate the mean (± s.e.m.). A representative of two independent experiments are shown.

Figure S3

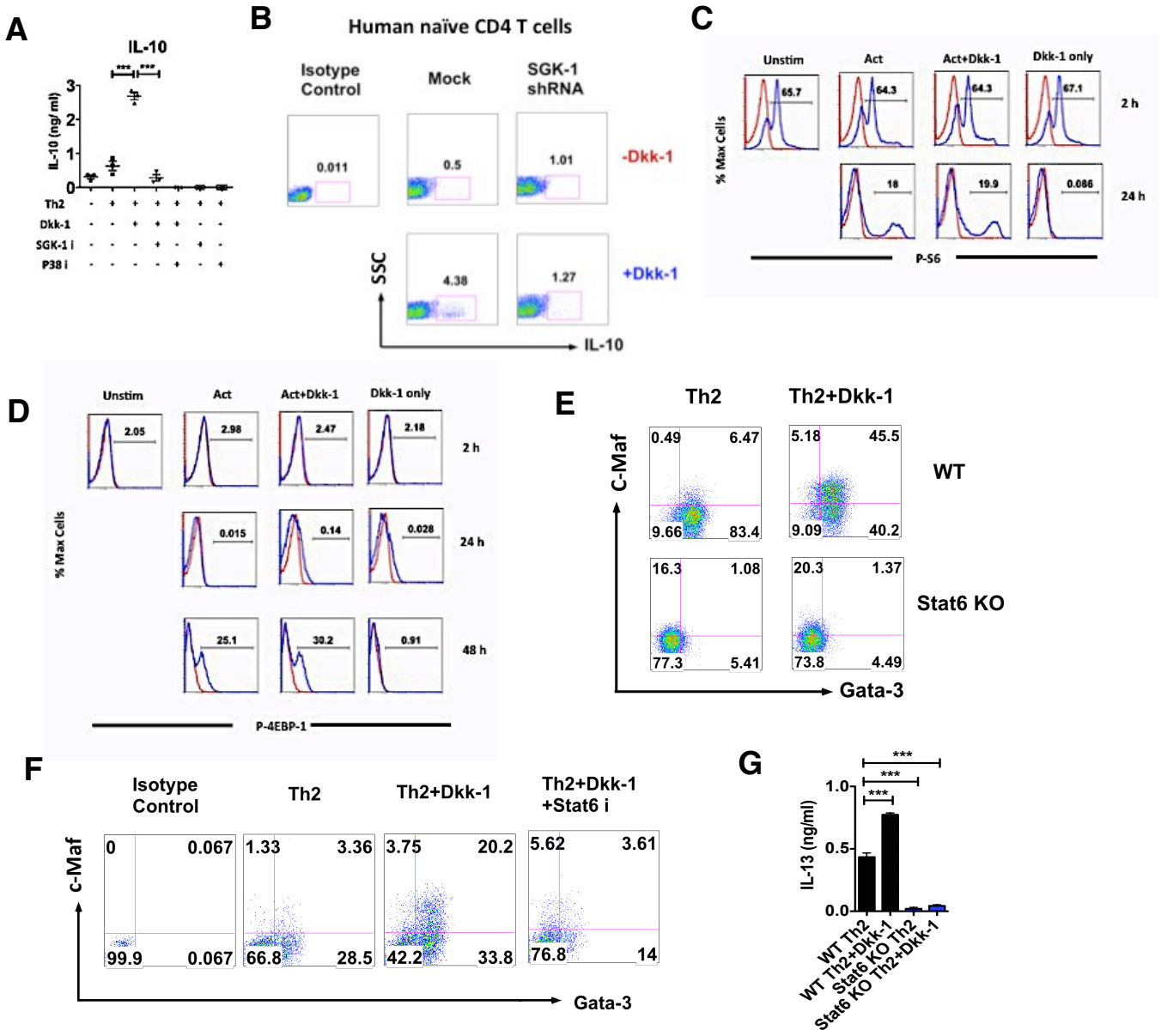

Figure S3, related to Figure 5. Dkk-1 induces IL-10 in CD4 T cells via SGK-1 and p38 MAPK

