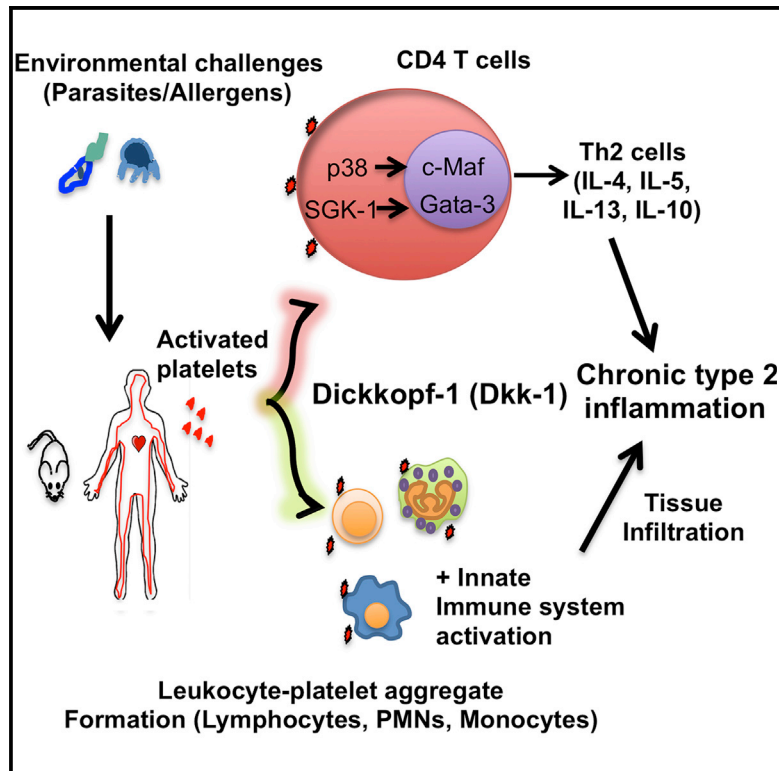


Immunity

The Wnt Antagonist Dickkopf-1 Promotes Pathological Type 2 Cell-Mediated Inflammation

Graphical Abstract



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In Brief

The role of Wnt antagonist Dickkopf-1 in immune responses is unknown. Bothwell and colleagues find that the elevation of Dkk-1 from platelets is critical to promote chronic and pathological type 2 inflammation via enhancing Th2 cell differentiation and leukocyte-platelet aggregate formation.

Highlights

- Reduced expression of Dkk-1 protects the host from house-dust-mite-induced asthma
- Dkk-1 induces Th2 cell polarization and potentiates Th2 cell cytokine expression
- Platelets are the major source of Dkk-1 in pathological type 2 inflammation
- Pharmacological inhibition of Dkk-1 protects host from cutaneous leishmaniasis



The Wnt Antagonist Dickkopf-1 Promotes Pathological Type 2 Cell-Mediated Inflammation

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SUMMARY

Exposure to a plethora of environmental challenges commonly triggers pathological type 2 cell-mediated inflammation. Here we report the pathological role of the Wnt antagonist Dickkopf-1 (Dkk-1) upon allergen challenge or non-healing parasitic infection. The increased circulating amounts of Dkk-1 polarized T cells to T helper 2 (Th2) cells, stimulating a marked simultaneous induction of the transcription factors c-Maf and Gata-3, mediated by the kinases p38 MAPK and SGK-1, resulting in Th2 cell cytokine production. Circulating Dkk-1 was primarily from platelets, and the increase of Dkk-1 resulted in formation of leukocyte-platelet aggregates (LPA) that facilitated leukocyte infiltration to the affected tissue. Functional inhibition of Dkk-1 impaired Th2 cell cytokine production and leukocyte infiltration, protecting mice from house dust mite (HDM)-induced asthma or *Leishmania major* infection. These results highlight that Dkk-1 from thrombocytes is an important regulator of leukocyte infiltration and polarization of immune responses in pathological type 2 cell-mediated inflammation.

INTRODUCTION

The primary goal of immune responses is to eliminate the main trigger of inflammation, contributing to the structural and functional repair of the affected tissue. During the process, the key roles for circulating blood leukocytes are to migrate to sites of infection or injury and to develop polarized immune responses (Abbas and Janeway, 2000; Ley et al., 2007). The canonical Wnt signaling pathway, which induces cell proliferation, is uti-

lized for tissue repair processes, and a Wnt antagonist might inhibit or delay such events in chronic inflammatory diseases (Whyte et al., 2012). Among the quintessential Wnt inhibitory ligands, Dickkopf-1 (Dkk-1) was originally found regulating head formation of *Xenopus laevis* and is known to inhibit the canonical Wnt signaling pathway (Cruciat and Niehrs, 2013; Glinka et al., 1998). The inhibition of canonical Wnt pathway activation by Dkk-1 is achieved by its competitive binding of the receptor LRP (low-density lipoprotein receptor)-5 and 6 complex with markedly higher affinity than its counterpart agonist Wnt3a (Cheng et al., 2011; Joiner et al., 2013).

Regarding its possible role in chronic inflammation, elevated Dkk-1 amounts in circulating blood have been reported in various types of cancers and bone diseases that are characterized by unhealed tissue lesions (Diarra et al., 2007; Sato et al., 2010; Tian et al., 2003). In addition, a previous study reports that the proliferation of intestinal epithelial cells in a DSS-colitis model is enhanced in Dkk-1 hypomorphic *doubleridge* mice (*Dkk1^{d/d}*), suggesting an inhibitory role of Dkk-1 in wound repair in a pro-inflammatory microenvironment (Koch et al., 2011). A diverse array of environmental stimuli also causes tissue damage and they commonly trigger type 2 cell-mediated inflammation or immune responses both in acute and chronic inflammation (Pulendran and Artis, 2012; Whyte et al., 2012). Epithelial cell-derived factors (e.g., TSLP, IL-25, and IL-33) can induce type 2 cell-mediated immune responses (Divekar and Kita, 2015). Cytokines such as IL-4, IL-5, IL-10, and IL-13 are the key features of type 2 cell-mediated immune responses after T helper 2 (Th2) cell differentiation (Allen and Wynn, 2011; Pulendran and Artis, 2012).

The role of the canonical Wnt pathway in T cell biology including T cell development, CD8⁺ memory T cell formation, and regulatory T cell function has been demonstrated in multiple experimental systems with varying results, depending on the model systems used (Ding et al., 2008; Guo et al., 2007; van Loosdregt et al., 2013; Xie et al., 2005). Although the role of canonical Wnt pathway components such as β -catenin or

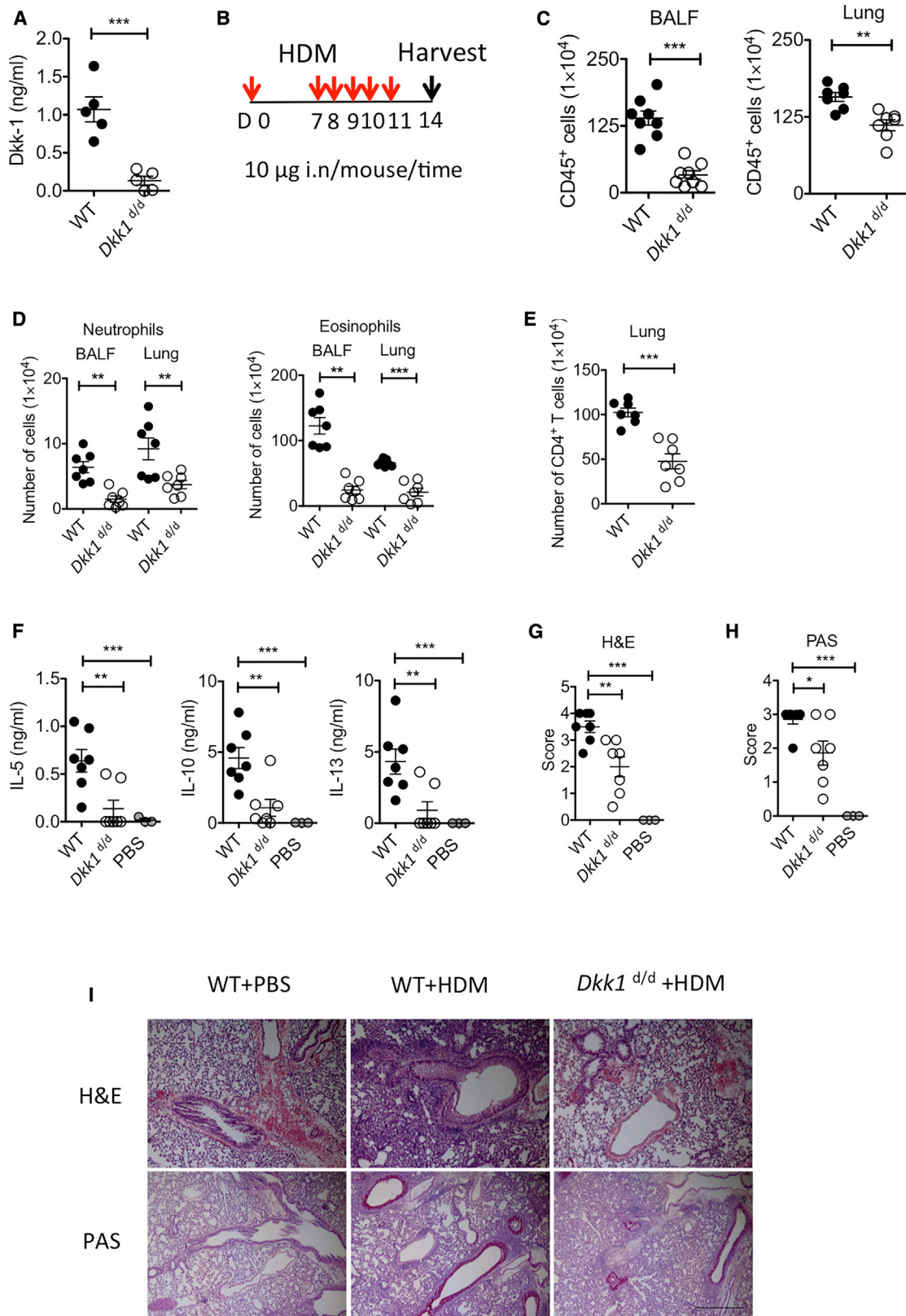


Figure 1. Reduced Expression of Dkk-1 Protects the Host from House Dust Mite-Induced Asthma

(A) Circulating concentrations of Dkk-1 in 8-week-old female *Dkk1^{d/d}* mice (n = 5) and their wild-type (WT) littermate control mice (n = 5).

(B) A scheme of HDM challenge protocol (10 µg/mouse/time).

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adenomatous polyposis coli (Apc) have been analyzed in these studies, the role of Wnt antagonists including Dkk-1 in the course of type 2 cell-mediated immune responses is poorly understood.

In recent years, the crucial role of platelets in inflammation has been widely emphasized (Morrell et al., 2014; Yeaman, 2014). Platelets are involved in coagulation upon various pathogenic challenges that protect surrounding tissues from both injury and invasion by microorganisms, and they also act as the reservoir of growth factors to recruit cells to repair tissue damage (Li et al., 2012). More recent studies show that platelets are important players in chronic inflammatory diseases by interacting with leukocytes in various pathological inflammatory conditions (Jenne and Kubes, 2015; Rondina and Garraud, 2014) and also selectively secreting immunomodulators to control T cell responses (Shi et al., 2014) and regulating leukocyte migration (Diacovo et al., 1996). It has been reported that activated platelets induce P-selectin expression, and P-selectin binds to P-selectin glycoprotein ligand (PSGL-1) in mature leukocytes to form leukocyte-platelet aggregates (León and Ardavin, 2008; Ley et al., 2007; Pitchford et al., 2005). Acute bacterial or viral infection can often lead to ITP (idiopathic thrombocytopenic purpura) with low platelet counts with the destruction of platelets (Cines et al., 2014; Zhang et al., 2009).

Here we demonstrate that the Wnt antagonist Dkk-1 induced Th2 cell polarization in physiologic models of type 2 cell-mediated inflammation or immune responses and utilized MAPK and mTOR pathway components to achieve potent Th2 cytokine production. Platelets were primarily responsible for the circulating Dkk-1 and its concentration was elevated by allergen or non-healing parasite challenge. Dkk-1 promoted interaction between leukocytes and platelets, facilitating the migration of leukocytes to the affected tissue.

RESULTS

Dkk-1 Hypomorphic Mice Are Protected from House-Dust-Mite-Induced Asthma

Th2-cell-mediated immune responses typically involve tissue damage and repair that requires the regulation of Wnt signaling (Pulendran and Artis, 2012; Whyte et al., 2012). We first explored the possibility that Dkk-1 functions as an immunomodulatory ligand in a standard model of house dust mite (HDM) extract allergen challenge, using the Dkk-1 hypomorphic *doubleridge* (*Dkk1^{d/d}*) mouse strain in which Dkk-1 expression is reduced by 90%. We first characterized hematological features of *doubleridge* mice. Of note, CD4⁺ T cells from *doubleridge* mice showed comparable cytokine production upon activation to those from WT littermate controls and the splenic CD4⁺ T cell population did not show significant differences in their cell surface markers of activation (Figures S1A–S1E). We also charac-

terized platelets from *Dkk1^{d/d}* mice and WT mice and determined that platelets from *Dkk1^{d/d}* mice were functionally comparable to WT platelets (Figures S2A–S2G). We confirmed the reduced amounts of Dkk-1 in plasma from *doubleridge* mice (Figure 1A) and challenged them with HDM extract (Figure 1B). CD45⁺ leukocytes including neutrophils, eosinophils, and CD4⁺ T cells were significantly increased in the lung and broncho-alveolar lavage (BAL) fluid of WT mice but this was substantially decreased in *Dkk1^{d/d}* mice, suggesting that the reduced expression of Dkk-1 after allergen challenge inhibited the infiltration of leukocytes to the lung (Figures 1C–1E). Th2 cytokines (IL-5, IL-10, and IL-13) were markedly induced in WT mice but notably decreased in *Dkk1^{d/d}* mice in ex vivo stimulation of mediastinal lymph node (medLN) cells with HDM allergen extract (Figure 1F). Hematoxylin and eosin (H&E) and periodic acid-Schiff base (PAS) staining and scoring of lung tissues also showed that *Dkk1^{d/d}* mice had reduced inflammation and leukocyte infiltration compared to WT mice (Figures 1G–1I). We also showed that airway resistance increase by HDM was notably decreased by using the Dkk-1 inhibitor (1-(4-(naphthalen-2-yl)pyrimidin-2-yl)piperidin-4-yl)methanamine (Pelletier et al., 2009) (WAY-262611) or in *Dkk1^{d/d}* mice (Figure S1F). Taken together, our results demonstrate that lack of Dkk-1 protects the host from chronic type 2 cell-mediated immune responses in the HDM-induced asthma model.

Functional Inhibition of Dkk-1 Protects Mice from *Leishmania major* Infection

We further questioned whether functional inhibition of Dkk-1 could impair chronic inflammation by different types of environmental pathogens via a different route of challenge such as skin. Parasite infections challenge the immune system to temper its activity with highly evolved immune evasion strategies (Redpath et al., 2014). Infection with the parasite *Leishmania major* causes chronic skin lesion formation with unresolved inflammation by Th2-cell-mediated immune responses in a murine model (Belkaid et al., 2001; Tacchini-Cottier et al., 2012). Many inbred mouse strains such as C57BL/6, C3H, and CBA/J are genetically resistant to *L. major* and spontaneously resolve infection because they mount a protective Th1-cell-type response. In contrast, susceptible BALB/c mice develop large nonhealing chronic lesions and mount a Th2-cell-type response that is associated with the production of the cytokines IL-4 and IL-10 (Reiner and Locksley, 1995; Scott, 1991). We assessed whether the inhibition of Dkk-1 function impairs Th2 cell polarization, ameliorating the development of cutaneous leishmaniasis. A Dkk-1 inhibitor, WAY-262611, was administered intraperitoneally during the course of *L. major* infection (Figure 2A). Both lesion size and parasite burden were greatly reduced at 42 days after infection, showing a 91% reduction of parasite survival in the group

(C and D) 9- to 12-week-old *Dkk1^{d/d}* (n = 7) and their littermate controls (n = 7) were analyzed. Total leukocytes (CD45⁺) (C), neutrophils and eosinophils from BAL fluid (BALF) (D), and lung tissue homogenates (D) were quantitated.

(E) CD4⁺ T cells from lung tissue homogenates were quantitated.

(F) Supernatants from mediastinal lymph node cells that were stimulated with HDM extract for 4 days were analyzed by ELISA.

(G–I) Lung tissues from each mouse were scored after H&E (G) and PAS (H) staining. Controls were challenged with PBS (n = 3) and one-way ANOVA analysis with Dunnett's post hoc test was performed. In (I), original magnification is 10×. Scale bar in lower right panel represents 10 μm.

Small horizontal lines indicate the mean (±SEM). A representative of two independent experiments is shown. Student's t test was performed. ***p < 0.0005, **p < 0.005, *p < 0.05. See also Figures S1 and S2.

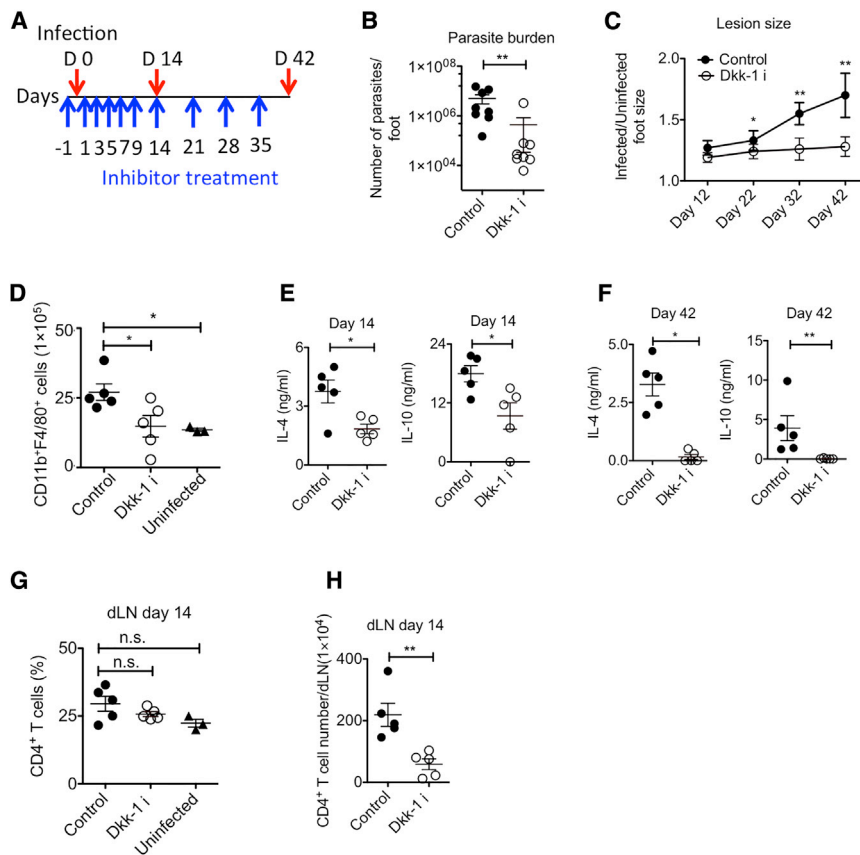


Figure 2. Functional Inhibition of Dkk-1 Protects the Host from Chronic Inflammation Caused by *L. major*

(A) A schematic diagram for Dkk-1 inhibitor treatment (10 µg/kg/time) in 6-week-old female BALB/c mice.

(B and C) The infected foot from each mouse was analyzed for parasite burden at day 42 (B) and lesion size was measured (n = 8/group) (C).

(D) 2 weeks after vehicle (n = 5) Dkk-1 inhibitor treatment (n = 5) based on (A), macrophages in the infected hindfoot were counted by flow cytometry and compared with infected (untreated) and uninfected (n = 3) BALB/c mice.

(E and F) 2 weeks (E) and 6 weeks (F) after infection, draining lymph node cells from each mouse (n = 5/group) were stimulated with sLMAG.

(G and H) CD4⁺ T cell numbers (G) and percentage (H) in draining lymph nodes from each group was determined by flow cytometry.