(A) Naïve CD4 T cell were differentiated into T_H2 cells with or without Dkk-1 (30 ng/ml), SGK-1 inhibitor (2 μM) or (B) p38 inhibitor (10 μM) for 96 hrs and then analyzed by ELISA. A representative of three independent experiments is shown. Student's t-test was performed. **, p<0.005, ***, p<0.0005. (**B**) Human naïve CD4 T cells ($CD4^+CD25^-CD45RA^+$) were transduced with SGK-1 shRNA and selected with puromycin (0.5 μg/ml) for 48 hr. The selected cells were then stimulated with αCD3 mAb (OKT3) and αCD28 mAb with or without Dkk-1 for 96 hr. For the last 5 hrs of culture, stimulation cocktail (PMA/Ionomycin/ Brefeldin A/Monensin) was added. Cells were then analyzed by flow cytometry. A representative of three independent experiments is shown. Small horizontal lines indicate the mean (± s.e.m.). **(C, D)** Splenic CD4+CD25- cells from 6 week-old C57BL/6 mice were isolated and activated with αCD3 and αCD28 mAb (2 μg/ml) with or without Dkk-1 (25 ng/ml) for indicated time points. Cells were analyzed by intracellular staining with antibodies recognizing phospho-S6 kinase (**C**) and phospho-4E-BP1 (**D**), respectively. A representative of two independent experiments is shown. (**E**) Splenic CD4 T cells or (**F**) naïve CD4 T cells from either 8 week-old C57Bl/6 or Stat6-deficient mice were stimulated with anti-CD3 and anti-CD28 antibody with or without Dkk-1 (30 ng/ml) for 96 hrs. In (**F**), Stat6 inhibitor (1 nM) was added for 96 hrs. Expression levels of c-Maf and Gata-3 were measured by flow cytometry and IL-13 was measured by ELISA (**G**). For (**G**), One-Way ANOVA analysis with Dunnet's post-hoc test was performed. ***, p<0.0005. A representative of two independent experiments is shown.

Figure S4

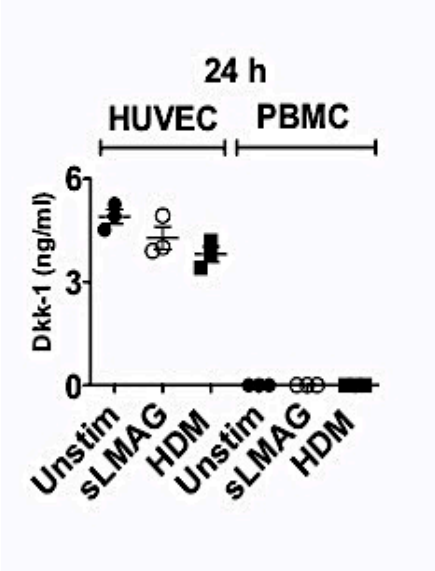

Figure S4, related to Figure 6. Dkk-1 is not induced by sLMAG or HDM in HUVECs or PBMCs.
HUVECs (2×10^5 /ml) (n=2) or human PBMCs (n=3) (2×10^6 /ml) were stimulated with either HDM extract (15 µg/ml) or sLMAG (1:50 as described in Materials and Methods) for 24 hrs in triplicate. Supernatants were harvested and measured by ELISA. A representative of two independent experiments is shown. All results were statistically not significant. One-Way ANOVA with Bonferroni's post-hoc test was performed. ***, $p<0.0005$. All the results were statistically non-significant.