Small horizontal lines indicate the mean (±SEM). A representative of two independent experiments is shown. Student's t test (B, C, E–H) and one-way ANOVA analysis with Dunnett's post hoc test (D, G) were performed. **p < 0.005, *p < 0.05.

treated with Dkk-1 inhibitor (Figures 2B and 2C). Macrophage accumulation after parasite infection was also diminished by 14 days after infection (Figure 2D). IL-4 and IL-10 production was notably reduced by Dkk-1 inhibitor treatment at days 14 and 42 after infection subsequent to ex vivo stimulation of draining lymph node (dLN) cells from the infected mice with soluble leishmania antigen (sLMAG), suggesting that type 2 cell cytokine production was promoted by Dkk-1 (Figures 2E and 2F). It has been known that *L. major* infection induces CD4⁺ T cell recruitment and proliferation with lymph node expansion as well as Th2 cell differentiation (Carvalho et al., 2012; Hsu and Scott, 2007). Dkk-1 inhibitor treatment did not reduce the percentage of CD4⁺ T cells in dLNs but reduced the number of CD4⁺ T cells in the dLNs (Figures 2G and 2H), indicating that CD4⁺ T cell proliferation or recruitment is markedly reduced by Dkk-1 inhibition and that this is concomitantly associated with reduced IL-4 and IL-10 production in dLNs. Taken together, these results suggest that different types and routes of environmental challenges induced elevation of circulating Dkk-1 to promote chronic tissue inflammation with robust Th2 cytokine production.

Dkk-1 Polarizes CD4⁺ T Cells to Th2 Cell Lineage

From the allergen exposure model, we observed that platelets and T cells were co-localized in the lung in WT mice after recurrent allergen challenges but that this was reduced in *Dkk1*^{d/d} mice (Figure 3A). This increased T cell infiltration in the lung in WT mice compared to *Dkk1*^{d/d} mice was consistent with the increased CD4⁺ T cell numbers and the H&E score in the lung (Figures 1E

and 1G). We further checked whether any residual Dkk-1 might be induced in *Dkk1*^{d/d} mice, but we could not detect any elevation of Dkk-1 after HDM challenge in *Dkk1*^{d/d} mice (Figure 3B). Next, we tested whether Dkk-1 induces Th2 cytokines from CD4⁺ T cells; Dkk-1 induced IL-4, IL-10, and IL-13 in total CD4⁺ T cell activation in vitro (Figure 3C). Other important effector T cell cytokines including Th1- and Th17-associated cytokines (e.g., IFN-γ, IL-17A, and IL-17F) were not induced by Dkk-1, suggesting that Dkk-1 preferentially induces Th2 cytokines (Figure 3D). This induction of Th2 cytokines was also observed when naive CD4⁺ T cells were activated with Dkk-1 in vitro (Figure 3E).

We further examined whether Dkk-1 could antagonize Th1 cell polarization. Dkk-1 suppressed IFN-γ expression under Th1-cell-polarization conditions while still elevating Gata-3 expression and IL-4, IL-5, IL-10, and IL-13 secretion, suggesting its strong ability to drive Th2 cell polarization (Figure 4A). Finally, Dkk-1 enhanced IL-4 expression in a dose-dependent manner and also potentiated IL-4, IL-5, and IL-10 production from cells undergoing Th2 cell differentiation (Figure 4B). We also observed that Dkk-1 could induce IL-10 in the presence of TGF-β while inhibiting TGF-β-mediated Foxp3 expression (Figure 4C). Of note, we tested the dose-dependent response of Dkk-1 on Gata-3 expression and compared it with Wnt3a. Interestingly, up to 30 ng/ml of Dkk-1 could induce Gata-3 expression similar to Wnt3a; however, at higher amounts, Dkk-1 inhibited Gata-3 expression (Figure 4D). It has been shown that the canonical Wnt pathway transcription factors such as Lef and TCF-1 (also known as Tcf7) act as repressors of Gata-3 transcription in naive CD4⁺ T cells, and IL-4 inhibits TCF-1 expression (Hebenstreit et al., 2008; Maier et al., 2011). As previously reported, naive CD4⁺ T cell activation downregulated Lef and T cell factor-1

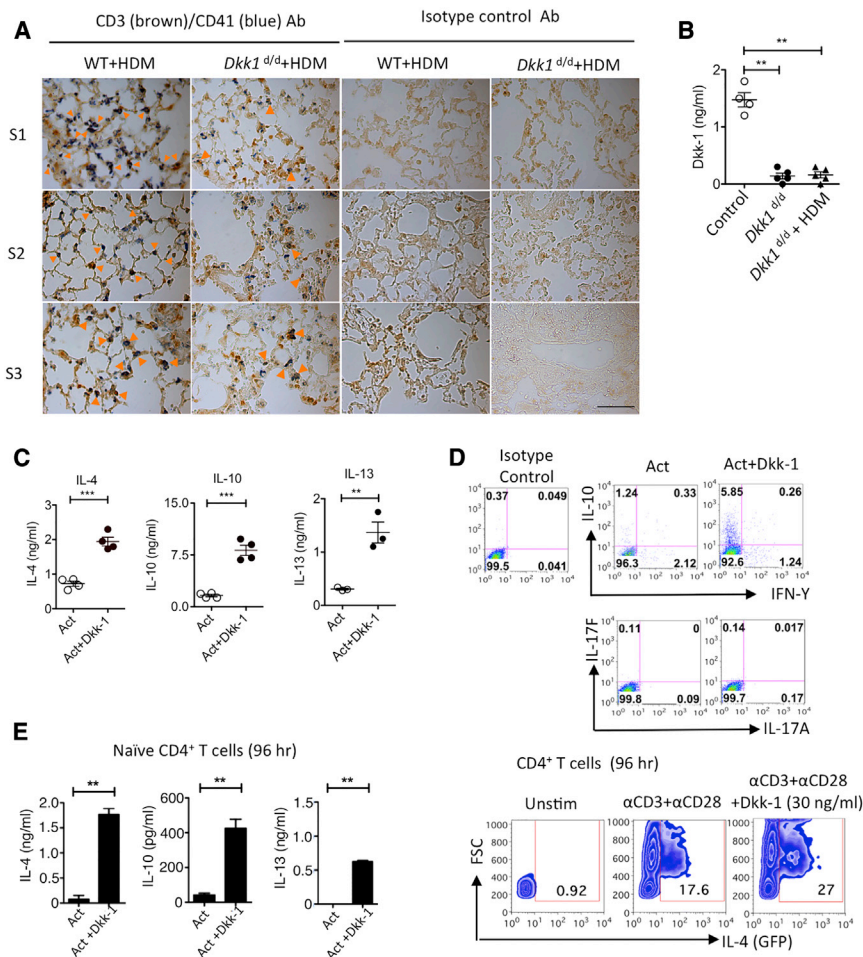


Figure 3. Dkk-1 Induces Th2 Cell Polarization

(A) *Dkk1*^{d/d} mice and their wild-type littermate controls were challenged with HDM extract allergen shown in Figure 1B. Mice were sacrificed and lungs were harvested for immunohistochemistry for CD3 (dark brown), CD41 (blue), and isotype control antibodies. Orange arrowheads in the images indicate co-localized CD3⁺ T cells with CD41⁺ platelets. S1, S2, and S3 indicate individual animals in each group. Original magnification is 63 $\times$. Scale bar in lower right panel represents 10 μ m.

(B) *Dkk1*^{d/d} mice ($n = 5$) were challenged with 50 μ g HDM extract per mouse and plasma samples were collected 24 hr later for ELISA.

(C and D) Splenic CD4⁺ T cells from 6- to 8-week-old C57BL/6 mice were activated with anti-CD3 and anti-CD28 antibodies (Act) with or without Dkk-1 (30 ng/ml unless indicated) for 4 days. Supernatants were analyzed by ELISA (C) and flow cytometry (D). For IL-4 in (D), splenic CD4 T cells from 8-week-old IL-4-GFP reporter mice were used.

(E) Naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 96 hr with or without Dkk-1 for flow cytometry analysis and ELISA.

Small horizontal lines and error bars indicate the mean ($\pm$ SEM). A representative of three independent experiments is shown. Student's t test was performed. *** $p < 0.0001$, ** $p < 0.005$, * $p < 0.05$.

T cell population in *Dkk1*^{d/d} mice was not related to a decreased percentage of CD4⁺ T cells in the medLNs (Figure 5A, right).

(TCF-1) expression. Dkk-1 did not induce TCF-1 or Lef expression (Figure 4E). Collectively, these results show that Dkk-1 induces Th2 cell polarization and potentiates Th2-cell-type cytokine expression.

Dkk-1 Utilizes SGK-1 and p38 MAPK to Potentiate Th2 Cell Cytokine Production

The Th2 cell differentiation program is mediated by Gata-3 and c-Maf (Farrar et al., 2001; Ho et al., 1996; Lee et al., 2001). Figure 4D shows that Dkk-1 could induce Gata-3 expression from naïve CD4⁺ T cells in a dose-dependent manner and hence Th2-cell-associated cytokines, so we further investigated how Dkk-1 potentiated Th2 cell cytokine production. Recent studies show that helper T cell differentiation signaling is mediated by mTOR (mammalian or mechanistic target of rapamycin) and MAPK signaling pathways in response to diverse extracellular cues including Wnts (Chi, 2012; Heikamp et al., 2014). In vivo, a CD4⁺ T cell population in medLNs that concomitantly expressed Th2 cell polarization transcription factors Gata-3 and c-Maf was significantly increased after ex vivo stimulation in WT mice after 2 weeks of recurrent allergen challenges. However, this population was only minimally present in *Dkk1*^{d/d} mice (Figure 5A), suggesting the role of Dkk-1 in inducing c-Maf. The decrease of Th2 cytokines and the Gata-3⁺c-Maf⁺CD4⁺

We observed that Dkk-1 increased the Gata-3⁺ and c-Maf⁺ CD4⁺ T cell population in vitro and increased the expression of IL-10 (Figure 5B). Blockade of IL-4R inhibited the expression of Gata-3 but not c-Maf, revealing that Dkk-1 can induce c-Maf independently of IL-4 signaling (Figure 5C). In the absence of Dkk-1, Th2 cell polarization with exogenous IL-4 was mainly induced by Gata-3, and both p38 MAPK and SGK-1 inhibition reduced Gata-3 expression (Figure 5D, top). The addition of Dkk-1 induced marked expression of both Gata-3 and c-Maf. The inhibition of p38 MAPK regulated c-Maf induction, whereas SGK (serum glucocorticoid kinase)-1 inhibition primarily reduced Gata-3 expression in c-Maf⁺Gata-3⁺ populations (Figure 5D, bottom). The marked potentiation of IL-10 secretion by Dkk-1 in Th2 cell polarization conditions was blocked either by SGK-1 or p38 MAPK inhibition, confirming that both kinases are required for Dkk-1-mediated Th2 cytokine secretion (Figure S3A). Although c-Maf has been known to be predominantly involved in IL-4 expression (Kim et al., 1999), our results suggest that Dkk-1 could potentiate other Th2-cell-type cytokines such as IL-10 production in a c-Maf-dependent manner. Based on high homology of the Dkk-1 amino acid sequence (>90%) between mice and humans, human CD4⁺ T cells were treated with mouse Dkk-1 with or without SGK-1 inhibitor. The inhibition of SGK-1 substantially reduced Gata-3 and IL-10 secretion

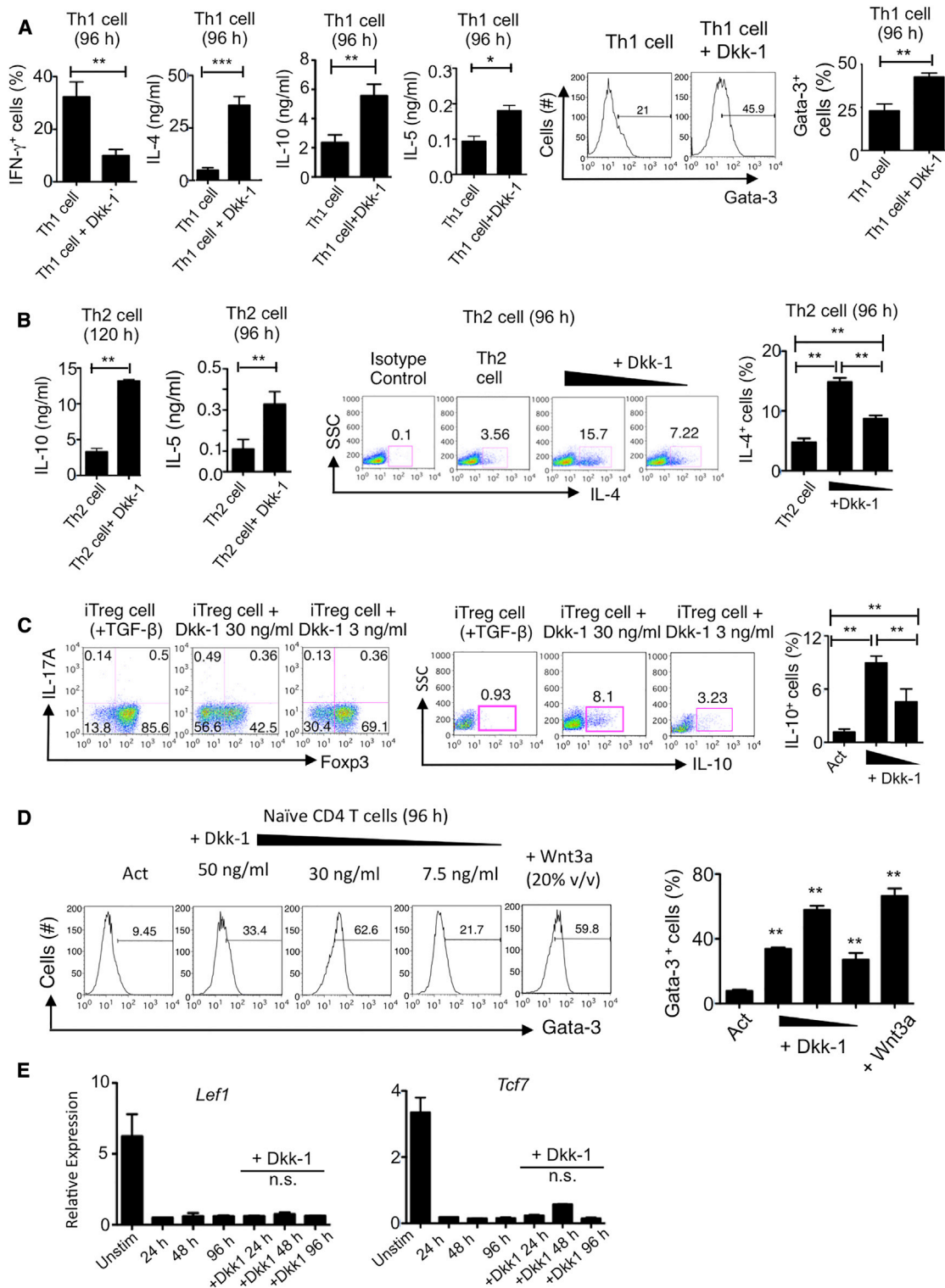


Figure 4. Dkk-1 Induces Th2 Cytokines in Various T Cell Differentiation Conditions

(A and B) Naïve CD4 T cells were stimulated under Th1 (A) or Th2 (B) polarization conditions for 96 hr with or without Dkk-1 for flow cytometry analysis and ELISA. (C) Splenic naïve CD4⁺ T cells were isolated and differentiated into iTreg cells for 5 days with 100 U/ml IL-2 and 1.5 ng/ml TGF- β with or without 30 ng/ml and 3 ng/ml of Dkk-1 was treated during the culture. Foxp3 and IL-17A expression (left) and IL-10 expression (right) were measured by flow cytometry. (D) Splenic naïve CD4⁺ T cells from 8-week-old C57BL/6 mice were treated with Dkk-1 with varying doses of Dkk-1 (50, 30, 7.5 ng/ml) or Wnt3a (20% v/v) for 96 hr in the presence of anti-CD3 and anti-CD28 antibody stimulation to measure Gata-3 expression amounts.

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(Figure 5E). Inhibition of SGK-1 expression by lentiviral shRNA transduction also diminished IL-10 induction by Dkk-1 in human CD4⁺ T cells (Figure S3B). Unlike the SGK-1 inhibition, the phosphorylation of S6 kinase or 4E-BP-1 was not altered by Dkk-1, indicating that the mTORC1 pathway was not affected by Dkk-1 (Figures S3C and S3D). In addition, we found that Stat6 was also required in Dkk-1-driven Th2 cell polarization (Figures S3E–S3G). These data, together with inhibition of Gata-3 expression (by IL-4R blocking antibody) demonstrated in Figure 4C, suggest that Dkk-1 promotes IL-4 induction and that naive CD4⁺ T-cell-derived IL-4 suppresses TCF-1 and/or Lef expression. Thus, our results show that Dkk-1 utilizes p38 MAPK and SGK-1 to potentiate Th2 cell polarization by increasing expression of c-Maf and Gata-3, respectively.

Dkk-1 Is Selectively Secreted from Platelets upon Allergen Challenge or Parasite Infection

Based on the pro-inflammatory effect of Dkk-1 in the HDM-induced asthma and the *L. major* infection model, we investigated the primary source of Dkk-1 in HDM-induced asthma. Initially, we found a marked increase of circulating amounts of Dkk-1 after recurrent allergen challenges (Figure 6A). We further tested whether a single intranasal allergen challenge could induce the elevation of circulating Dkk-1 and found that Dkk-1 was readily elevated in plasma of C57BL/6 mice (Figure 6B). This increase of Dkk-1 was also detected in the infiltrated lungs from WT littermate control mice although it was markedly reduced in *Dkk1*^{d/d} mice, suggesting that the elevation of Dkk-1 does occur both locally and systemically (Figure 6C). It has been implied that an increase of circulating Dkk-1 in multiple myeloma might be due to platelets (Trikalinos et al., 2009). While we were collecting plasma from C57BL/6 mice, we observed high Dkk-1 amounts even in the homeostatic conditions when samples were collected without anti-coagulant by cardiac puncture (Figure 6D). Because platelets sense mechanical shear stress by aggregating to form clots and release soluble mediators (Butler, 1995; McGrath et al., 2011), we questioned whether the elevated amounts of Dkk-1 are released from platelets. We depleted platelets in intact C57BL/6 mice and found that depletion of platelets abolished circulating amounts of Dkk-1 in 12 hr (Figure 6E). This marked reduction of Dkk-1 by platelet depletion was also observed with HDM allergen challenge (as in Figure 1B), suggesting that the primary circulating source of Dkk-1 in during both homeostatic and allergen-challenged conditions is platelets (Figure 6F). Next, we investigated whether the elevation of Dkk-1 by HDM is a specific response to the allergen. To this end, we challenged various types of compounds known as pathogen-associated molecular patterns (PAMPs) intranasally. Unlike the PAMPs that were tested, HDM could uniquely increase Dkk-1 in circulating blood (Figure 6G).

We further checked whether systemic concentrations of Dkk-1 were increased by different types of environmental pathogens via skin to promote Th2 cell responses. Subcutaneous infection

of the parasite *L. major* in the hindfoot of BALB/c mice increased circulating amounts of Dkk-1 that persisted 42 days after infection (Figure 6H). Upon parasitic infection, depletion of platelets using platelet-specific anti-mouse GPIIb/IIIa (CD42b) antibody markedly ablated elevation of circulating Dkk-1 amounts, even below homeostatic amounts of Dkk-1 in 72 hr (Figure 6I). At 10 weeks after infection, we showed that Dkk-1 is still maintained at a high concentration, and platelet depletion resulted in the complete loss of Dkk-1 in 12 hr, suggesting that platelets are continuously secreting Dkk-1 (Figure 6J). Activation of human platelets with sLMAG induced Dkk-1 within 1 hr in a dose-dependent manner (Figure 6K). Inhibition of PKC α (Figure 6L) blocked secretion of Dkk-1 upon sLMAG stimulation of human platelets, confirming that activated platelets secrete Dkk-1 from α -granules (Moncada de la Rosa et al., 2013). In contrast, stimulation of human peripheral blood mononuclear cells (PBMCs) or vascular endothelial cells with sLMAG or HDM extract failed to induce secretion of Dkk-1 (Figure S4). Taken together, our results suggest that Dkk-1 is primarily secreted from platelets upon allergen challenge or non-healing parasite infection, promoting type 2 cell-mediated immune responses.

Dkk-1 Facilitates Leukocyte Infiltration and Plays an Important Role in Chronic Type 2 Cell-Mediated Inflammation

Given that the source of circulating Dkk-1 was platelets and that *Dkk1*^{d/d} mice showed reduced leukocyte infiltration in the lung, we considered the possibility that Dkk-1 might regulate leukocyte-platelet interactions and leukocyte migration into the lung upon HDM extract challenge. LPA (leukocyte-platelet aggregate) formation in human and WT mouse peripheral blood has been considered a prerequisite for the increases of infiltrating immune cells to the affected tissues to develop immune responses (Ley et al., 2007; Li et al., 1997; Pitchford et al., 2005; Tamagawa-Mineoka et al., 2007; Zarbock et al., 2006). We observed that infiltration of CD45⁺ immune cells into the lung was significantly increased in WT mice but that this was impaired in *Dkk1*^{d/d} mice at 72 hr after a single allergen challenge (Figure 7A). Consistent with the marked decrease of leukocyte migration into the lung, a single challenge of allergen induced elevation of LPA (CD45⁺CD41⁺ population) in the peripheral blood of WT mice at 4 and 24 hr from the steady-state amounts but not in *Dkk1*^{d/d} mice (Figures 7B, S5A, and S5B). Pretreatment with Dkk-1 inhibitor 24 hr prior to infection with the parasite *L. major* similarly reduced the elevation of LPA formation at 4 hr after infection (Figure S5C). We further tested whether exogenous Dkk-1 could induce LPA formation and increase leukocyte infiltration in the lung. Exogenous administration of Dkk-1 did not increase the number of infiltrated leukocytes in the lung 72 hr after Dkk-1 protein administration (Figure 7C) although increased LPA formation was observed without allergen or parasite challenge (Figure S5D). Combined with results from Figure 7A, this suggests that infiltration of leukocytes to the lung requires both

(E) Splenic naive CD4⁺ T cells from 8-week-old C57BL/6 mice were stimulated with or without Dkk-1 (30 ng/ml) for 96 hr. RNA was harvested and analyzed by real-time qPCR.

All results were statistically non-significant. All experiments are representative of two to three independent experiments. For (A), Student's t test was performed. For (B)–(E), one-way ANOVA analysis with Bonferroni's post hoc test was performed. Small horizontal lines and error bars indicate the mean ($\pm$ SEM). ***p < 0.0001, **p < 0.005, *p < 0.05.

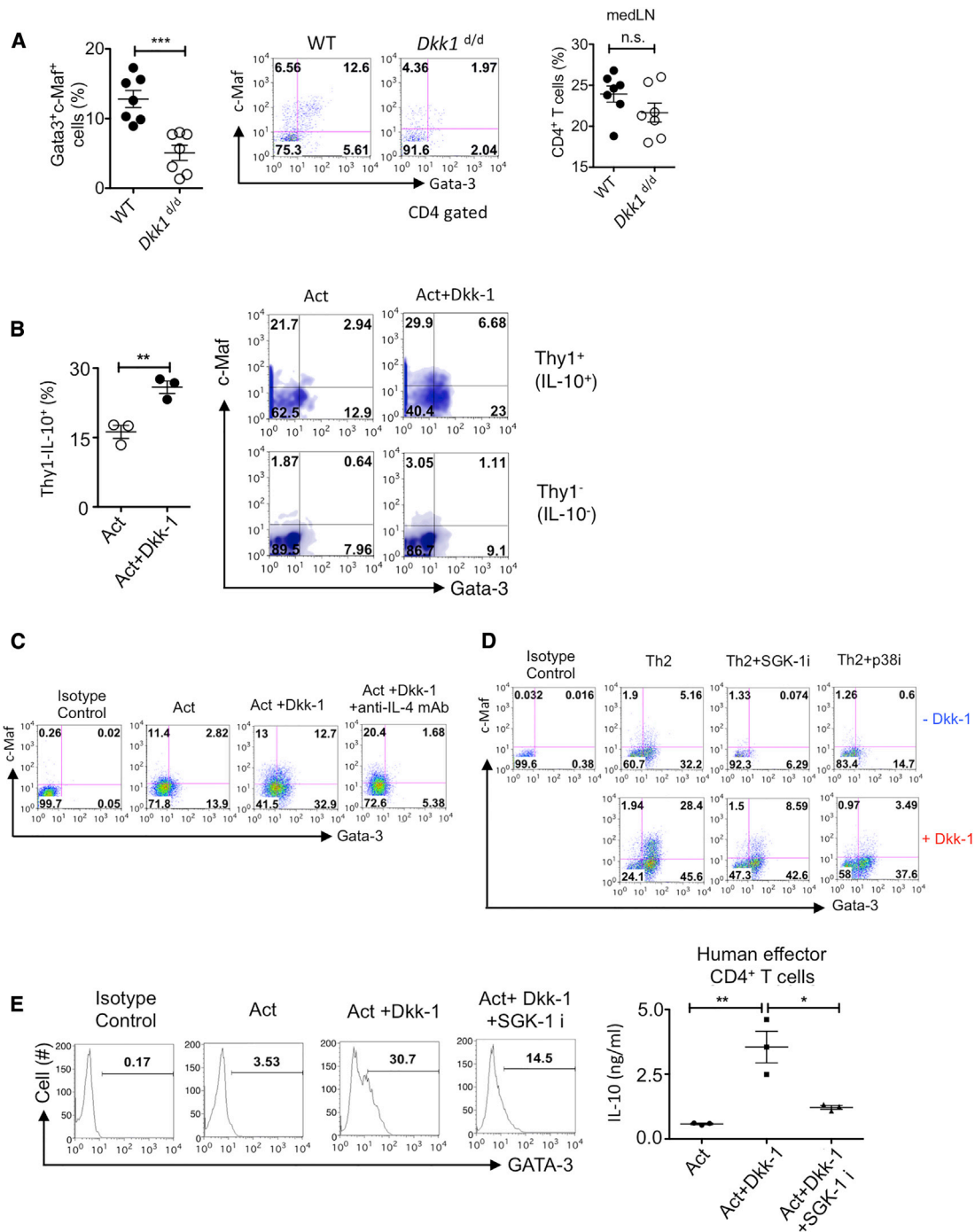


Figure 5. Dkk-1 Directs Th2 Cell Polarization by p38 MAPK and SGK-1 to Induce c-Maf and Gata-3

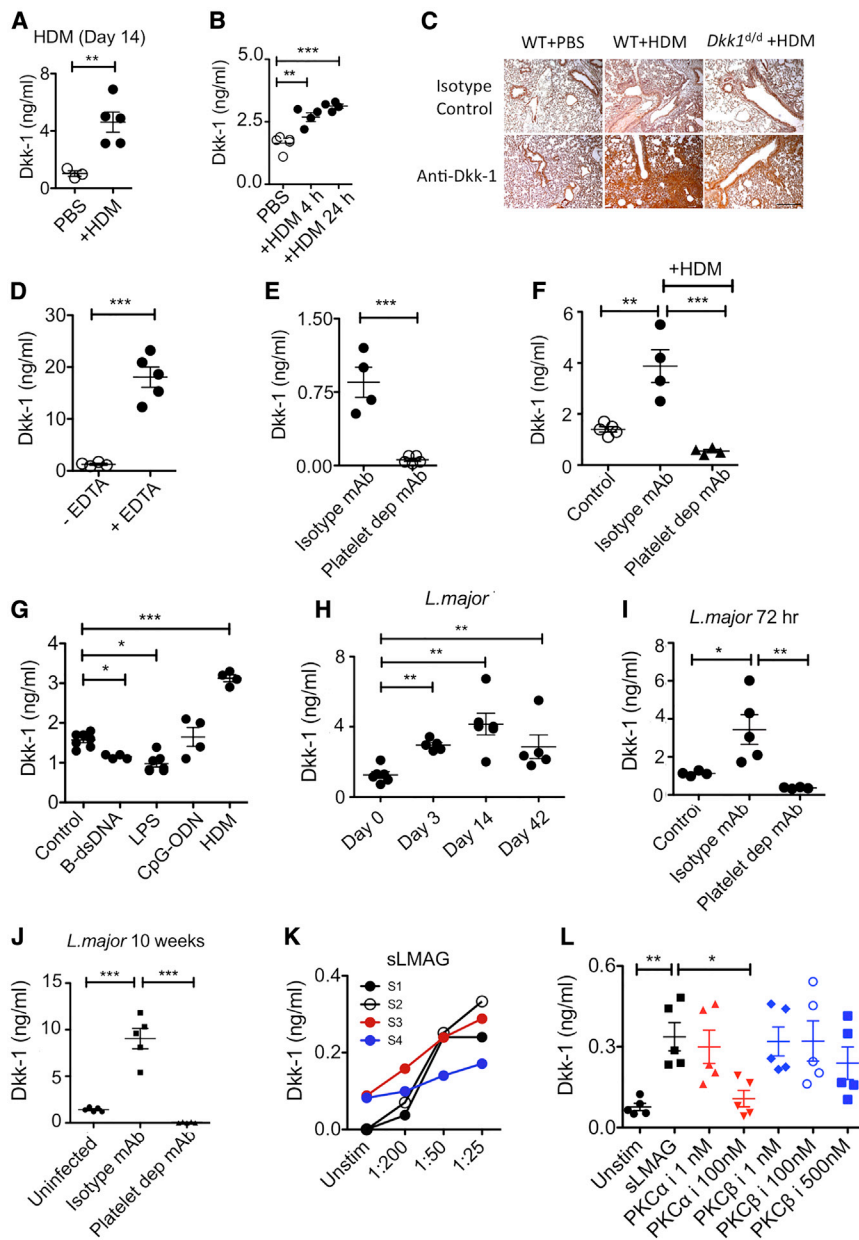
(A) Mediastinal lymph node cells from Figure 1D were stimulated with HDM extract for 4 days and the percentages of Gata3⁺ and c-Maf⁺ in CD4⁺ T cells were quantitated by flow cytometry.

(B) Splenic CD4⁺ T cells from 12-week-old Thy1-IL-10 reporter mice were activated with or without Dkk-1 (30 ng/ml) for 84 hr. Cells were gated for Thy1⁺ and Thy1⁻. (C) Splenic CD4⁺ T cells were activated with or without Dkk-1 or anti-IL-4 mAb (10 μ g/ml) for 4 days and analyzed by flow cytometry.

(D) Naive CD4⁺ T cells were stimulated under Th2 cell polarization conditions for 96 hr with or without Dkk-1 for flow cytometry analysis. SGK-1 inhibitor (2 μ M) or p38 inhibitor (10 μ M) was added at 0 hr.

(E) PBMCs from healthy volunteers were used to isolate human CD4⁺ T cells and then stimulated with human anti-CD3 mAb (OKT3) and anti-CD28 mAb for 4 days with or without Dkk-1 (30 ng/ml) and SGK-1 inhibitor GSK653094 (2 μ M). Cells were analyzed by flow cytometry. Supernatants were harvested and analyzed by ELISA (right).

A representative of three independent experiments is shown. Student's t test was performed. *** p < 0.0001, ** p < 0.005, * p < 0.05, n.s., not significant. For (E), one-way ANOVA analysis with Bonferroni's post hoc test was performed. See also Figure S3.



A representative of two independent experiments is shown. Small horizontal lines indicate the mean ($\pm$ SEM). Student's *t* test (A, D, E), one-way ANOVA with Dunnett's post hoc test (B, I), and one-way ANOVA with Bonferroni's post hoc test (F–H, J, L) was performed. ****p* < 0.0005, ***p* < 0.005, **p* < 0.05. See also Figure S4.

Dkk-1 and further innate immune responses for leukocyte migration upon allergen challenge. In addition, in contrast to WT mice, we observed that leukocytes from peripheral blood in *Dkk1*^{d/d} mice failed to sustain the elevation of PSGL-1 expression and its ligand P-selectin on platelets upon HDM allergen challenges. Similarly, inhibition of Dkk-1 in *L. major* infection blocked the increase of PSGL-1 expression (Figures S5E–S5G).

Next, we evaluated the importance of Dkk-1 either at the challenge stage or at the sensitization stage in the HDM-induced asthma model. To this end, we treated Dkk-1 inhibitor either at the challenge stage (ET, early treatment) or at the sensitization stage (LT, late treatment) (Figure 7D). Leukocyte infiltration

including CD4⁺ T cells, neutrophils, and eosinophils was all markedly decreased in BAL fluid and lung tissues in both the ET and LT treatment protocols (Figures 7E, 7F, and S6A). The number of CD4⁺ T cells was also reduced in mediastinal lymph nodes by inhibiting Dkk-1, but the percentage of CD4⁺ T cells in the lymph nodes did not change (Figures 7G and S6B). Th2 cytokine secretion from mediastinal lymph nodes was diminished (Figure 7H). Consistent with these results, histopathological scores also showed that the functional inhibition of Dkk-1 in either the challenge stage or the sensitization stage resulted in inhibition of type 2 cell inflammatory responses in HDM-induced asthma model (Figures 7I, 7J, and S6C). Taken together,

Figure 6. Circulating Dkk-1 Is from Platelets upon Allergen Challenge or Parasitic Infection

(A) Female C57BL/6 mice were challenged with PBS (*n* = 3) or HDM extract (10 μ g/challenge/mouse, *n* = 5) as in Figure 1B.

(B) 8-week-old female C57BL/6 mice were challenged with HDM extract (30 μ g/challenge, *n* = 4) or PBS (*n* = 5) for 4 and 24 hr. Plasma samples were analyzed by ELISA.

(C) *Dkk1*^{d/d} mice (*n* = 7) and their wild-type littermate controls (*n* = 7) were challenged with HDM extract as in Figure 1B. Harvested lungs were analyzed by immunohistochemistry. Original magnification is 10 $\times$. Scale bar in lower right panel represents 10 μ m.

(D) Peripheral blood was collected by cardiac puncture with or without EDTA.

(E) 8-week-old female C57BL/6 mice were injected with platelet depletion antibody (*n* = 5) or isotype control antibody (*n* = 4). 24 hr later, plasma samples were measured.

(F) HDM allergen was challenged as in Figure 1B, and platelets were depleted for 12 hr.

(G) Bacterial dsDNA from *E. coli* (B-ds DNA) (30 μ g/mouse, *n* = 4), LPS (3 μ g/mouse, *n* = 5), CpG-ODN 1585 (25 μ g/mouse, *n* = 4), and HDM allergen extract (50 μ g/mouse, *n* = 4) were challenged intranasally in 6-week-old C57BL/6 mice. 24 hr later, plasma samples were collected and Dkk1 concentrations were determined by ELISA. Control (*n* = 7) mice were given 20 μ l of 0.9% NaCl saline.

(H) At each time point, plasma from female BALB/c mice (6 weeks old) that were infected with *L. major* was measured.

(I) Platelets were depleted 4 hr prior to parasitic infection. Plasma samples from 5-week-old female BALB/c mice were analyzed 72 hr after infection.

(J) 10 weeks after infection, platelets were depleted for 12 hr in BALB/c mice.

(K) Human platelets from four healthy volunteers were activated with sLMAG for 1 hr. S1–S4 designates healthy donors. x axis shows dilution of sLMAG in the culture. 1:50 is equivalent to 1 $\times$ 10⁶ parasites.

(L) Human platelets (1 $\times$ 10⁶/ml, *n* = 5) were activated with sLMAG (1:50) in the presence of PKC α inhibitor or PKC β inhibitor for 1 hr.

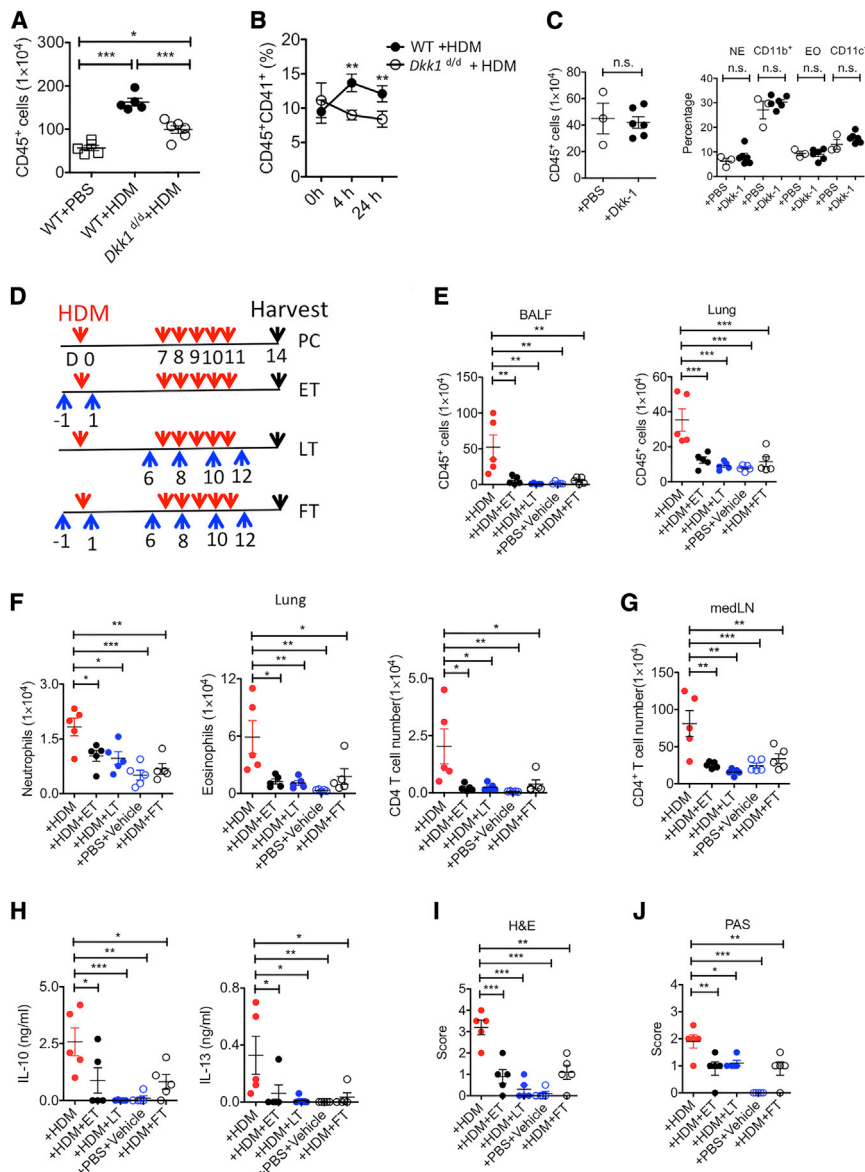


Figure 7. Dkk-1 Facilitates Leukocyte Migration and Regulates Leukocyte-Platelet Aggregate Formation in Type 2 Cell-Mediated Inflammation

(A) 9-week-old *Dkk1*^{d/d} mice and wild-type littermate controls ($n = 5$ –6/group) were challenged with 30 μ g HDM allergen extract intranasally. Lung homogenates were quantitated and analyzed by flow cytometry 72 hr after HDM challenge and CD45⁺ cells were analyzed by flow cytometry.

(B) Peripheral blood from 9-week-old *Dkk1*^{d/d} mice and wild-type littermate controls were collected at 4 and 24 hr after allergen challenge and analyzed for the percentages of CD45⁺CD41⁺ cells.

(C) Dkk-1 protein (300 ng) was injected intraperitoneally. CD45⁺ cells were analyzed by flow cytometry. Abbreviations are as follows: NE, neutrophils; EO, eosinophils.

(D) Schematic diagram of Dkk-1 inhibitor treatment protocol for each group ($n = 5$ for each group) in HDM-induced asthma model. HDM allergen extract (10 μ g) was challenged intranasally at the indicated time points.

(E–J) CD45⁺ leukocytes numbers in BALF and lung (E), neutrophils, eosinophils, and CD4⁺ T cell numbers in the lungs (F), and the number of CD4⁺ T cells in mediastinal LNs were counted (G). After 4 days of stimulation of medLN cells, cytokines were measured by ELISA (H). H&E (I) and PAS (J) staining were scored. Abbreviations are as follows: ET, early treatment; LT, late treatment; FT, full treatment; PC, positive control.

Small horizontal lines and error bars indicate the mean ($\pm$ SEM). A representative of two independent experiments is shown. One-way ANOVA analysis with Dunnett's post hoc test was performed. *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$, n.s., not significant. See also Figures S5 and S6.

our results suggest that Dkk-1 plays an important proinflammatory role for Th2 cell differentiation and leukocyte migration in each stage of HDM allergen challenge to promote chronic type 2 cell-mediated inflammation.

DISCUSSION

The increased amounts of Dkk-1 in peripheral blood have been reported in chronic inflammatory diseases such as various types of cancers, rheumatoid arthritis, and lupus (Diarra et al., 2007; Sato et al., 2010; Wang et al., 2014), and the role of Dkk-1 has been studied as a Wnt antagonist in various model systems. Our results demonstrate an important immunomodulatory role of Dkk-1 in type 2 cell-mediated immune responses caused by environmental challenges.

Although multiple roles of the canonical Wnt signaling pathway have been studied in the context of intracellular mediators such

and mTOR signaling pathway components to induce type 2 cell-mediated immune responses and/or inflammation upon allergen or non-healing parasite infection.