Figure S5

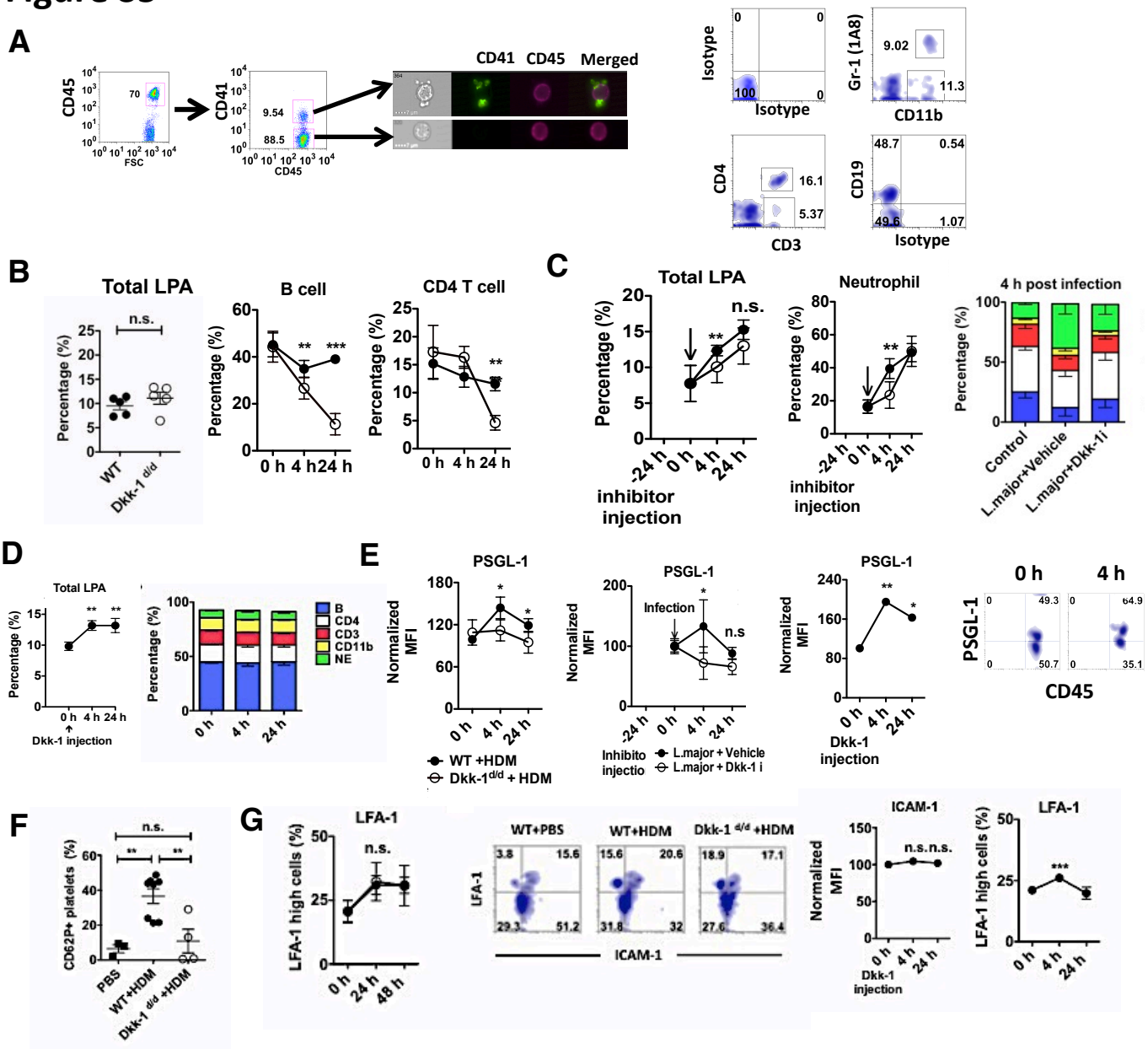

Figure S5, related to Figure 7. Regulation of LPA formation by Dkk-1 (**A**) Peripheral blood from 6-week old C57BL/6 male mice was analyzed by AMNIS. (**B**) (**Left panel**) The basal LPA population ($CD45^+CD41^+$) was measured in Dkk-$1^{d/d}$ mice (n=5) and their wildtype littermate controls (n=5). (**Right panels**) Dkk-$1^{d/d}$ mice and their littermate controls (n=5) were challenged with 30 μg HDM extract/mouse, and B cells and CD4 T cells among $CD45^+CD41^+$ cell population were monitored.(**C**) Dkk-1 inhibitor (n=5) or DMSO vehicle (n=5) was injected 24 hr prior to infection with *L.major*. LPA formation in total CD45+ cells (**left panel**) and neutrophils was monitored (**middle panels**) and each subset of leukocytes was analyzed (**right panel**). Small horizontal lines indicate the mean (± s.e.m.). (**D**) Nine-week old C57BL/6 mice (n=5) were injected with 300 ng of Dkk-1. Peripheral blood was analyzed for LPA formation among $CD45^+$ leukocytes. (**E**) PSGL-1 expressions in $CD45^+$ leukocyte population in peripheral blood from Figure 7B, figure S7C, and Figure 7C were measured, respectively. MFI (Mean fluorescence intensity) was normalized to unchallenged C57BL/6 mice. Small horizontal lines and error bars indicate the mean (± s.e.m.). A representative of three independent experiments is shown. Student's t-test was performed. ***, $p < 0.0005$, **, $p< 0.005$. (**F**) WT mice (n=8) and Dkk-$1^{d/d}$ mice (n=4) were challenged with HDM allergen (10 μg/time) as described in Figure 1B. Nine-week old C57BL/6 mice (n=3) that were injected with vehicle (PBS) were used as controls. Peripheral whole blood was collected and stained with CD41 and CD62P for flow cytometry. (**G**) (**Upper panels**) Dkk-$1^{d/d}$ mice (open circle) and WT mice (closed circle) were challenged with HDM allergen and analyzed for the percentages of LFA-1 high cells in LPA population. A representative flow cytometry plots for ICAM-1 and LFA-1 expression in LPA population is shown. (**Lower panels**) Normalized MFI for ICAM-1 expression and the percentages of LFA-1 high cells were measured by flow cytometry following injection of 300 ng of Dkk-1 intraperitoneally. Student's t-test was used. Small horizontal lines indicate the mean (± s.e.m.). ***, $p<0.0005$, **, $p<0.005$, n.s., not significant. A representative of two independent experiments are shown. For Figure S5F, One-way ANOVA analysis with Bonferroni's post-hoc test was performed.