A previous study has addressed the role of the canonical Wnt pathway that is mediated by β -catenin in Th2 cell differentiation (Notani et al., 2010), focusing on early progenitor T cells (e.g., thymocytes and naive CD4 T cells) in vitro. The prediction based on these in vitro studies utilizing high concentrations of Dkk-1 or Wnt3a would have been inhibition of Th2 cell differentiation by Dkk-1 and hence the inhibition of HDM-induced asthma or *L. major* infection. However, we have shown that the function of Dkk-1 in vivo at physiological concentrations is, in fact, the opposite and that Dkk-1 potentiates Th2 cell polarization. Physiologic concentrations of Dkk-1 (up to 30 ng/ml) in fact promote type 2 cell-mediated inflammation and Th2 cell differentiation primarily through SGK-1 and p38 MAPK. Of note, we also observed that Wnt3a induced Gata-3 expression and that

non-physiologic high concentrations of Dkk-1 (>50 ng/ml) inhibited Gata-3 expression similar to the previous report. There are signaling pathways identified which Dkk-1 utilizes other than the well-known canonical Wnt pathway (Fukuda et al., 2010; Krause et al., 2014). It is notable that Dkk-1 has a colipase domain in its carboxy-terminal region that is responsible for Wnt antagonist activity, but the function and role of the N-terminal domain of Dkk-1 is largely unknown (Brott and Sokol, 2002). More detailed characterization of Dkk-1 molecular structure affecting Th2 cell differentiation is warranted in the future. Collectively, our findings suggest that physiological concentrations of Dkk-1 utilized SGK-1 and p38 MAPK under type 2 cell-mediated immune responses or inflammation.

Recent studies point out the specificity of platelet activation and its immunomodulatory role in addition to its traditional role in coagulation events and hemostasis (Rondina and Garraud, 2014). Platelets do express a variety of receptors for PAMPs, and recent studies identify more receptors and ligands that are expressed in immune cells (Semple et al., 2011). We demonstrated that platelets are an important source of Dkk-1 in our models and that the release of Dkk-1 is specific to environmental challenges that lead to chronic type 2 cell-mediated immune responses. Platelets are the most abundant source of TGF- β 1 (Kim et al., 2013). Although the release of TGF- β 1 could be assumed in platelet activation and thus further assumed to increase Foxp3⁺CD4⁺ T cells, it should be noted that platelets also possess ligands such as PF4 (CXCL4) that is abundantly present and can inhibit TGF- β 1-mediated T cell differentiation (Shi et al., 2014). The inhibition of TGF- β 1-mediated Foxp3 expression by Dkk-1 suggests that the immunosuppressive function of TGF- β in a given microenvironment would be potently inhibited, and hence Dkk-1 would be able to generate robust Th2 cytokines. Together with the inhibitory role of Dkk-1 on TGF- β 1-mediated Foxp3⁺ T cell differentiation, it is unlikely that TGF- β 1 would affect T cell differentiation in our models. Additionally, we did not observe any increased Foxp3⁺ T cells in *doubleridge* mice, confirming that platelet activation and subsequent Dkk-1 release from platelets is specific to a given environmental challenge.

Our findings might suggest that immunomodulation of Dkk-1 from platelets is an example of the development of the mammalian immune system from the hemocyte system that exists in *Drosophila* (Lavine and Strand, 2002; Wood and Jacinto, 2007). Our results from exogenous Dkk-1 injection and HDM-induced asthma and *Leishmania major* infection models suggest that platelet activation and subsequent release of Dkk-1 facilitates leukocyte migration to the site of environmental challenges, and this should be coordinated with the recognition of such challenges by innate immune cells. Because hemocytes play roles of both platelets and innate immune cells, our results might show the specialized role of platelets and leukocytes to coordinate type 2 cell-mediated immune responses in mammals. It is notable that the potent induction of IL-10 by Dkk-1 in the *L. major* infection model demonstrates that the parasite utilizes Dkk-1 as a strategy to reconcile immune activation and immunosuppression to favor parasite survival, avoiding complete eradication and maintaining lesions.

Taken together, our findings highlight the importance of the orchestrating role of Dkk-1 from platelets to facilitate leukocyte migration and further to polarize immune responses by inducing

Th2 cell polarization, placing Dkk-1 as a highly attractive target for controlling type 2 cell-mediated immune responses.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J mice were purchased from the Jackson Laboratory and have been bred in our mouse facility. BALB/c mice were purchased from NCI. The animals were kept under normal light and dark cycle (12/12). *Doubleridge* mice (*Dkk1*^{del/d}) were kindly provided by Asma Nusrat (Emory University). Foxp3-IRES-RFP mice and Stat6-deficient mice were bred in our mouse facility. Thy1-IRES-IL-10 reporter mice were provided by Casey Weaver (University of Alabama). IL-4-GFP reporter (4Get) mice on the BALB/c background were kindly provided by Ruslan Medzhitov (Yale University). All mouse protocols were approved by the Yale University Institutional Animal Care and Use Committee (IACUC) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Flow Cytometry, Cell Culture, Cytokine ELISA, and Immunohistochemistry

For CD4⁺ T cell differentiation, naive CD4⁺ T cells (CD4⁺CD62L^{hi}CD44^{lo} Foxp3(RFP)-) were isolated from 6- to 7-week-old Foxp3-IRES-RFP mice. For Th1 cell differentiation, cells were activated with plate-bound anti-CD3 (2 μ g/ml), anti-CD28 (2 μ g/ml) in the presence of IL-2 (25 U/ml), and IL-12 (20 ng/ml) for 96 hr in RPMI-1640 media (5% FBS, 1% penicillin/streptomycin, and 10 mM HEPES). For Th2 cell differentiation, cells were activated with plate bound anti-CD3 (2 μ g/ml), anti-CD28 (2 μ g/ml) in the presence of IL-2 (25 U/ml), anti-IFN- γ (XMG1.2) mAb (10 μ g/ml), and IL-4 (100 U/ml) for 96 hr. For the isolation of CD4⁺ T cells, spleens from 6- to 7-week-old male Foxp3-IRES-RFP mice were prepared as single-cell suspensions. After RBC (red blood cell) lysis, cells were stained with CD16/CD32 FcR (Fc Receptor) blocking antibody (clone 2.4G2). For intracellular staining, cells were stimulated with cell stimulation cocktail with protein transport inhibitors (eBioscience) for 5 hr. Intracellular staining was performed as described in the manufacturer's protocol (eBioscience). FACS Calibur or Stratedigm (BD Biosciences) was used for flow cytometry and data were analyzed by FlowJo software (Treestar). Human and mouse Dkk-1 ELISA kits were purchased from R&D Systems. The human IL-10 ELISA, mouse Th1 and Th2 cytokine panel ELISA kit, and mouse IL-5/IL-13 ELISA kit were purchased from eBioscience. Cytokines were measured by ELISA according to the manufacturer's protocol.

House Dust Mite Asthma Model

House dust mite extract (*Dermatophagoides pteronyssinus*; Greer Labs) (10 μ g) was dissolved in 20 μ l of PBS for each mouse per intranasal challenge. 6- to 10-week-old *doubleridge* mice and their littermate controls were challenged intranasally on days 0, 7, 8, 9, 10, and 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. For histological analysis, lungs were fixed with 10% formalin at least for 24 hr, embedded in paraffin, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) reagent and were assigned scores by established methods. For lymphocyte-platelet aggregation assay, 50 μ g of HDM extract was dissolved in 25 μ l PBS and then intranasal challenge was performed. Lymphocyte-platelet aggregation was measured at 4 and 24 hr after the challenge. At 72 hr, lung homogenate was prepared by collagenase digestion. Cells were counted and lymphocytes were analyzed by flow cytometry. For more details, see [Supplemental Experimental Procedures](#).

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 μ l DMSO) was injected on days -1, +1, +3, +5, +7, +9, +11, and

weekly afterward until harvest. Draining lymph nodes, non-draining lymph nodes, and plasma were collected. For ex vivo analysis, soluble *L. major* antigen (sLMAG) was employed. For depleting platelets, 80 μ g of anti-CD42 mAb (Emfret Analytical) was injected intravenously in 100 μ l PBS. For more details, see [Supplemental Experimental Procedures](#).

Human or Mouse Platelet Isolation and Functional Characterization and Lymphocyte-Platelet Aggregate Assay

Human platelets were isolated as described from healthy volunteers (Tang et al., 2011). All procedures were in accordance with HIC protocol (protocol # 1005006865). All volunteers consented to donate their blood. In brief, 3.2% citrate buffer was used to prevent coagulation. Blood was centrifuged at 250 $\times$ 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 and resuspended in Tyrode's buffer. 100 million platelets/ml were used for activation. House dust mite extract (50 μ g) was 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 at indicated ratio for 1 hr. Platelets were resuspended in Tyrode's buffer. Supernatant was collected for human Dkk-1 ELISA. For details, see [Supplemental Experimental Procedures](#).

Statistical Significance

Statistically significant differences were determined by one-way ANOVA with Bonferroni's post hoc test, Dunnett's post hoc test, or Student's t test with Graph Pad Prism software (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.01.008>.

AUTHOR CONTRIBUTIONS

W.-J.C., A.K.E., D.M.-P., and K.G.-P. performed *L. major* experiments and analyzed results. W.-J.C. and P.Y.C. performed HDM asthma experiments and analyzed results. W.-J.C. and A.M.T. performed mouse platelet experiments. L.H. scored histology samples. W.H.T., J.H., D.S.K., and A.M.T. contributed experimental advice for human platelet experiments. P.S. and P.J.L. advised and helped with the Flexivent assay. J.H.S. performed immunohistochemistry. S.-T.K. and J.-H.P. assisted with in vitro experiments. S.E.M. gave technical help. C.V.R. provided experimental materials and advised the writing of the manuscript. D.M.-P. conceived the *Leishmania* study, provided experimental materials, and wrote the paper. O.H. analyzed results and provided experimental advice with discussion. W.-J.C. and A.L.M.B. conceived all experimental studies and wrote the paper.

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Supplemental Information

The Wnt Antagonist Dickkopf-1 Promotes

Pathological Type 2 Cell-Mediated Inflammation

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Figure S1

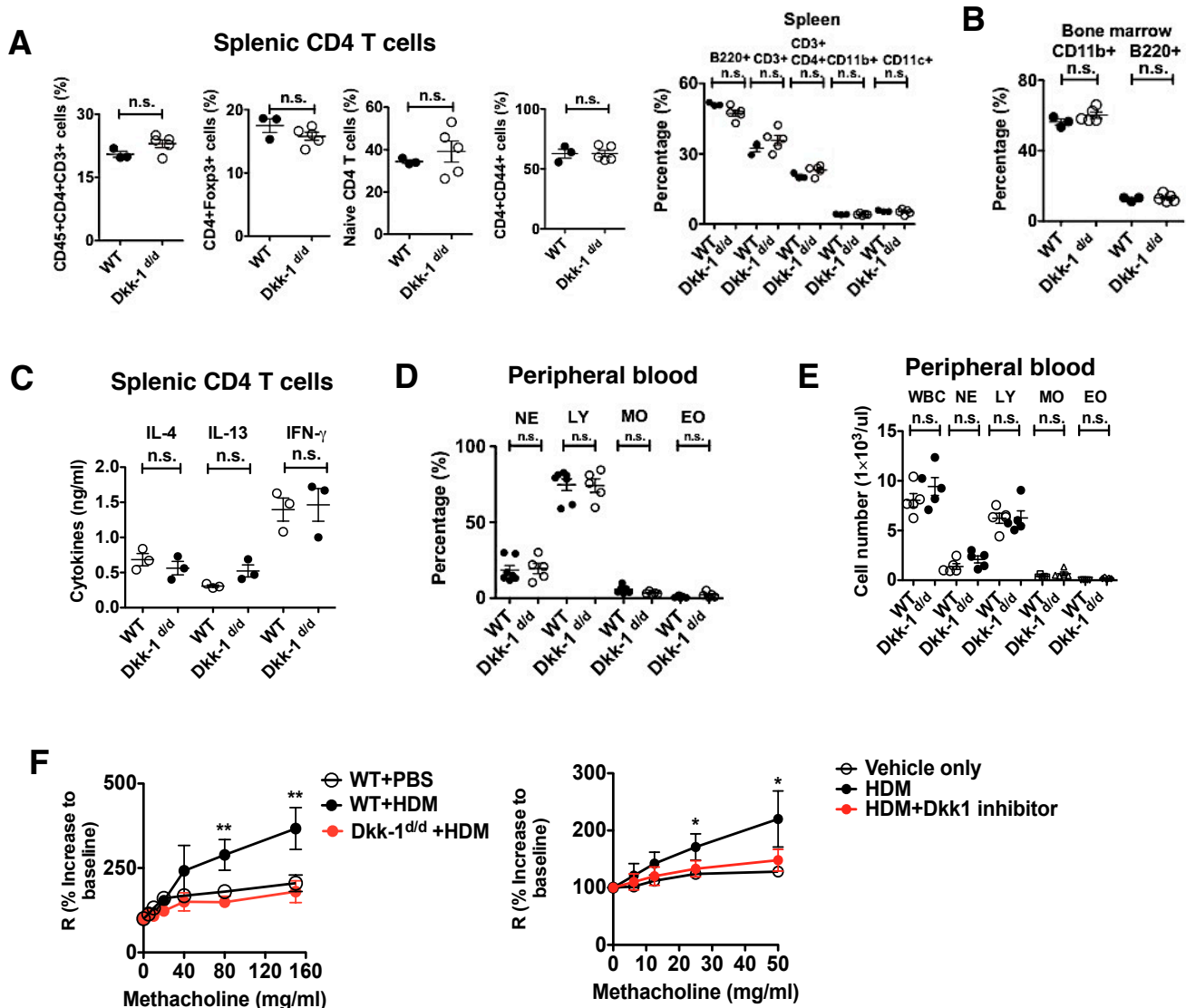


Figure S1, related to Figure 1. Characterization of Dkk-1^{d/d} mice (A, B) Six to ten week-old Dkk-1^{d/d} mice (n=5) and their wildtype littermate controls (n=3) were used. Single cell suspensions from spleen and bone marrow (B) were prepared and analyzed by flow cytometry. (C) Splenic CD4 T cells were isolated by MACS and stimulated with α CD3 and α CD28 mAb (2 μ g/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 μ g 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 ($\pm$ s.e.m.). *, p< 0.05

Figure S2

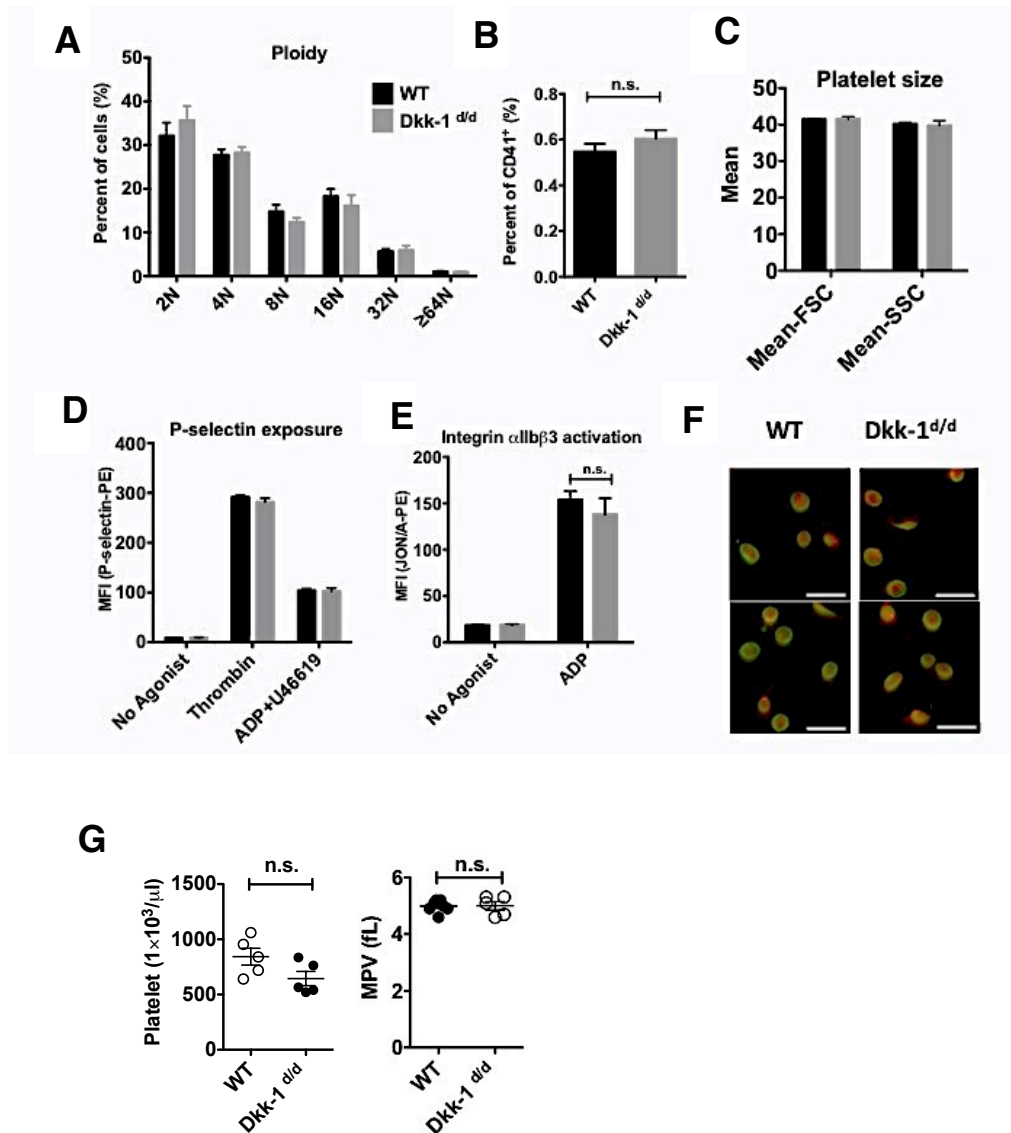


Figure S2, related to Figure 1. Dkk-1^{d/d} mice have normal megakaryocyte ploidy, platelet size and platelet activation Six to ten week-old Dkk-1^{d/d} mice (n=3) and their wildtype littermate controls (n=3) were used. **(A)** Ploidy profile of CD41⁺ bone marrow cells assessed by propidium iodide staining (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 α IIb β 3 receptor in response to ADP **(E)** at comparable levels to WT (n=3 per genotype). **(F)** Platelet cytoskeletal architecture assessed by b-tubulin and Phalloidin (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 ($\pm$ s.e.m.). A representative of two independent experiments are shown.