Figure S6

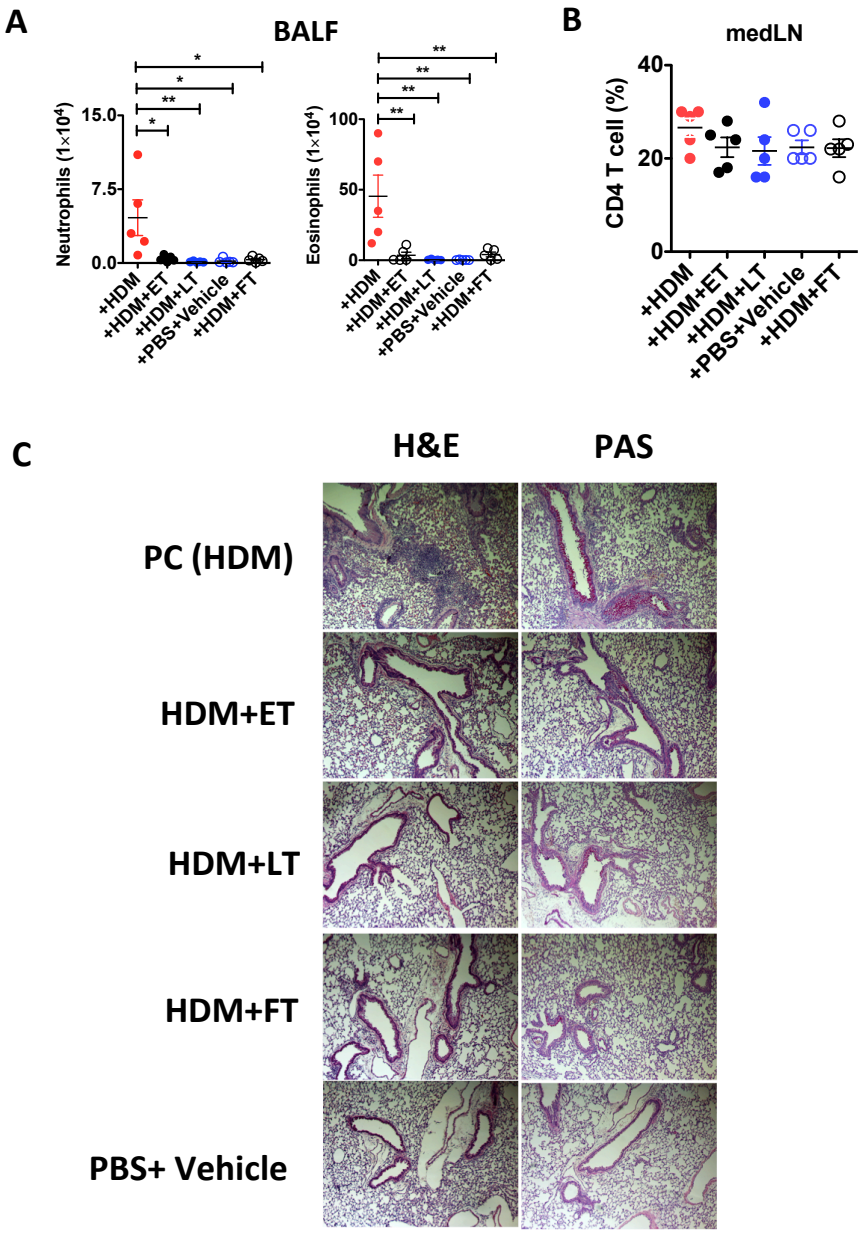

Figure S6, related to Figure 7. Pharmacological antagonism of Dkk-1 (A) Neutrophils and eosinophils in BALF were counted for each group (n=5) as described in Figure 7E. (**B**) CD4 T cell percentages in med LNs were measured by flow cytometry. (**C**) Representative images H&E and PAS staining were shown. ET, early treatment of Dkk-1 inhibitor, LT, late treatment of Dkk-1 inhibitor, FT, full treatment of Dkk-1 inhibitor, PC, positive control (HDM only). Small horizontal lines and error bars indicate the mean (± s.e.m.). A representative of two independent experiments is shown. One-way ANOVA analysis with Dunnet's post-hoc test was performed. **, $p< 0.005$, *, $p<0.05$.

Supplementary Text

Supplementary Experimental Procedures

Antibodies and Reagents

Anti-mouse CD4 (clone RM4-5), anti-mouse CD8β (clone eBIOH35-17.2), anti-mouse CD11c (clone N418), anti-mouse CD19 (clone eBio1D3), anti-mouse CD3 (clone eBio500A2), anti-mouse CD11b (clone M1/70), anti-mouse F4/80 (clone BM8), anti-mouse Ly6G (clone 1A8), anti-mouse B220 (clone RA3-6B2), anti-mouse Foxp3 (clone FJK-16s), anti-human/mouse GATA-3 (clone TWAJ), anti-human/mouse c-Maf (clone sym0F1), anti-IL-17A (clone eBio 17B7), anti-IFN-γ (clone XMG1.2), anti-IL-4 (clone 11B11), anti-IL-10 (clone JES5-16E3), anti-CD62L (clone Mel-14), anti-CD69 (clone H12F3), anti-CD25 (clone 7D4), anti-CD44 (clone IM7), Rag IgG2a isotype control (clone eBR2a), anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51), anti-mouse CD45 (clone 30-F11), anti-mouse CD41 (clone eBioMWReg30), anti-mouse CD42d (clone 1C2), and anti-human/mouse CD62P (clone P.Sel.K02.3) antibodies were purchased from eBioscience. Anti-siglecF (clone ES22-10D8) antibody was purchased from Miltenyi Biotec. SGK1 inhibitor (GSK650394) was purchased from GSK (GlaxoSmithKline). Dkk1 inhibitor (EMD Millipore), p38 inhibitor SB203528 (Tocris), and Stat6 inhibitor (AxonMedchem) were purchased from the indicated vendors.

Cell lines and plasmids

Dkk-1 cDNA was cloned into pFRSV-SRα expression vector (Slanetz et al., 1991) Briefly, CHO (Chinese Hamster Ovary) cells were transfected with Dkk-1-pFRSV-SRα and then Dkk-1 expression was amplified by methotrexate (MTX) treatment. Before harvest, MTX was removed and cells were washed. As a control, pFRSV-SRα expression vector was transfected and then the supernatant was also harvested and used in the experiment as a control. The amount of Dkk-1 in the culture supernatant was determined by Dkk-1 ELISA (R&D systems). To determine the biological activity of Dkk-1, Tcf reporter (TOP FLASH) luciferase and *Renilla* reporter plasmids were co-transfected in HEK 293T cells with Lipofectamine 2000 (Invitrogen), and then luciferase activity was measured. by luciferase assay kit (Promega) after 24 hr of Wnt3a-conditioned media treatment. 10 mM LiCl (Lithium Chloride) was also used to activate Wnt pathway in HEK 293T cells. Luciferase activity was measured using a fluorometer with GEN5 software (Biotek). Dkk-1 was added to inhibit Wnt pathway and relative luciferase activity was measured. A GSK-3β inhibitor BIO was purchased from Sigma. HEK 293T cells were maintained in 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine DMEM media.

Immunohistochemistry

These sections were de-paraffinized by immersion in xylene and re-hydrated. Antigen retrieval was performed using 10 mM sodium citrate buffer. Endogenous peroxidase activity was inhibited by immersion in 3% H_2O_2 PBS. The sections were blocked for 30 min at room temperature followed by overnight incubation with the specific primary antibody at 4°C. The primary antibodies used were: anti-mouse DKK-1 antibody (R&D Systems), anti-mouse CD41 antibody (clone: MWReg30, abcam) and anti-CD3ε antibody (clone: SP7, Pierce). The sections were rinsed in PBS and incubated with the biotinylated-secondary antibody (Vector Laboratories) for 30 min at room temperature. Sections were washed in PBS and ABC reagent (Vector Laboratories) was added for 30 min at room temperature. Sections were again washed in PBS and bound peroxidase detected by adding DAB substrate (Vector Laboratories) for 15 min at room temperature. Counterstaining was performed using Mayer's haematoxylin for 5 min in Dkk-1 staining. For double staining, Vectastain-ABC-AP kit (Vector Laboratories) was added for 30 min at room temperature for the second staining. Sections were washed in PBS and incubated with Vector Blue substrate (Vector Laboratories) for 30 min at room temperature in a humid chamber. No counterstaining was used on the double stained slides.

Ploidy/DNA Content analysis

DNA content analysis was performed as previously described with minor modifications (Smith et al., 2012). Briefly, whole bone marrow was stained with FITC-CD41 antibody (BD Biosciences), then fixed with 70% ethanol overnight. Samples were digested for four hours on ice with 200 μg/ml RNase (Sigma-Aldrich), then stained with 10 μg/ml propidium iodide (Sigma-Aldrich). Data was collected on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Platelet Immunofluorescence

Platelet immunofluorescence was performed as previously described with modifications (Smith et al., 2012). Blood was collected via the retro-orbital sinus into tubes containing 3.2% sodium citrate (Medicago, Sweden). Platelet-rich plasma was obtained by centrifugation of whole blood at 200 x *g* for 8 min. Platelet-richplasma was washed (140mM NaCl, 5mM KCl, 12mM $Na_3C_6H_5O_7$, 10mM dextrose, 12.5mM sucrose [pH 6.0]) and platelets were isolated at 900*g* and resuspended (10mM HEPES, 140mM NaCl, 3mM KCl, 0.5mM $MgCl_2$, 5mM $NaHCO_3$, 10mM dextrose [pH 7.4]). Resting platelets were centrifuged onto poly-L-lysine coated coverslips, fixed (15 min,4% paraformaldehyde), permeabilized (15 min, 0.5% Triton X-100), and blocked overnight. Coverslips were incubated with anti β-tubulin (1:250; Sigma-Aldrich) for 2-3 hours at room temperature, followed by incubation with secondary antibody (1:500; Life Technologies) and Texas

Red-XPhalloidin (Life Technologies) for one hour. After washing, coverslips were mounted with Aqua Poly/ Mount (Polysciences, Inc.). Images were captured using an Olympus BX51 microscope with a Sensicam^{QE} CCD camera (Cooke) and analyzed using IPLab software (BD, V 4.0.8).