Figure S3

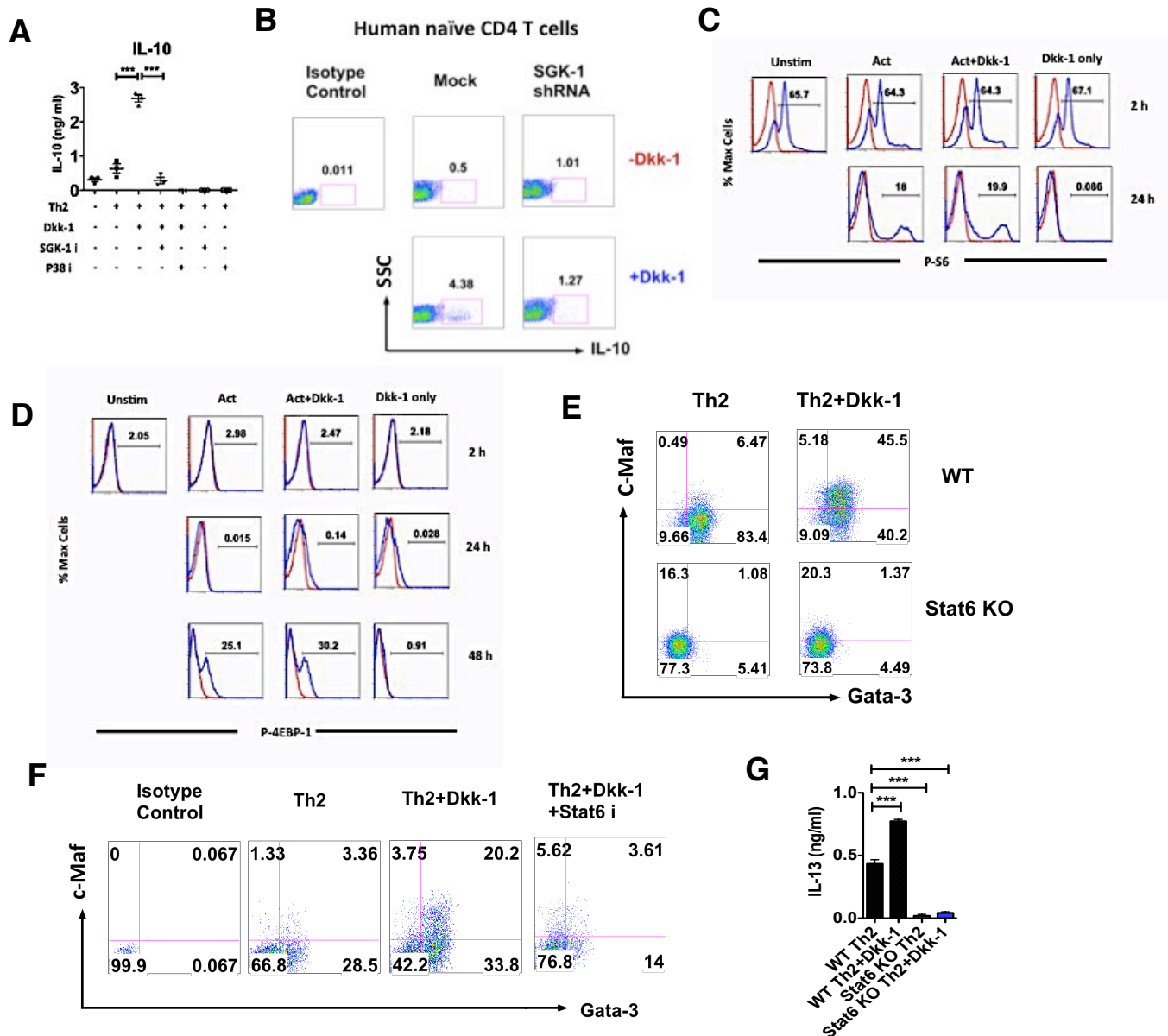


Figure S3, related to Figure 5. Dkk-1 induces IL-10 in CD4 T cells via SGK-1 and p38 MAPK

(A) Naïve CD4 T cells were differentiated into Th₂ cells with or without Dkk-1 (30 ng/ml), SGK-1 inhibitor (2 μ M) or (B) p38 inhibitor (10 μ M) 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 μ g/ml) for 48 hr. The selected cells were then stimulated with α CD3 mAb (OKT3) and α CD28 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 ($\pm$ s.e.m.). (C, D) Splenic CD4⁺CD25⁻ cells from 6 week-old C57BL/6 mice were isolated and activated with α CD3 and α CD28 mAb (2 μ g/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 Dunnett's post-hoc test was performed. ***, $p < 0.0005$. A representative of two independent experiments is shown.

Figure S4

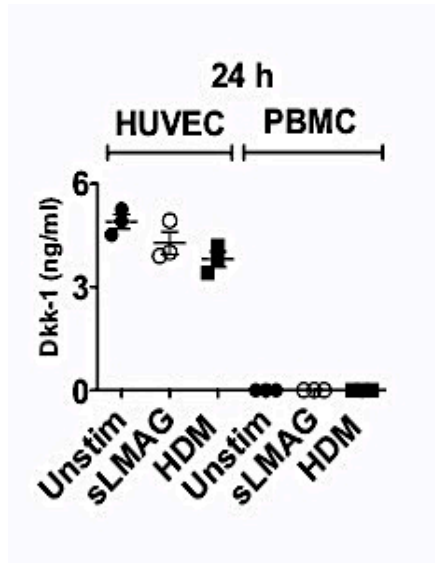


Figure S4, related to Figure 6. Dkk-1 is not induced by sLMAG or HDM in HUVECs or PBMCs.

HUVECs (2×10^5 /ml) (n=2) or human PBMCs (n=3) (2×10^6 /ml) were stimulated with either HDM extract (15 μ g/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

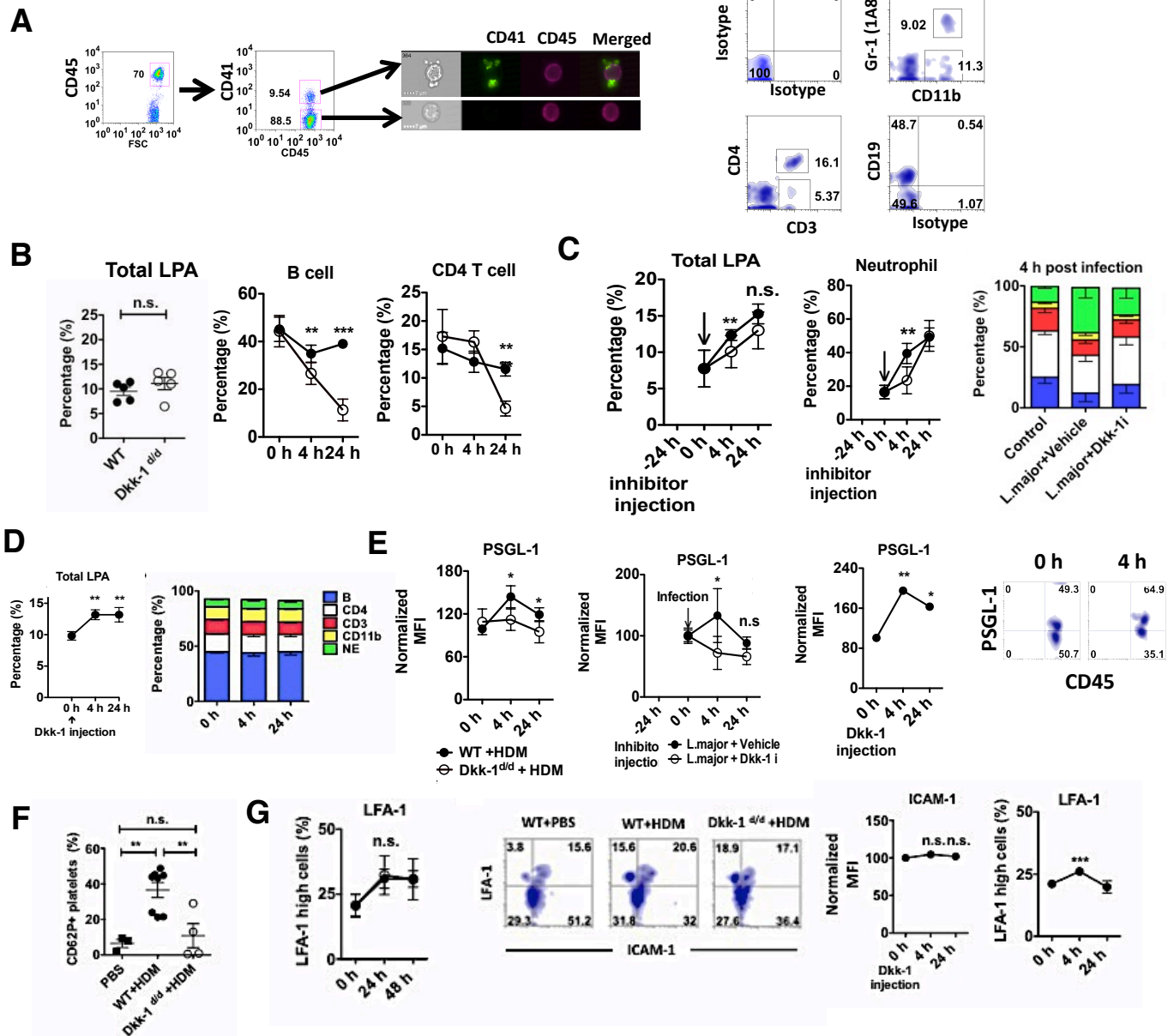


Figure S5, related to Figure 7. Regulation of LPA formation by Dkk-1 (A) Peripheral blood from 6-week old C57BL/6 male mice was analyzed by AMNIS. (B) (Left panel) The basal LPA population (CD45⁺CD41⁺) was measured in Dkk-1^{d/d} mice (n=5) and their wildtype littermate controls (n=5). (Right panels) Dkk-1^{d/d} mice and their littermate controls (n=5) were challenged with 30 μ g 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 ($\pm$ 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 ($\pm$ 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 μ g/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 ($\pm$ 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

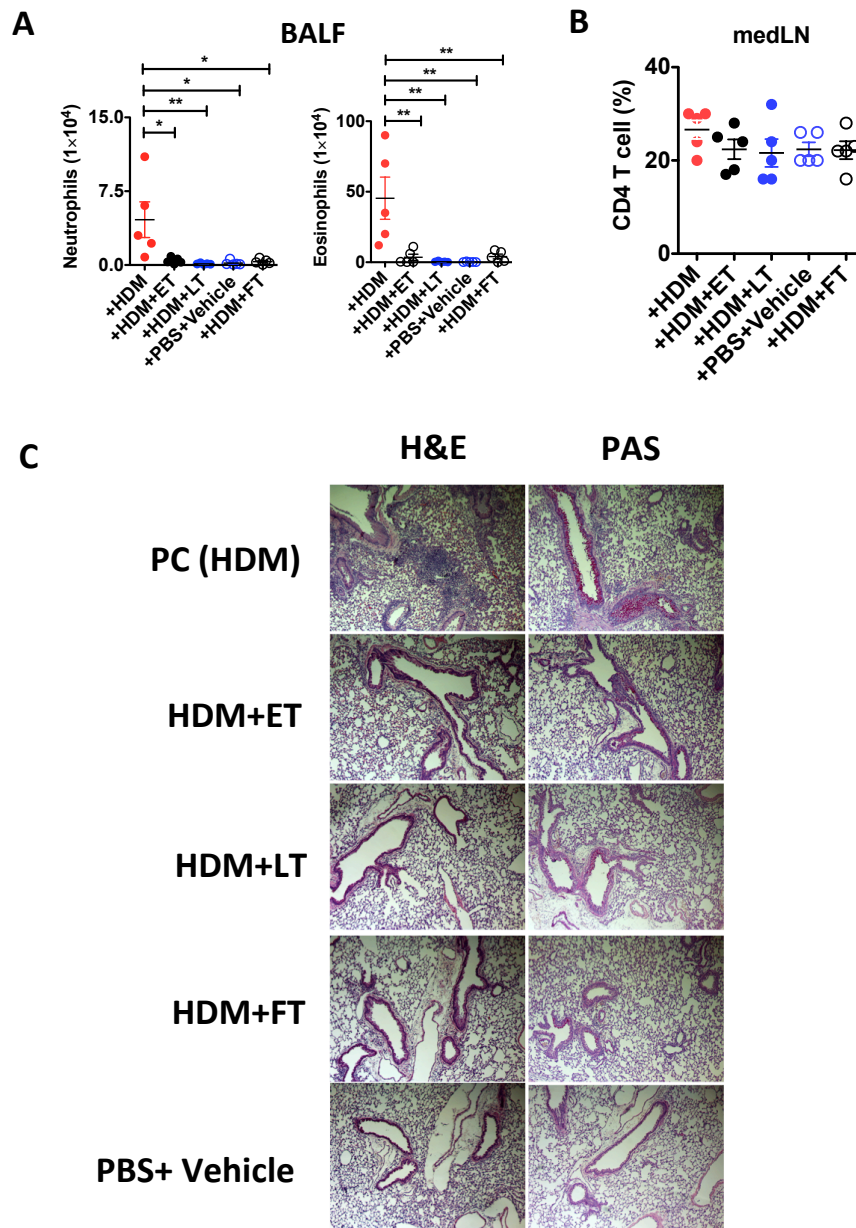


Figure S6, related to Figure 7. Pharmacological antagonism of Dkk-1 (A) Neutrophils and eosinophils in BALF were counted for each group (n=5) as described in Figure 7E. **(B)** CD4 T cell percentages in med LNs were measured by flow cytometry. **(C)** Representative images H&E and PAS staining were shown. 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 ($\pm$ 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 CD8 β (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-SR α expression vector (Slanetz et al., 1991) Briefly, CHO (Chinese Hamster Ovary) cells were transfected with Dkk-1-pFRSV-SR α and then Dkk-1 expression was amplified by methotrexate (MTX) treatment. Before harvest, MTX was removed and cells were washed. As a control, pFRSV-SR α 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-3 β 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% H₂O₂ 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-rich plasma was washed (140mM NaCl, 5mM KCl, 12mM Na₃C₆H₅O₇, 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 MgCl₂, 5mM NaHCO₃, 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 MgCl₂, 5 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 5.5 mM dextrose, 0.42 mM Na₂HPO₄, 12 mM NaHCO₃ [pH 7.4]), spinning at 2800rpm to discard the supernatant. For staining, washed blood was resuspended in Tyrodes-HEPES with 2 mM CaCl₂. 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 (DynaBeads). 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, 2×10⁶ frozen PBMCs were thawed and stimulated with soluble leishmania antigen (equivalent to 5×10⁶ 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, 2×10⁴ 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 and resuspended

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 *doublerridge* 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.

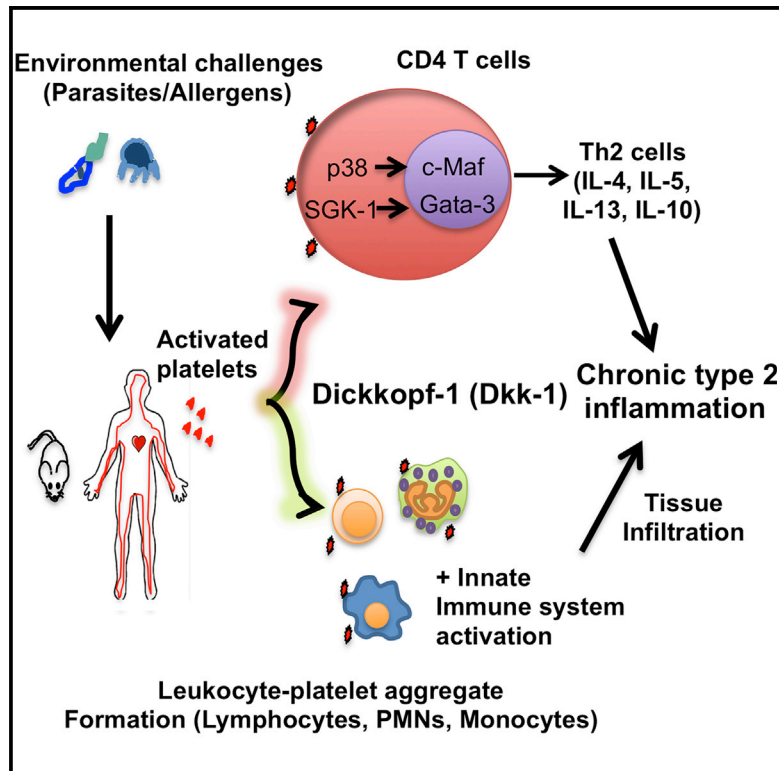
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Immunity

The Wnt Antagonist Dickkopf-1 Promotes Pathological Type 2 Cell-Mediated Inflammation

Graphical Abstract



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In Brief

The role of Wnt antagonist Dickkopf-1 in immune responses is unknown. Bothwell and colleagues find that the elevation of Dkk-1 from platelets is critical to promote chronic and pathological type 2 inflammation via enhancing Th2 cell differentiation and leukocyte-platelet aggregate formation.

Highlights

- Reduced expression of Dkk-1 protects the host from house-dust-mite-induced asthma
- Dkk-1 induces Th2 cell polarization and potentiates Th2 cell cytokine expression
- Platelets are the major source of Dkk-1 in pathological type 2 inflammation
- Pharmacological inhibition of Dkk-1 protects host from cutaneous leishmaniasis



The Wnt Antagonist Dickkopf-1 Promotes Pathological Type 2 Cell-Mediated Inflammation

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SUMMARY

Exposure to a plethora of environmental challenges commonly triggers pathological type 2 cell-mediated inflammation. Here we report the pathological role of the Wnt antagonist Dickkopf-1 (Dkk-1) upon allergen challenge or non-healing parasitic infection. The increased circulating amounts of Dkk-1 polarized T cells to T helper 2 (Th2) cells, stimulating a marked simultaneous induction of the transcription factors c-Maf and Gata-3, mediated by the kinases p38 MAPK and SGK-1, resulting in Th2 cell cytokine production. Circulating Dkk-1 was primarily from platelets, and the increase of Dkk-1 resulted in formation of leukocyte-platelet aggregates (LPA) that facilitated leukocyte infiltration to the affected tissue. Functional inhibition of Dkk-1 impaired Th2 cell cytokine production and leukocyte infiltration, protecting mice from house dust mite (HDM)-induced asthma or *Leishmania major* infection. These results highlight that Dkk-1 from thrombocytes is an important regulator of leukocyte infiltration and polarization of immune responses in pathological type 2 cell-mediated inflammation.

INTRODUCTION

The primary goal of immune responses is to eliminate the main trigger of inflammation, contributing to the structural and functional repair of the affected tissue. During the process, the key roles for circulating blood leukocytes are to migrate to sites of infection or injury and to develop polarized immune responses (Abbas and Janeway, 2000; Ley et al., 2007). The canonical Wnt signaling pathway, which induces cell proliferation, is uti-

lized for tissue repair processes, and a Wnt antagonist might inhibit or delay such events in chronic inflammatory diseases (Whyte et al., 2012). Among the quintessential Wnt inhibitory ligands, Dickkopf-1 (Dkk-1) was originally found regulating head formation of *Xenopus laevis* and is known to inhibit the canonical Wnt signaling pathway (Cruciat and Niehrs, 2013; Glinka et al., 1998). The inhibition of canonical Wnt pathway activation by Dkk-1 is achieved by its competitive binding of the receptor LRP (low-density lipoprotein receptor)-5 and 6 complex with markedly higher affinity than its counterpart agonist Wnt3a (Cheng et al., 2011; Joiner et al., 2013).

Regarding its possible role in chronic inflammation, elevated Dkk-1 amounts in circulating blood have been reported in various types of cancers and bone diseases that are characterized by unhealed tissue lesions (Diarra et al., 2007; Sato et al., 2010; Tian et al., 2003). In addition, a previous study reports that the proliferation of intestinal epithelial cells in a DSS-colitis model is enhanced in Dkk-1 hypomorphic *doubleridge* mice (*Dkk1^{d/d}*), suggesting an inhibitory role of Dkk-1 in wound repair in a pro-inflammatory microenvironment (Koch et al., 2011). A diverse array of environmental stimuli also causes tissue damage and they commonly trigger type 2 cell-mediated inflammation or immune responses both in acute and chronic inflammation (Pulendran and Artis, 2012; Whyte et al., 2012). Epithelial cell-derived factors (e.g., TSLP, IL-25, and IL-33) can induce type 2 cell-mediated immune responses (Divekar and Kita, 2015). Cytokines such as IL-4, IL-5, IL-10, and IL-13 are the key features of type 2 cell-mediated immune responses after T helper 2 (Th2) cell differentiation (Allen and Wynn, 2011; Pulendran and Artis, 2012).