Platelet Activation Flow Cytometry

Peripheral blood was collected from the retro-orbital sinus into tubes coated with lithium heparin (BD). Whole blood was washed three times in Tyrodes-HEPES (1 mM $MgCl_2$, 5 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 5.5 mM dextrose, 0.42 mM Na_2HPO_4, 12 mM $NaHCO_3$ [pH 7.4]), spinning at 2800rpm to discard the supernatant. For staining, washed blood was resuspended in Tyrodes-HEPES with 2 mM $CaCl_2$. Platelets were stimulated with 10μM adenosine 5'-diphosphate (ADP; CHRONO-LOG), 3 μM U-46619 (Cayman Chemical), or 0.1 U/ml Thrombin (Roche), and activation was assessed by CD41/61, JON/A, and P-selectin/CD62P expression (Emfret Analytics). Data was collected on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Human PBMC, HUVEC and lentiviral transduction

Healthy frozen human PBMCs were prepared by a Ficoll-Hypaque method. Human CD4 T cells were isolated using a CD4 T cell isolation kit (Miltenyi Biotec) from four independent donors. CD4 T cells were further sorted for naïve and effector T cells. Naïve CD4 T cells were activated for 24 hr with T cell expander kit (Dynalbeads). Lentivirus particles were packaged using HEK 293T cells. At 48 hr T cells were infected with shRNA lentivirus particles (MOI=5). shRNA vector for SGK-1 was purchased from Addgene. Cells were selected with puromycin at 0.3 μg/ml. Selected T cells were further stimulated with anti-CD3 and anti-CD28. IL-10 and Gata-3 were measured by intracellular cytokine staining after stimulating cells with cell stimulation cocktail with protein transport inhibitors (eBioscience) for 5 hr. For Dkk-1 measurement by ELISA, 2×10^6 frozen PBMCs were thawed and stimulated with soluble leishmania antigen (equivalent to 5×10^6 parasites/ml) or HDM extract (20 μg/ml) for 24 and 48 hrs. HUVECs (passage 2 to 9) were kindly provided by Jordan Pober (Yale University). Briefly, 2×10^4 HUVECs were stimulated with sLMAG or HDM for 24 hrs. HUVECs were maintained with M199 media with 20% FCS and ECGS (15 μg/ml). All procedures were in accordance with HIC (Human Investigation Committee) protocol (protocol # 0008011950).

Human platelet isolation and stimulation

Human platelets were isolated as described from healthy volunteers (Tang et al., 2011). All volunteers consented to donate their blood. Briefly, 3.2% citrate buffer was used to prevent coagulation. Blood was centrifuged at 250 *g* for 15 min. The upper layer was taken as platelet rich plasma (PRP). The Hemavet 950FS (Drew Scientific) was used to count platelet numbers. Platelets were washed twice with wash buffer andresuspended

in Tyrode's buffer. 100 million platelets/ml were used for activation. House dust mite extract (50 μg) added for activation of platelets for 1 hr. For soluble leishmania antigen (sLMAG), *L.major* parasites were prepared in 5×10^8 parasites/ml Schneider's culture medium. sLMAG was prepared by repeated freeze and thaw cycles. This stock was added to platelet culture indicated ratio for 1 hr. Platelets were resuspended in Tyrode's buffer. Supernatant was collected for human Dkk-1 ELISA. All procedures were in accordance with HIC protocol (protocol # 1005006865).

Lymphocyte-platelet aggregate assay

Lymphocyte-platelet aggregate detection was performed as described previously with minor modifications (Li et al., 1997; Pitchford et al., 2005). For details, see supplemental experiment procedures. Briefly, 90 μl blood was collected via the retro-orbital sinus into tubes containing 10 μl 0.5 M EDTA (pH 8.0) at 4 and 24 hours after house dust mite extract challenge, *L. major* infection, or recombinant Dkk1 injection. Peripheral blood (8 μl) was stained with anti-CD16/32 antibody for 15 min at room temperature. The peripheral blood was further incubated with each antibody to detect each subset of lymphocyte subset and platelets for 15 min at room temperature in the dark.Fix/red blood cell lysis buffer (Biolegend) (450 μl) was added and incubated for 15 min in the dark. Samples were analyzed by flow cytometry within 4 to 6 hours. Live gating was performed on leukocyte-sized events to exclude single platelets. Leukocytes were identified by their forward and side scatter characteristics and also CD45 expression. Lymphocyte-platelet aggregates were identified by the $CD41^+$ population. Each subset of lymphocyte was further gated based on this $CD45^+CD41^+$ population.

House dust mite asthma model

6-10 week old *doubleridge* mice and their littermate controls were challenged intranasally on day 0, 7, 8, 9, 10, 11. Mice were sacrificed on day 14. Broncho-alveolar lavage (BAL) fluid was collected and BAL cells were counted and analyzed by flow cytometry. Mediastinal lymph nodes (medLNs) were harvested and a single cell suspension was prepared. Subsequently, med LNs cells were stimulated with 15 μg/ml of HDM extract for 96 hr. Supernatant was harvested for ELISA assays and cells were analyzed for flow cytometry. Lung homogenate was prepared by collagenase digestion. Cells were counted and lymphocytes were analyzed by flow cytometry.Lymphocytes were gated on $CD45^+$ cells. Neutrophils ($CD45^+CD11b^+Ly6G^+$) and Eosinophils ($CD45^+CD11c^-SiglecF^+$) in lung tissue homogenates and BAL fluid were measured by flow cytometry. For histological analysis, lungs were fixed with 10% formalin at least for 24 hrs, embedded in paraffin and stained with hematoxylin and eosin (H&E), or periodic acid–Schiff (PAS) reagent and were assigned scores by established methods. Peribronchiolar and perivascular inflammation was determined by a semiquantitatively graded scale as follows:

0, no detectable airway inflammation; 1, less than 25% bronchials and surrounding vasculature were found to have either perivascular or peribronchial inflammatory cell infiltration; 2, approximately 25–50% of bronchials and surrounding vasculature were affected; 3, approximately 50–75% bronchials and surrounding vasculature were affected; 4, more than 75% of bronchials and surrounding vasculature were affected. Histology scoring was performed in a double-blinded manner by a certified pathologist. For lymphocyte-platelet aggregation assay, 50 μg of HDM extract was dissolved in 25 μl PBS and then intranasal challenge was performed. Lymphocyte-platelet aggregation was measured at 4 h and 24 h after the challenge. At 72 h, lung homogenate was prepared by collagenase digestion. Cells were counted and lymphocytes were analyzed by flow cytometry as described above.

***Leishmania* infection model**

L. major WR309 strain (MHOM/IL/79/LRC-L251) was originally isolated from a human case of cutaneous leishmaniasis in Israel and has been maintained through culture and frequent passages in mice to maintain virulence. BALB/c mice were infected with 2×10^6 late stationary promastigotes isolated from a Percoll gradient. For monitoring 2 week and 6 week experiments, Dkk-1 inhibitor (10 mg/kg) or vehicle (50 μl DMSO) was injected on day -1, +1, +3, +5, +7, +9, +11 and weekly afterwards till harvest. Draining lymph nodes, non-draining lymph nodes, and plasma were collected. For ex vivo analysis, soluble *Leishmania major* antigen (sLMAg) was employed. Single cell suspension of draining and non-draining lymph node cells was incubated with sLMAg (equivalent to 5×10^6 organisms/ml final) for 96 hr. Supernatants were harvested and analyzed by ELISA assays. For depleting platelets, 80 μg of anti-CD42 mAb (Emfret Analytical) was injected intravenously in 100 μl PBS. Equal amounts of isotype-matched control Ab (eBioscience) was used for control group animals. Tissues from mice were harvested on day 3 post-infection for analysis.

Supplementary reference

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