The role of the canonical Wnt pathway in T cell biology including T cell development, CD8⁺ memory T cell formation, and regulatory T cell function has been demonstrated in multiple experimental systems with varying results, depending on the model systems used (Ding et al., 2008; Guo et al., 2007; van Loosdregt et al., 2013; Xie et al., 2005). Although the role of canonical Wnt pathway components such as β -catenin or

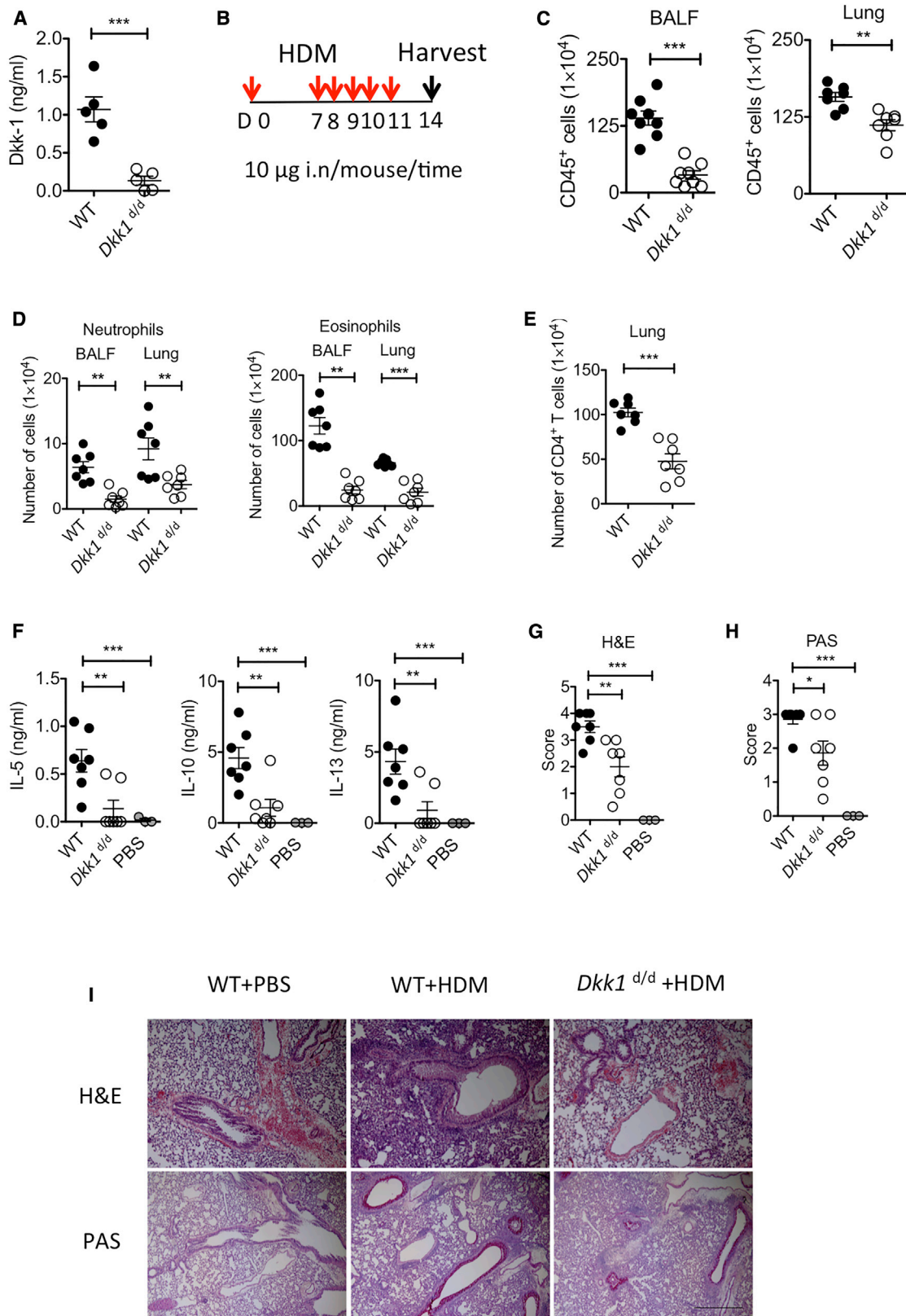


Figure 1. Reduced Expression of Dkk-1 Protects the Host from House Dust Mite-Induced Asthma

(A) Circulating concentrations of Dkk-1 in 8-week-old female *Dkk1^{d/d}* mice (n = 5) and their wild-type (WT) littermate control mice (n = 5).

(B) A scheme of HDM challenge protocol (10 µg/mouse/time).

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adenomatous polyposis coli (Apc) have been analyzed in these studies, the role of Wnt antagonists including Dkk-1 in the course of type 2 cell-mediated immune responses is poorly understood.

In recent years, the crucial role of platelets in inflammation has been widely emphasized (Morrell et al., 2014; Yeaman, 2014). Platelets are involved in coagulation upon various pathogenic challenges that protect surrounding tissues from both injury and invasion by microorganisms, and they also act as the reservoir of growth factors to recruit cells to repair tissue damage (Li et al., 2012). More recent studies show that platelets are important players in chronic inflammatory diseases by interacting with leukocytes in various pathological inflammatory conditions (Jenne and Kubes, 2015; Rondina and Garraud, 2014) and also selectively secreting immunomodulators to control T cell responses (Shi et al., 2014) and regulating leukocyte migration (Diacovo et al., 1996). It has been reported that activated platelets induce P-selectin expression, and P-selectin binds to P-selectin glycoprotein ligand (PSGL-1) in mature leukocytes to form leukocyte-platelet aggregates (León and Ardavin, 2008; Ley et al., 2007; Pitchford et al., 2005). Acute bacterial or viral infection can often lead to ITP (idiopathic thrombocytopenic purpura) with low platelet counts with the destruction of platelets (Cines et al., 2014; Zhang et al., 2009).

Here we demonstrate that the Wnt antagonist Dkk-1 induced Th2 cell polarization in physiologic models of type 2 cell-mediated inflammation or immune responses and utilized MAPK and mTOR pathway components to achieve potent Th2 cytokine production. Platelets were primarily responsible for the circulating Dkk-1 and its concentration was elevated by allergen or non-healing parasite challenge. Dkk-1 promoted interaction between leukocytes and platelets, facilitating the migration of leukocytes to the affected tissue.

RESULTS

Dkk-1 Hypomorphic Mice Are Protected from House-Dust-Mite-Induced Asthma

Th2-cell-mediated immune responses typically involve tissue damage and repair that requires the regulation of Wnt signaling (Pulendran and Artis, 2012; Whyte et al., 2012). We first explored the possibility that Dkk-1 functions as an immunomodulatory ligand in a standard model of house dust mite (HDM) extract allergen challenge, using the Dkk-1 hypomorphic *doubleridge* (*Dkk1^{d/d}*) mouse strain in which Dkk-1 expression is reduced by 90%. We first characterized hematological features of *doubleridge* mice. Of note, CD4⁺ T cells from *doubleridge* mice showed comparable cytokine production upon activation to those from WT littermate controls and the splenic CD4⁺ T cell population did not show significant differences in their cell surface markers of activation (Figures S1A–S1E). We also charac-

terized platelets from *Dkk1^{d/d}* mice and WT mice and determined that platelets from *Dkk1^{d/d}* mice were functionally comparable to WT platelets (Figures S2A–S2G). We confirmed the reduced amounts of Dkk-1 in plasma from *doubleridge* mice (Figure 1A) and challenged them with HDM extract (Figure 1B). CD45⁺ leukocytes including neutrophils, eosinophils, and CD4⁺ T cells were significantly increased in the lung and broncho-alveolar lavage (BAL) fluid of WT mice but this was substantially decreased in *Dkk1^{d/d}* mice, suggesting that the reduced expression of Dkk-1 after allergen challenge inhibited the infiltration of leukocytes to the lung (Figures 1C–1E). Th2 cytokines (IL-5, IL-10, and IL-13) were markedly induced in WT mice but notably decreased in *Dkk1^{d/d}* mice in ex vivo stimulation of mediastinal lymph node (medLN) cells with HDM allergen extract (Figure 1F). Hematoxylin and eosin (H&E) and periodic acid-Schiff base (PAS) staining and scoring of lung tissues also showed that *Dkk1^{d/d}* mice had reduced inflammation and leukocyte infiltration compared to WT mice (Figures 1G–1I). We also showed that airway resistance increase by HDM was notably decreased by using the Dkk-1 inhibitor (1-(4-(naphthalen-2-yl)pyrimidin-2-yl)piperidin-4-yl)methanamine (Pelletier et al., 2009) (WAY-262611) or in *Dkk1^{d/d}* mice (Figure S1F). Taken together, our results demonstrate that lack of Dkk-1 protects the host from chronic type 2 cell-mediated immune responses in the HDM-induced asthma model.

Functional Inhibition of Dkk-1 Protects Mice from *Leishmania major* Infection

We further questioned whether functional inhibition of Dkk-1 could impair chronic inflammation by different types of environmental pathogens via a different route of challenge such as skin. Parasite infections challenge the immune system to temper its activity with highly evolved immune evasion strategies (Redpath et al., 2014). Infection with the parasite *Leishmania major* causes chronic skin lesion formation with unresolved inflammation by Th2-cell-mediated immune responses in a murine model (Belkaid et al., 2001; Tacchini-Cottier et al., 2012). Many inbred mouse strains such as C57BL/6, C3H, and CBA/J are genetically resistant to *L. major* and spontaneously resolve infection because they mount a protective Th1-cell-type response. In contrast, susceptible BALB/c mice develop large nonhealing chronic lesions and mount a Th2-cell-type response that is associated with the production of the cytokines IL-4 and IL-10 (Reiner and Locksley, 1995; Scott, 1991). We assessed whether the inhibition of Dkk-1 function impairs Th2 cell polarization, ameliorating the development of cutaneous leishmaniasis. A Dkk-1 inhibitor, WAY-262611, was administered intraperitoneally during the course of *L. major* infection (Figure 2A). Both lesion size and parasite burden were greatly reduced at 42 days after infection, showing a 91% reduction of parasite survival in the group

(C and D) 9- to 12-week-old *Dkk1^{d/d}* (n = 7) and their littermate controls (n = 7) were analyzed. Total leukocytes (CD45⁺) (C), neutrophils and eosinophils from BAL fluid (BALF) (D), and lung tissue homogenates (D) were quantitated.

(E) CD4⁺ T cells from lung tissue homogenates were quantitated.

(F) Supernatants from mediastinal lymph node cells that were stimulated with HDM extract for 4 days were analyzed by ELISA.

(G–I) Lung tissues from each mouse were scored after H&E (G) and PAS (H) staining. Controls were challenged with PBS (n = 3) and one-way ANOVA analysis with Dunnett's post hoc test was performed. In (I), original magnification is 10×. Scale bar in lower right panel represents 10 μm.

Small horizontal lines indicate the mean (±SEM). A representative of two independent experiments is shown. Student's t test was performed. ***p < 0.0005, **p < 0.005, *p < 0.05. See also Figures S1 and S2.

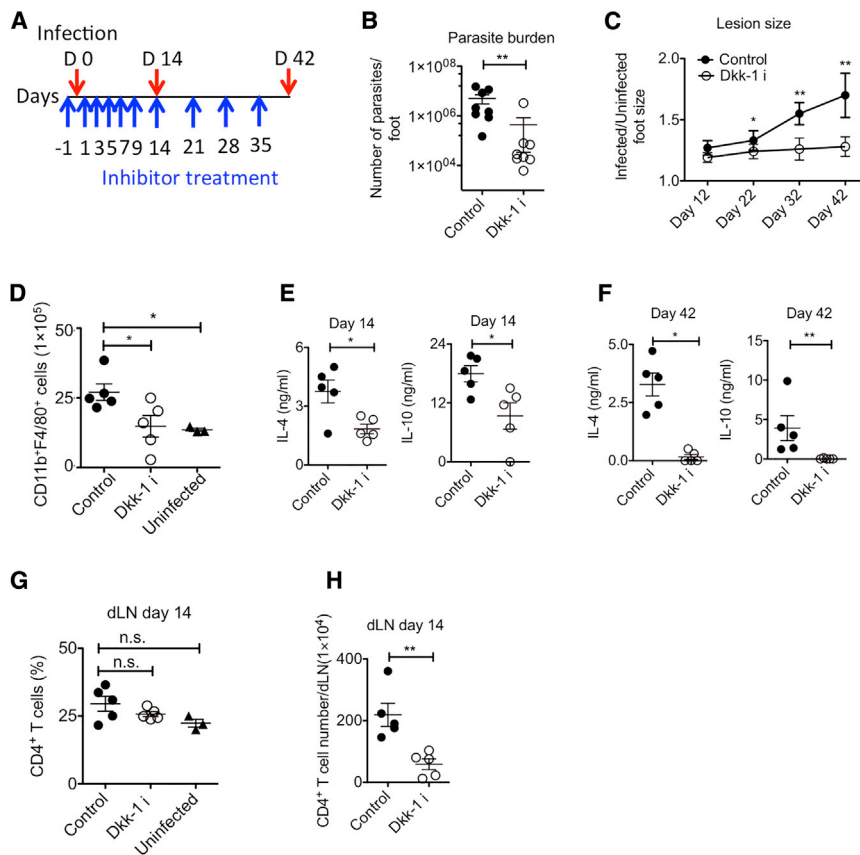


Figure 2. Functional Inhibition of Dkk-1 Protects the Host from Chronic Inflammation Caused by *L. major*

(A) A schematic diagram for Dkk-1 inhibitor treatment (10 µg/kg/time) in 6-week-old female BALB/c mice.

(B and C) The infected foot from each mouse was analyzed for parasite burden at day 42 (B) and lesion size was measured (n = 8/group) (C).

(D) 2 weeks after vehicle (n = 5) Dkk-1 inhibitor treatment (n = 5) based on (A), macrophages in the infected hindfoot were counted by flow cytometry and compared with infected (untreated) and uninfected (n = 3) BALB/c mice.

(E and F) 2 weeks (E) and 6 weeks (F) after infection, draining lymph node cells from each mouse (n = 5/group) were stimulated with sLMAG.

(G and H) CD4⁺ T cell numbers (G) and percentage (H) in draining lymph nodes from each group was determined by flow cytometry.

Small horizontal lines indicate the mean (±SEM). A representative of two independent experiments is shown. Student's t test (B, C, E–H) and one-way ANOVA analysis with Dunnett's post hoc test (D, G) were performed. **p < 0.005, *p < 0.05.

treated with Dkk-1 inhibitor (Figures 2B and 2C). Macrophage accumulation after parasite infection was also diminished by 14 days after infection (Figure 2D). IL-4 and IL-10 production was notably reduced by Dkk-1 inhibitor treatment at days 14 and 42 after infection subsequent to ex vivo stimulation of draining lymph node (dLN) cells from the infected mice with soluble leishmania antigen (sLMAG), suggesting that type 2 cell cytokine production was promoted by Dkk-1 (Figures 2E and 2F). It has been known that *L. major* infection induces CD4⁺ T cell recruitment and proliferation with lymph node expansion as well as Th2 cell differentiation (Carvalho et al., 2012; Hsu and Scott, 2007). Dkk-1 inhibitor treatment did not reduce the percentage of CD4⁺ T cells in dLNs but reduced the number of CD4⁺ T cells in the dLNs (Figures 2G and 2H), indicating that CD4⁺ T cell proliferation or recruitment is markedly reduced by Dkk-1 inhibition and that this is concomitantly associated with reduced IL-4 and IL-10 production in dLNs. Taken together, these results suggest that different types and routes of environmental challenges induced elevation of circulating Dkk-1 to promote chronic tissue inflammation with robust Th2 cytokine production.

Dkk-1 Polarizes CD4⁺ T Cells to Th2 Cell Lineage

From the allergen exposure model, we observed that platelets and T cells were co-localized in the lung in WT mice after recurrent allergen challenges but that this was reduced in *Dkk1*^{d/d} mice (Figure 3A). This increased T cell infiltration in the lung in WT mice compared to *Dkk1*^{d/d} mice was consistent with the increased CD4⁺ T cell numbers and the H&E score in the lung (Figures 1E

and 1G). We further checked whether any residual Dkk-1 might be induced in *Dkk1*^{d/d} mice, but we could not detect any elevation of Dkk-1 after HDM challenge in *Dkk1*^{d/d} mice (Figure 3B). Next, we tested whether Dkk-1 induces Th2 cytokines from CD4⁺ T cells; Dkk-1 induced IL-4, IL-10, and IL-13 in total CD4⁺ T cell activation in vitro (Figure 3C). Other important effector T cell cytokines including Th1- and Th17-associated cytokines (e.g., IFN-γ, IL-17A, and IL-17F) were not induced by Dkk-1, suggesting that Dkk-1 preferentially induces Th2 cytokines (Figure 3D). This induction of Th2 cytokines was also observed when naive CD4⁺ T cells were activated with Dkk-1 in vitro (Figure 3E).

We further examined whether Dkk-1 could antagonize Th1 cell polarization. Dkk-1 suppressed IFN-γ expression under Th1-cell-polarization conditions while still elevating Gata-3 expression and IL-4, IL-5, IL-10, and IL-13 secretion, suggesting its strong ability to drive Th2 cell polarization (Figure 4A). Finally, Dkk-1 enhanced IL-4 expression in a dose-dependent manner and also potentiated IL-4, IL-5, and IL-10 production from cells undergoing Th2 cell differentiation (Figure 4B). We also observed that Dkk-1 could induce IL-10 in the presence of TGF-β while inhibiting TGF-β-mediated Foxp3 expression (Figure 4C). Of note, we tested the dose-dependent response of Dkk-1 on Gata-3 expression and compared it with Wnt3a. Interestingly, up to 30 ng/ml of Dkk-1 could induce Gata-3 expression similar to Wnt3a; however, at higher amounts, Dkk-1 inhibited Gata-3 expression (Figure 4D). It has been shown that the canonical Wnt pathway transcription factors such as Lef and TCF-1 (also known as Tcf7) act as repressors of Gata-3 transcription in naive CD4⁺ T cells, and IL-4 inhibits TCF-1 expression (Hebenstreit et al., 2008; Maier et al., 2011). As previously reported, naive CD4⁺ T cell activation downregulated Lef and T cell factor-1

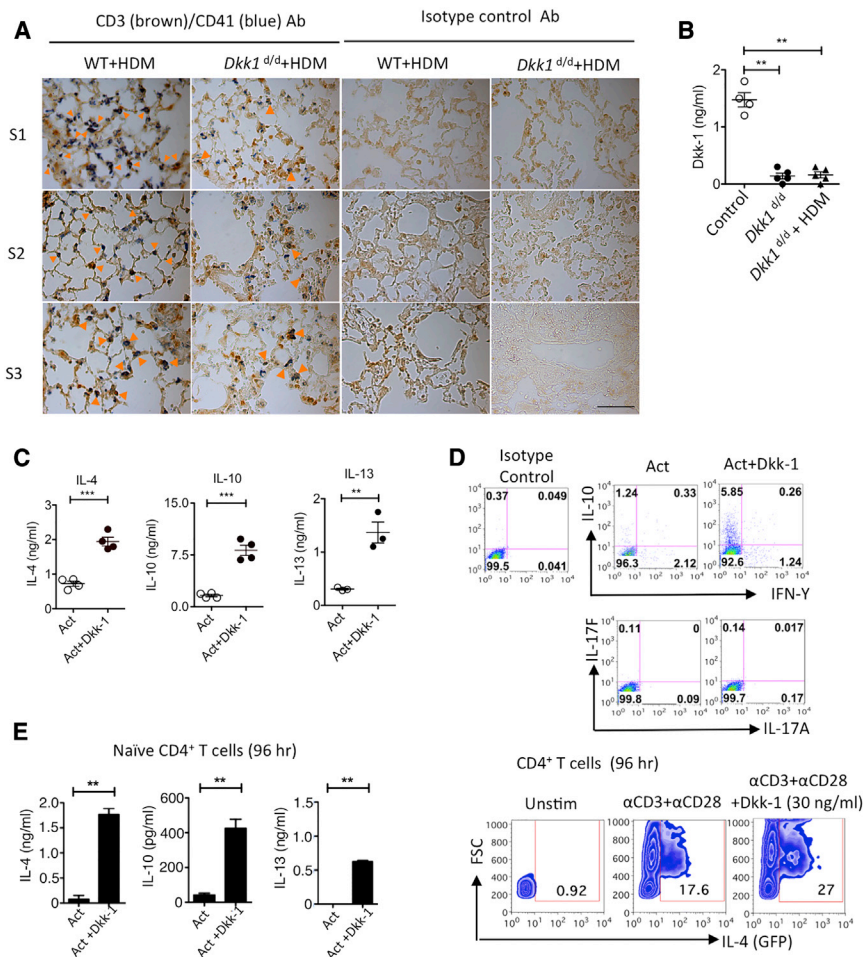


Figure 3. Dkk-1 Induces Th2 Cell Polarization

(A) *Dkk1*^{d/d} mice and their wild-type littermate controls were challenged with HDM extract allergen shown in Figure 1B. Mice were sacrificed and lungs were harvested for immunohistochemistry for CD3 (dark brown), CD41 (blue), and isotype control antibodies. Orange arrowheads in the images indicate co-localized CD3⁺ T cells with CD41⁺ platelets. S1, S2, and S3 indicate individual animals in each group. Original magnification is 63 $\times$. Scale bar in lower right panel represents 10 μ m.

(B) *Dkk1*^{d/d} mice (n = 5) were challenged with 50 μ g HDM extract per mouse and plasma samples were collected 24 hr later for ELISA.

(C and D) Splenic CD4⁺ T cells from 6- to 8-week-old C57BL/6 mice were activated with anti-CD3 and anti-CD28 antibodies (Act) with or without Dkk-1 (30 ng/ml unless indicated) for 4 days. Supernatants were analyzed by ELISA (C) and flow cytometry (D). For IL-4 in (D), splenic CD4 T cells from 8-week-old IL-4-GFP reporter mice were used.

(E) Naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 96 hr with or without Dkk-1 for flow cytometry analysis and ELISA.

Small horizontal lines and error bars indicate the mean ($\pm$ SEM). A representative of three independent experiments is shown. Student's t test was performed. ***p < 0.0001, **p < 0.005, *p < 0.05.

T cell population in *Dkk1*^{d/d} mice was not related to a decreased percentage of CD4⁺ T cells in the medLNs (Figure 5A, right).

(TCF-1) expression. Dkk-1 did not induce TCF-1 or Lef expression (Figure 4E). Collectively, these results show that Dkk-1 induces Th2 cell polarization and potentiates Th2-cell-type cytokine expression.

Dkk-1 Utilizes SGK-1 and p38 MAPK to Potentiate Th2 Cell Cytokine Production

The Th2 cell differentiation program is mediated by Gata-3 and c-Maf (Farrar et al., 2001; Ho et al., 1996; Lee et al., 2001). Figure 4D shows that Dkk-1 could induce Gata-3 expression from naïve CD4⁺ T cells in a dose-dependent manner and hence Th2-cell-associated cytokines, so we further investigated how Dkk-1 potentiated Th2 cell cytokine production. Recent studies show that helper T cell differentiation signaling is mediated by mTOR (mammalian or mechanistic target of rapamycin) and MAPK signaling pathways in response to diverse extracellular cues including Wnts (Chi, 2012; Heikamp et al., 2014). In vivo, a CD4⁺ T cell population in medLNs that concomitantly expressed Th2 cell polarization transcription factors Gata-3 and c-Maf was significantly increased after ex vivo stimulation in WT mice after 2 weeks of recurrent allergen challenges. However, this population was only minimally present in *Dkk1*^{d/d} mice (Figure 5A), suggesting the role of Dkk-1 in inducing c-Maf. The decrease of Th2 cytokines and the Gata-3⁺c-Maf⁺CD4⁺

We observed that Dkk-1 increased the Gata-3⁺ and c-Maf⁺ CD4⁺ T cell population in vitro and increased the expression of IL-10 (Figure 5B). Blockade of IL-4R inhibited the expression of Gata-3 but not c-Maf, revealing that Dkk-1 can induce c-Maf independently of IL-4 signaling (Figure 5C). In the absence of Dkk-1, Th2 cell polarization with exogenous IL-4 was mainly induced by Gata-3, and both p38 MAPK and SGK-1 inhibition reduced Gata-3 expression (Figure 5D, top). The addition of Dkk-1 induced marked expression of both Gata-3 and c-Maf. The inhibition of p38 MAPK regulated c-Maf induction, whereas SGK (serum glucocorticoid kinase)-1 inhibition primarily reduced Gata-3 expression in c-Maf⁺Gata-3⁺ populations (Figure 5D, bottom). The marked potentiation of IL-10 secretion by Dkk-1 in Th2 cell polarization conditions was blocked either by SGK-1 or p38 MAPK inhibition, confirming that both kinases are required for Dkk-1-mediated Th2 cytokine secretion (Figure S3A). Although c-Maf has been known to be predominantly involved in IL-4 expression (Kim et al., 1999), our results suggest that Dkk-1 could potentiate other Th2-cell-type cytokines such as IL-10 production in a c-Maf-dependent manner. Based on high homology of the Dkk-1 amino acid sequence (>90%) between mice and humans, human CD4⁺ T cells were treated with mouse Dkk-1 with or without SGK-1 inhibitor. The inhibition of SGK-1 substantially reduced Gata-3 and IL-10 secretion

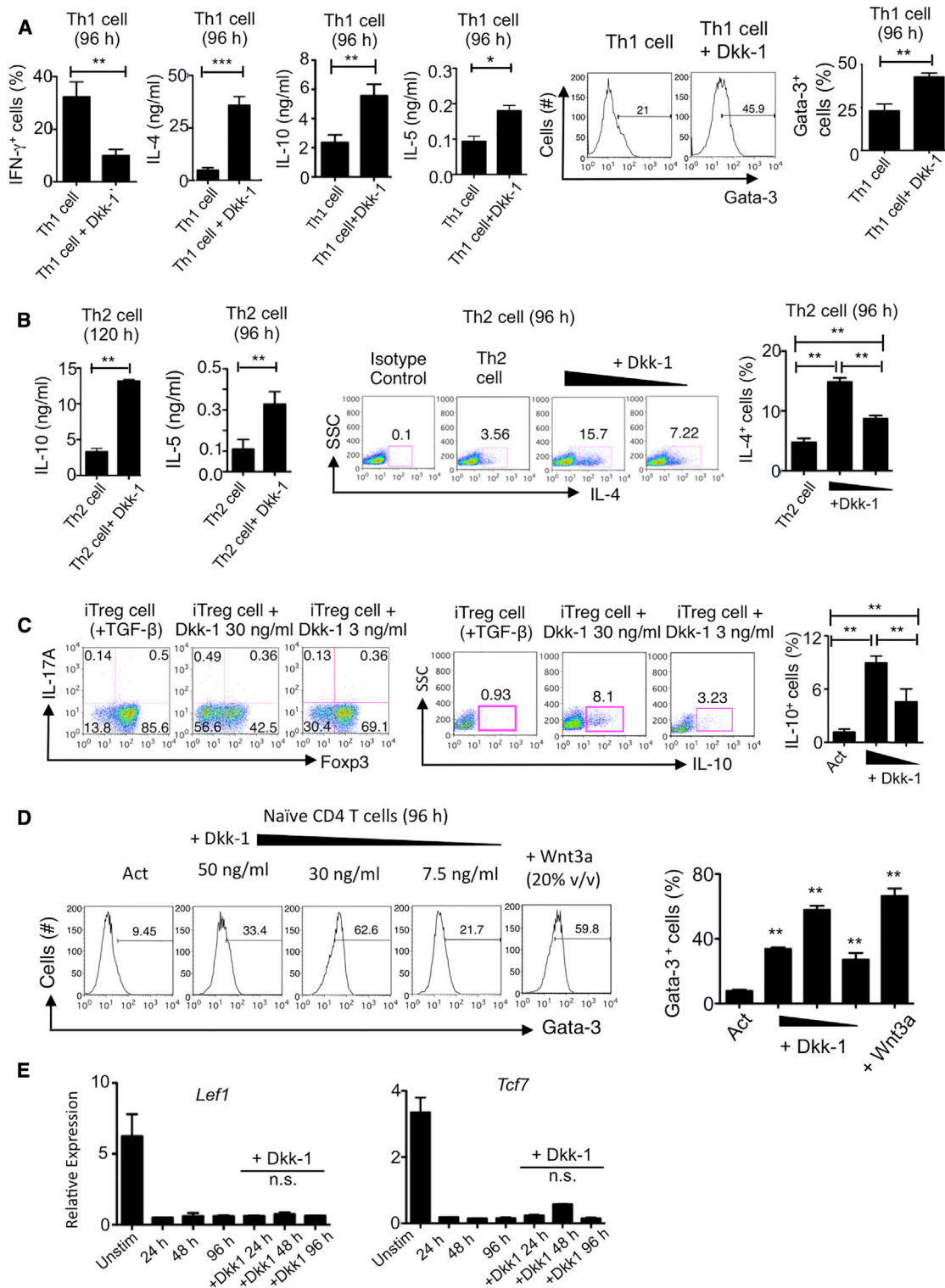


Figure 4. Dkk-1 Induces Th2 Cytokines in Various T Cell Differentiation Conditions

(A and B) Naïve CD4 T cells were stimulated under Th1 (A) or Th2 (B) polarization conditions for 96 hr with or without Dkk-1 for flow cytometry analysis and ELISA. (C) Splenic naïve CD4⁺ T cells were isolated and differentiated into iTreg cells for 5 days with 100 U/ml IL-2 and 1.5 ng/ml TGF- β with or without 30 ng/ml and 3 ng/ml of Dkk-1 was treated during the culture. Foxp3 and IL-17A expression (left) and IL-10 expression (right) were measured by flow cytometry. (D) Splenic naïve CD4⁺ T cells from 8-week-old C57BL/6 mice were treated with Dkk-1 with varying doses of Dkk-1 (50, 30, 7.5 ng/ml) or Wnt3a (20% v/v) for 96 hr in the presence of anti-CD3 and anti-CD28 antibody stimulation to measure Gata-3 expression amounts.

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(Figure 5E). Inhibition of SGK-1 expression by lentiviral shRNA transduction also diminished IL-10 induction by Dkk-1 in human CD4⁺ T cells (Figure S3B). Unlike the SGK-1 inhibition, the phosphorylation of S6 kinase or 4E-BP-1 was not altered by Dkk-1, indicating that the mTORC1 pathway was not affected by Dkk-1 (Figures S3C and S3D). In addition, we found that Stat6 was also required in Dkk-1-driven Th2 cell polarization (Figures S3E–S3G). These data, together with inhibition of Gata-3 expression (by IL-4R blocking antibody) demonstrated in Figure 4C, suggest that Dkk-1 promotes IL-4 induction and that naive CD4⁺ T-cell-derived IL-4 suppresses TCF-1 and/or Lef expression. Thus, our results show that Dkk-1 utilizes p38 MAPK and SGK-1 to potentiate Th2 cell polarization by increasing expression of c-Maf and Gata-3, respectively.

Dkk-1 Is Selectively Secreted from Platelets upon Allergen Challenge or Parasite Infection

Based on the pro-inflammatory effect of Dkk-1 in the HDM-induced asthma and the *L. major* infection model, we investigated the primary source of Dkk-1 in HDM-induced asthma. Initially, we found a marked increase of circulating amounts of Dkk-1 after recurrent allergen challenges (Figure 6A). We further tested whether a single intranasal allergen challenge could induce the elevation of circulating Dkk-1 and found that Dkk-1 was readily elevated in plasma of C57BL/6 mice (Figure 6B). This increase of Dkk-1 was also detected in the infiltrated lungs from WT littermate control mice although it was markedly reduced in *Dkk1*^{d/d} mice, suggesting that the elevation of Dkk-1 does occur both locally and systemically (Figure 6C). It has been implied that an increase of circulating Dkk-1 in multiple myeloma might be due to platelets (Trikalinos et al., 2009). While we were collecting plasma from C57BL/6 mice, we observed high Dkk-1 amounts even in the homeostatic conditions when samples were collected without anti-coagulant by cardiac puncture (Figure 6D). Because platelets sense mechanical shear stress by aggregating to form clots and release soluble mediators (Butler, 1995; McGrath et al., 2011), we questioned whether the elevated amounts of Dkk-1 are released from platelets. We depleted platelets in intact C57BL/6 mice and found that depletion of platelets abolished circulating amounts of Dkk-1 in 12 hr (Figure 6E). This marked reduction of Dkk-1 by platelet depletion was also observed with HDM allergen challenge (as in Figure 1B), suggesting that the primary circulating source of Dkk-1 in during both homeostatic and allergen-challenged conditions is platelets (Figure 6F). Next, we investigated whether the elevation of Dkk-1 by HDM is a specific response to the allergen. To this end, we challenged various types of compounds known as pathogen-associated molecular patterns (PAMPs) intranasally. Unlike the PAMPs that were tested, HDM could uniquely increase Dkk-1 in circulating blood (Figure 6G).

We further checked whether systemic concentrations of Dkk-1 were increased by different types of environmental pathogens via skin to promote Th2 cell responses. Subcutaneous infection

of the parasite *L. major* in the hindfoot of BALB/c mice increased circulating amounts of Dkk-1 that persisted 42 days after infection (Figure 6H). Upon parasitic infection, depletion of platelets using platelet-specific anti-mouse GPIIb/IIIa (CD42b) antibody markedly ablated elevation of circulating Dkk-1 amounts, even below homeostatic amounts of Dkk-1 in 72 hr (Figure 6I). At 10 weeks after infection, we showed that Dkk-1 is still maintained at a high concentration, and platelet depletion resulted in the complete loss of Dkk-1 in 12 hr, suggesting that platelets are continuously secreting Dkk-1 (Figure 6J). Activation of human platelets with sLMAG induced Dkk-1 within 1 hr in a dose-dependent manner (Figure 6K). Inhibition of PKC α (Figure 6L) blocked secretion of Dkk-1 upon sLMAG stimulation of human platelets, confirming that activated platelets secrete Dkk-1 from α -granules (Moncada de la Rosa et al., 2013). In contrast, stimulation of human peripheral blood mononuclear cells (PBMCs) or vascular endothelial cells with sLMAG or HDM extract failed to induce secretion of Dkk-1 (Figure S4). Taken together, our results suggest that Dkk-1 is primarily secreted from platelets upon allergen challenge or non-healing parasite infection, promoting type 2 cell-mediated immune responses.

Dkk-1 Facilitates Leukocyte Infiltration and Plays an Important Role in Chronic Type 2 Cell-Mediated Inflammation

Given that the source of circulating Dkk-1 was platelets and that *Dkk1*^{d/d} mice showed reduced leukocyte infiltration in the lung, we considered the possibility that Dkk-1 might regulate leukocyte-platelet interactions and leukocyte migration into the lung upon HDM extract challenge. LPA (leukocyte-platelet aggregate) formation in human and WT mouse peripheral blood has been considered a prerequisite for the increases of infiltrating immune cells to the affected tissues to develop immune responses (Ley et al., 2007; Li et al., 1997; Pitchford et al., 2005; Tamagawa-Mineoka et al., 2007; Zarbock et al., 2006). We observed that infiltration of CD45⁺ immune cells into the lung was significantly increased in WT mice but that this was impaired in *Dkk1*^{d/d} mice at 72 hr after a single allergen challenge (Figure 7A). Consistent with the marked decrease of leukocyte migration into the lung, a single challenge of allergen induced elevation of LPA (CD45⁺CD41⁺ population) in the peripheral blood of WT mice at 4 and 24 hr from the steady-state amounts but not in *Dkk1*^{d/d} mice (Figures 7B, S5A, and S5B). Pretreatment with Dkk-1 inhibitor 24 hr prior to infection with the parasite *L. major* similarly reduced the elevation of LPA formation at 4 hr after infection (Figure S5C). We further tested whether exogenous Dkk-1 could induce LPA formation and increase leukocyte infiltration in the lung. Exogenous administration of Dkk-1 did not increase the number of infiltrated leukocytes in the lung 72 hr after Dkk-1 protein administration (Figure 7C) although increased LPA formation was observed without allergen or parasite challenge (Figure S5D). Combined with results from Figure 7A, this suggests that infiltration of leukocytes to the lung requires both

(E) Splenic naive CD4⁺ T cells from 8-week-old C57BL/6 mice were stimulated with or without Dkk-1 (30 ng/ml) for 96 hr. RNA was harvested and analyzed by real-time qPCR.

All results were statistically non-significant. All experiments are representative of two to three independent experiments. For (A), Student's t test was performed. For (B)–(E), one-way ANOVA analysis with Bonferroni's post hoc test was performed. Small horizontal lines and error bars indicate the mean ($\pm$ SEM). ***p < 0.0001, **p < 0.005, *p < 0.05.

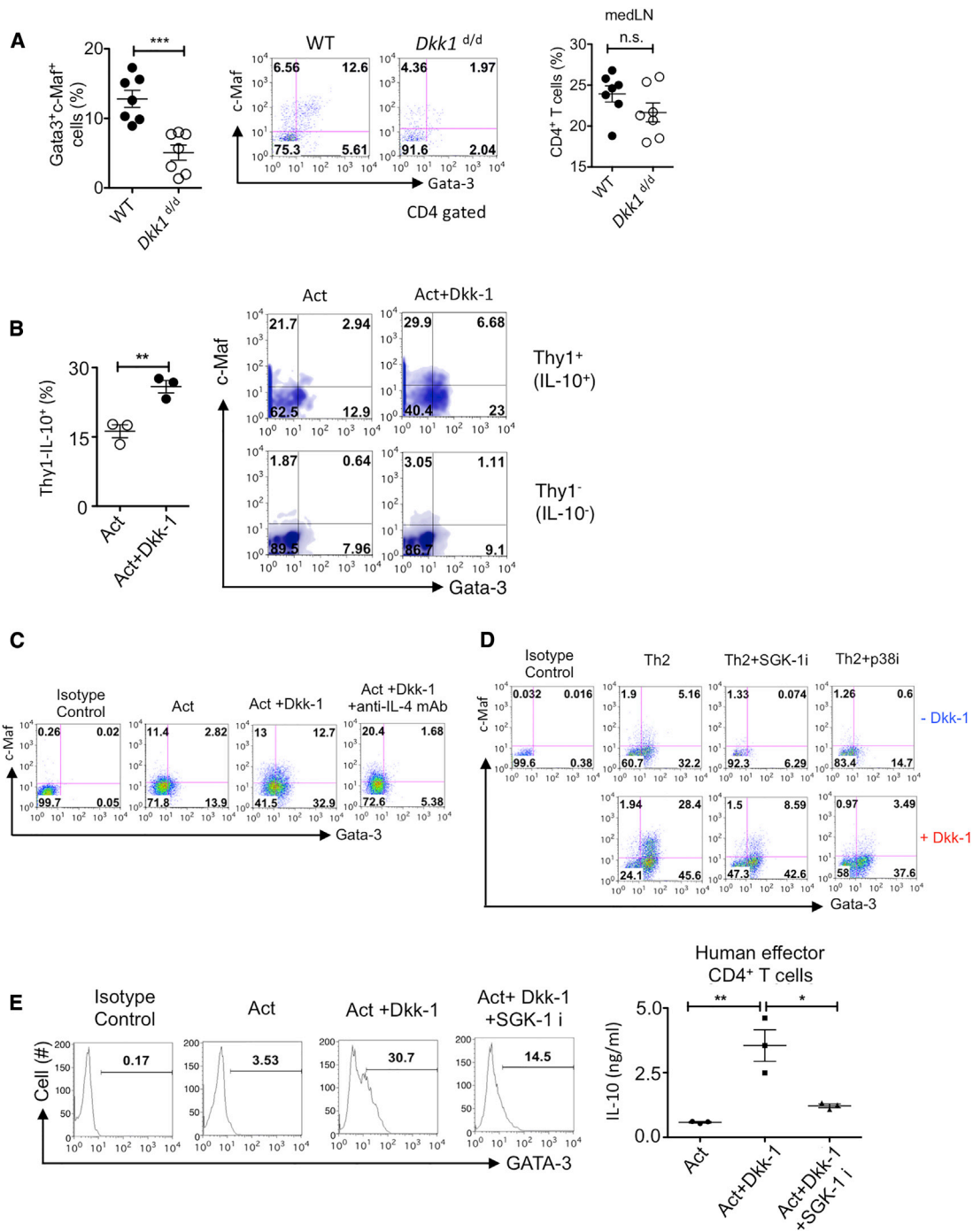


Figure 5. Dkk-1 Directs Th2 Cell Polarization by p38 MAPK and SGK-1 to Induce c-Maf and Gata-3

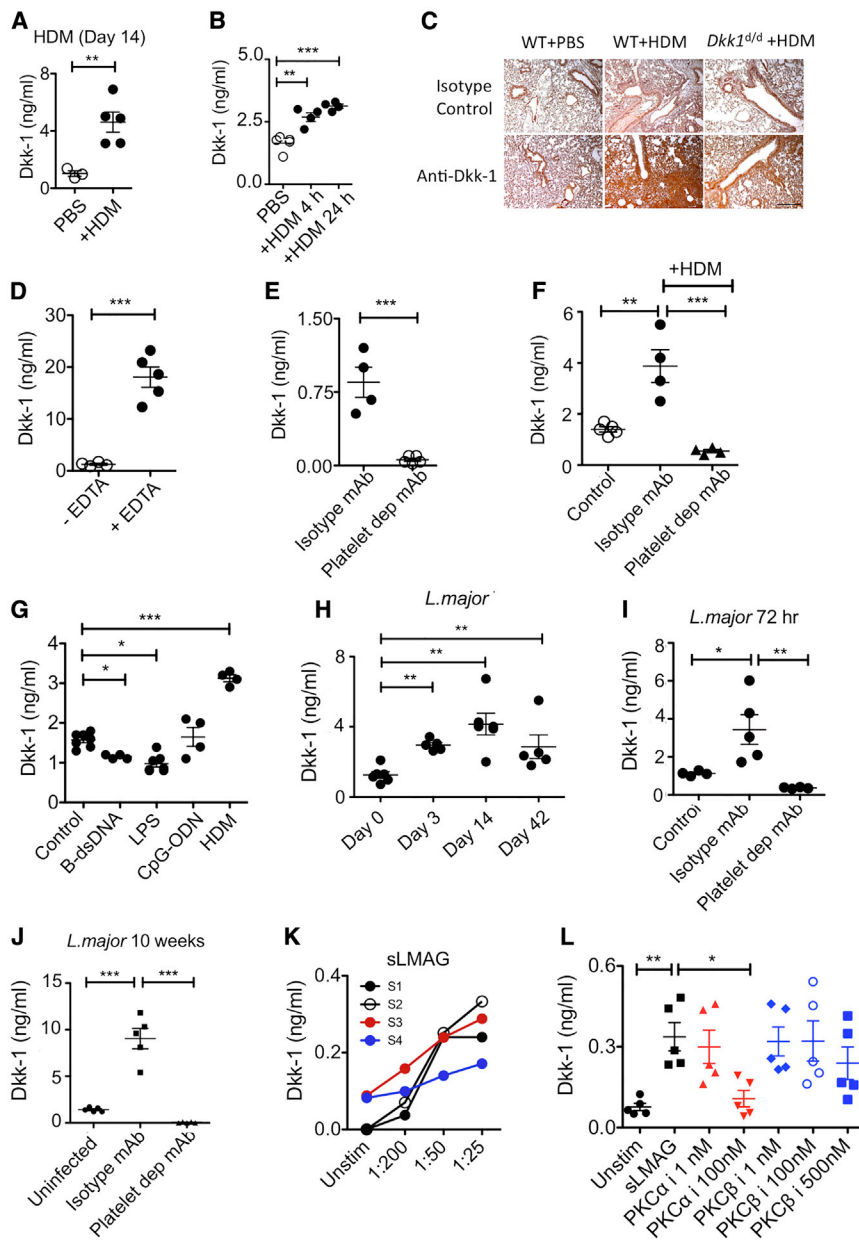
(A) Mediastinal lymph node cells from Figure 1D were stimulated with HDM extract for 4 days and the percentages of Gata3⁺ and c-Maf⁺ in CD4⁺ T cells were quantitated by flow cytometry.

(B) Splenic CD4⁺ T cells from 12-week-old Thy1-IL-10 reporter mice were activated with or without Dkk-1 (30 ng/ml) for 84 hr. Cells were gated for Thy1⁺ and Thy1⁻. (C) Splenic CD4⁺ T cells were activated with or without Dkk-1 or anti-IL-4 mAb (10 μ g/ml) for 4 days and analyzed by flow cytometry.

(D) Naive CD4⁺ T cells were stimulated under Th2 cell polarization conditions for 96 hr with or without Dkk-1 for flow cytometry analysis. SGK-1 inhibitor (2 μ M) or p38 inhibitor (10 μ M) was added at 0 hr.

(E) PBMCs from healthy volunteers were used to isolate human CD4⁺ T cells and then stimulated with human anti-CD3 mAb (OKT3) and anti-CD28 mAb for 4 days with or without Dkk-1 (30 ng/ml) and SGK-1 inhibitor GSK653094 (2 μ M). Cells were analyzed by flow cytometry. Supernatants were harvested and analyzed by ELISA (right).

A representative of three independent experiments is shown. Student's t test was performed. *** p < 0.0001, ** p < 0.005, * p < 0.05, n.s., not significant. For (E), one-way ANOVA analysis with Bonferroni's post hoc test was performed. See also Figure S3.



A representative of two independent experiments is shown. Small horizontal lines indicate the mean ($\pm$ SEM). Student's *t* test (A, D, E), one-way ANOVA with Dunnett's post hoc test (B, I), and one-way ANOVA with Bonferroni's post hoc test (F–H, J, L) was performed. ****p* < 0.0005, ***p* < 0.005, **p* < 0.05. See also Figure S4.

Dkk-1 and further innate immune responses for leukocyte migration upon allergen challenge. In addition, in contrast to WT mice, we observed that leukocytes from peripheral blood in *Dkk1*^{d/d} mice failed to sustain the elevation of PSGL-1 expression and its ligand P-selectin on platelets upon HDM allergen challenges. Similarly, inhibition of Dkk-1 in *L. major* infection blocked the increase of PSGL-1 expression (Figures S5E–S5G).

Next, we evaluated the importance of Dkk-1 either at the challenge stage or at the sensitization stage in the HDM-induced asthma model. To this end, we treated Dkk-1 inhibitor either at the challenge stage (ET, early treatment) or at the sensitization stage (LT, late treatment) (Figure 7D). Leukocyte infiltration

including CD4⁺ T cells, neutrophils, and eosinophils was all markedly decreased in BAL fluid and lung tissues in both the ET and LT treatment protocols (Figures 7E, 7F, and S6A). The number of CD4⁺ T cells was also reduced in mediastinal lymph nodes by inhibiting Dkk-1, but the percentage of CD4⁺ T cells in the lymph nodes did not change (Figures 7G and S6B). Th2 cytokine secretion from mediastinal lymph nodes was diminished (Figure 7H). Consistent with these results, histopathological scores also showed that the functional inhibition of Dkk-1 in either the challenge stage or the sensitization stage resulted in inhibition of type 2 cell inflammatory responses in HDM-induced asthma model (Figures 7I, 7J, and S6C). Taken together,

Figure 6. Circulating Dkk-1 Is from Platelets upon Allergen Challenge or Parasitic Infection

(A) Female C57BL/6 mice were challenged with PBS (*n* = 3) or HDM extract (10 μ g/challenge/mouse, *n* = 5) as in Figure 1B.

(B) 8-week-old female C57BL/6 mice were challenged with HDM extract (30 μ g/challenge, *n* = 4) or PBS (*n* = 5) for 4 and 24 hr. Plasma samples were analyzed by ELISA.

(C) *Dkk1*^{d/d} mice (*n* = 7) and their wild-type littermate controls (*n* = 7) were challenged with HDM extract as in Figure 1B. Harvested lungs were analyzed by immunohistochemistry. Original magnification is 10 $\times$. Scale bar in lower right panel represents 10 μ m.

(D) Peripheral blood was collected by cardiac puncture with or without EDTA.

(E) 8-week-old female C57BL/6 mice were injected with platelet depletion antibody (*n* = 5) or isotype control antibody (*n* = 4). 24 hr later, plasma samples were measured.

(F) HDM allergen was challenged as in Figure 1B, and platelets were depleted for 12 hr.

(G) Bacterial dsDNA from *E. coli* (B-ds DNA) (30 μ g/mouse, *n* = 4), LPS (3 μ g/mouse, *n* = 5), CpG-ODN 1585 (25 μ g/mouse, *n* = 4), and HDM allergen extract (50 μ g/mouse, *n* = 4) were challenged intranasally in 6-week-old C57BL/6 mice. 24 hr later, plasma samples were collected and Dkk1 concentrations were determined by ELISA. Control (*n* = 7) mice were given 20 μ l of 0.9% NaCl saline.

(H) At each time point, plasma from female BALB/c mice (6 weeks old) that were infected with *L. major* was measured.

(I) Platelets were depleted 4 hr prior to parasitic infection. Plasma samples from 5-week-old female BALB/c mice were analyzed 72 hr after infection.

(J) 10 weeks after infection, platelets were depleted for 12 hr in BALB/c mice.

(K) Human platelets from four healthy volunteers were activated with sLMAG for 1 hr. S1–S4 designates healthy donors. x axis shows dilution of sLMAG in the culture. 1:50 is equivalent to 1 $\times$ 10⁶ parasites.

(L) Human platelets (1 $\times$ 10⁵/ml, *n* = 5) were activated with sLMAG (1:50) in the presence of PKC α inhibitor or PKC β inhibitor for 1 hr.

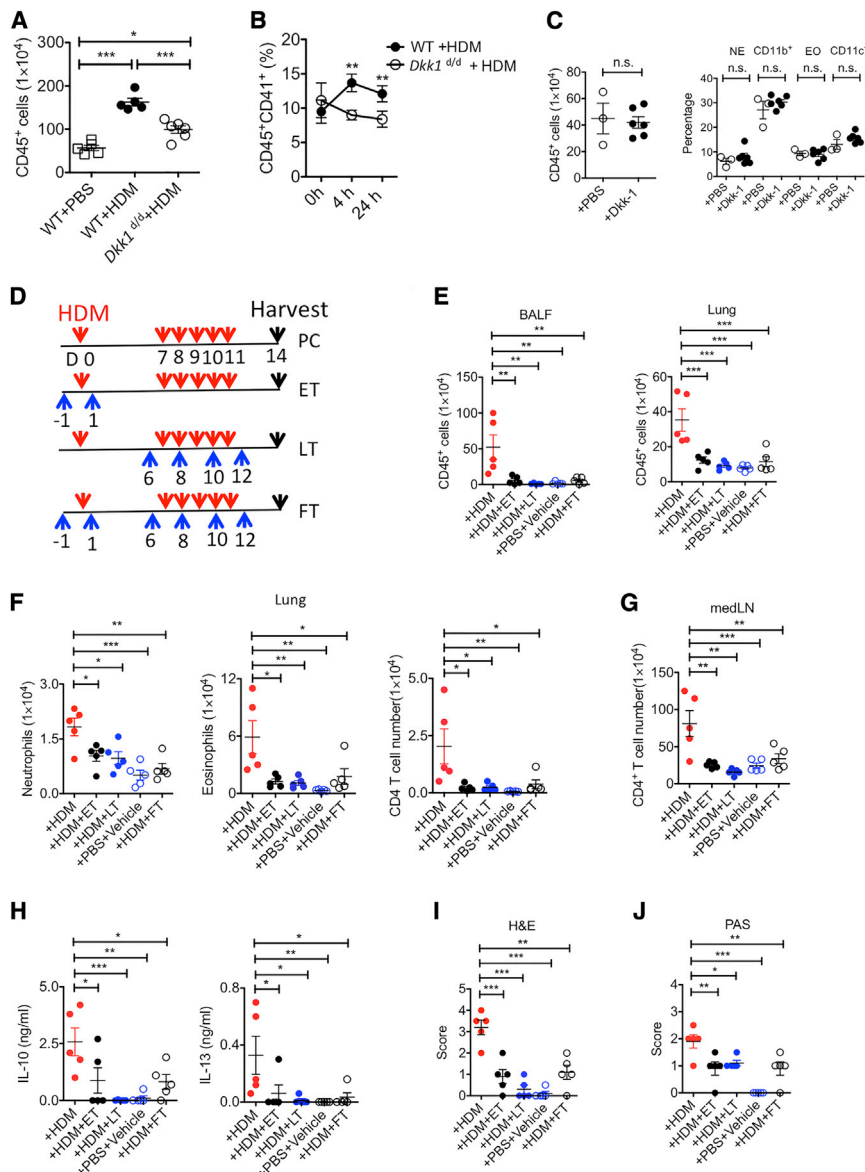


Figure 7. Dkk-1 Facilitates Leukocyte Migration and Regulates Leukocyte-Platelet Aggregate Formation in Type 2 Cell-Mediated Inflammation

(A) 9-week-old *Dkk1*^{d/d} mice and wild-type littermate controls ($n = 5$ –6/group) were challenged with 30 μ g HDM allergen extract intranasally. Lung homogenates were quantitated and analyzed by flow cytometry 72 hr after HDM challenge and CD45⁺ cells were analyzed by flow cytometry.

(B) Peripheral blood from 9-week-old *Dkk1*^{d/d} mice and wild-type littermate controls were collected at 4 and 24 hr after allergen challenge and analyzed for the percentages of CD45⁺CD41⁺ cells.

(C) Dkk-1 protein (300 ng) was injected intraperitoneally. CD45⁺ cells were analyzed by flow cytometry. Abbreviations are as follows: NE, neutrophils; EO, eosinophils.

(D) Schematic diagram of Dkk-1 inhibitor treatment protocol for each group ($n = 5$ for each group) in HDM-induced asthma model. HDM allergen extract (10 μ g) was challenged intranasally at the indicated time points.

(E–J) CD45⁺ leukocytes numbers in BALF and lung (E), neutrophils, eosinophils, and CD4⁺ T cell numbers in the lungs (F), and the number of CD4⁺ T cells in mediastinal LNs were counted (G). After 4 days of stimulation of medLN cells, cytokines were measured by ELISA (H). H&E (I) and PAS (J) staining were scored. Abbreviations are as follows: ET, early treatment; LT, late treatment; FT, full treatment; PC, positive control.

Small horizontal lines and error bars indicate the mean ($\pm$ SEM). A representative of two independent experiments is shown. One-way ANOVA analysis with Dunnett's post hoc test was performed. *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$, n.s., not significant. See also Figures S5 and S6.

our results suggest that Dkk-1 plays an important proinflammatory role for Th2 cell differentiation and leukocyte migration in each stage of HDM allergen challenge to promote chronic type 2 cell-mediated inflammation.

DISCUSSION

The increased amounts of Dkk-1 in peripheral blood have been reported in chronic inflammatory diseases such as various types of cancers, rheumatoid arthritis, and lupus (Diarra et al., 2007; Sato et al., 2010; Wang et al., 2014), and the role of Dkk-1 has been studied as a Wnt antagonist in various model systems. Our results demonstrate an important immunomodulatory role of Dkk-1 in type 2 cell-mediated immune responses caused by environmental challenges.

Although multiple roles of the canonical Wnt signaling pathway have been studied in the context of intracellular mediators such

and mTOR signaling pathway components to induce type 2 cell-mediated immune responses and/or inflammation upon allergen or non-healing parasite infection.

A previous study has addressed the role of the canonical Wnt pathway that is mediated by β -catenin in Th2 cell differentiation (Notani et al., 2010), focusing on early progenitor T cells (e.g., thymocytes and naive CD4 T cells) in vitro. The prediction based on these in vitro studies utilizing high concentrations of Dkk-1 or Wnt3a would have been inhibition of Th2 cell differentiation by Dkk-1 and hence the inhibition of HDM-induced asthma or *L. major* infection. However, we have shown that the function of Dkk-1 in vivo at physiological concentrations is, in fact, the opposite and that Dkk-1 potentiates Th2 cell polarization. Physiologic concentrations of Dkk-1 (up to 30 ng/ml) in fact promote type 2 cell-mediated inflammation and Th2 cell differentiation primarily through SGK-1 and p38 MAPK. Of note, we also observed that Wnt3a induced Gata-3 expression and that

non-physiologic high concentrations of Dkk-1 (>50 ng/ml) inhibited Gata-3 expression similar to the previous report. There are signaling pathways identified which Dkk-1 utilizes other than the well-known canonical Wnt pathway (Fukuda et al., 2010; Krause et al., 2014). It is notable that Dkk-1 has a colipase domain in its carboxy-terminal region that is responsible for Wnt antagonist activity, but the function and role of the N-terminal domain of Dkk-1 is largely unknown (Brott and Sokol, 2002). More detailed characterization of Dkk-1 molecular structure affecting Th2 cell differentiation is warranted in the future. Collectively, our findings suggest that physiological concentrations of Dkk-1 utilized SGK-1 and p38 MAPK under type 2 cell-mediated immune responses or inflammation.

Recent studies point out the specificity of platelet activation and its immunomodulatory role in addition to its traditional role in coagulation events and hemostasis (Rondina and Garraud, 2014). Platelets do express a variety of receptors for PAMPs, and recent studies identify more receptors and ligands that are expressed in immune cells (Semple et al., 2011). We demonstrated that platelets are an important source of Dkk-1 in our models and that the release of Dkk-1 is specific to environmental challenges that lead to chronic type 2 cell-mediated immune responses. Platelets are the most abundant source of TGF- β 1 (Kim et al., 2013). Although the release of TGF- β 1 could be assumed in platelet activation and thus further assumed to increase Foxp3⁺CD4⁺ T cells, it should be noted that platelets also possess ligands such as PF4 (CXCL4) that is abundantly present and can inhibit TGF- β 1-mediated T cell differentiation (Shi et al., 2014). The inhibition of TGF- β 1-mediated Foxp3 expression by Dkk-1 suggests that the immunosuppressive function of TGF- β in a given microenvironment would be potently inhibited, and hence Dkk-1 would be able to generate robust Th2 cytokines. Together with the inhibitory role of Dkk-1 on TGF- β 1-mediated Foxp3⁺ T cell differentiation, it is unlikely that TGF- β 1 would affect T cell differentiation in our models. Additionally, we did not observe any increased Foxp3⁺ T cells in *doubleridge* mice, confirming that platelet activation and subsequent Dkk-1 release from platelets is specific to a given environmental challenge.

Our findings might suggest that immunomodulation of Dkk-1 from platelets is an example of the development of the mammalian immune system from the hemocyte system that exists in *Drosophila* (Lavine and Strand, 2002; Wood and Jacinto, 2007). Our results from exogenous Dkk-1 injection and HDM-induced asthma and *Leishmania major* infection models suggest that platelet activation and subsequent release of Dkk-1 facilitates leukocyte migration to the site of environmental challenges, and this should be coordinated with the recognition of such challenges by innate immune cells. Because hemocytes play roles of both platelets and innate immune cells, our results might show the specialized role of platelets and leukocytes to coordinate type 2 cell-mediated immune responses in mammals. It is notable that the potent induction of IL-10 by Dkk-1 in the *L. major* infection model demonstrates that the parasite utilizes Dkk-1 as a strategy to reconcile immune activation and immunosuppression to favor parasite survival, avoiding complete eradication and maintaining lesions.

Taken together, our findings highlight the importance of the orchestrating role of Dkk-1 from platelets to facilitate leukocyte migration and further to polarize immune responses by inducing

Th2 cell polarization, placing Dkk-1 as a highly attractive target for controlling type 2 cell-mediated immune responses.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J mice were purchased from the Jackson Laboratory and have been bred in our mouse facility. BALB/c mice were purchased from NCI. The animals were kept under normal light and dark cycle (12/12). *Doubleridge* mice (*Dkk1^{del/d}*) were kindly provided by Asma Nusrat (Emory University). Foxp3-IRES-RFP mice and Stat6-deficient mice were bred in our mouse facility. Thy1-IRES-IL-10 reporter mice were provided by Casey Weaver (University of Alabama). IL-4-GFP reporter (4Get) mice on the BALB/c background were kindly provided by Ruslan Medzhitov (Yale University). All mouse protocols were approved by the Yale University Institutional Animal Care and Use Committee (IACUC) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Flow Cytometry, Cell Culture, Cytokine ELISA, and Immunohistochemistry

For CD4⁺ T cell differentiation, naive CD4⁺ T cells (CD4⁺CD62L^{hi}CD44^{lo} Foxp3(RFP)-) were isolated from 6- to 7-week-old Foxp3-IRES-RFP mice. For Th1 cell differentiation, cells were activated with plate-bound anti-CD3 (2 μ g/ml), anti-CD28 (2 μ g/ml) in the presence of IL-2 (25 U/ml), and IL-12 (20 ng/ml) for 96 hr in RPMI-1640 media (5% FBS, 1% penicillin/streptomycin, and 10 mM HEPES). For Th2 cell differentiation, cells were activated with plate bound anti-CD3 (2 μ g/ml), anti-CD28 (2 μ g/ml) in the presence of IL-2 (25 U/ml), anti-IFN- γ (XMG1.2) mAb (10 μ g/ml), and IL-4 (100 U/ml) for 96 hr. For the isolation of CD4⁺ T cells, spleens from 6- to 7-week-old male Foxp3-IRES-RFP mice were prepared as single-cell suspensions. After RBC (red blood cell) lysis, cells were stained with CD16/CD32 FcR (Fc Receptor) blocking antibody (clone 2.4G2). For intracellular staining, cells were stimulated with cell stimulation cocktail with protein transport inhibitors (eBioscience) for 5 hr. Intracellular staining was performed as described in the manufacturer's protocol (eBioscience). FACS Calibur or Stratedigm (BD Biosciences) was used for flow cytometry and data were analyzed by FlowJo software (TreeStar). Human and mouse Dkk-1 ELISA kits were purchased from R&D Systems. The human IL-10 ELISA, mouse Th1 and Th2 cytokine panel ELISA kit, and mouse IL-5/IL-13 ELISA kit were purchased from eBioscience. Cytokines were measured by ELISA according to the manufacturer's protocol.

House Dust Mite Asthma Model

House dust mite extract (*Dermatophagoides pteronyssinus*; Greer Labs) (10 μ g) was dissolved in 20 μ l of PBS for each mouse per intranasal challenge. 6- to 10-week-old *doubleridge* mice and their littermate controls were challenged intranasally on days 0, 7, 8, 9, 10, and 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. For histological analysis, lungs were fixed with 10% formalin at least for 24 hr, embedded in paraffin, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) reagent and were assigned scores by established methods. For lymphocyte-platelet aggregation assay, 50 μ g of HDM extract was dissolved in 25 μ l PBS and then intranasal challenge was performed. Lymphocyte-platelet aggregation was measured at 4 and 24 hr after the challenge. At 72 hr, lung homogenate was prepared by collagenase digestion. Cells were counted and lymphocytes were analyzed by flow cytometry. For more details, see [Supplemental Experimental Procedures](#).

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 μ l DMSO) was injected on days -1, +1, +3, +5, +7, +9, +11, and

weekly afterward until harvest. Draining lymph nodes, non-draining lymph nodes, and plasma were collected. For ex vivo analysis, soluble *L. major* antigen (sLMAG) was employed. For depleting platelets, 80 μ g of anti-CD42 mAb (Emfret Analytical) was injected intravenously in 100 μ l PBS. For more details, see [Supplemental Experimental Procedures](#).

Human or Mouse Platelet Isolation and Functional Characterization and Lymphocyte-Platelet Aggregate Assay

Human platelets were isolated as described from healthy volunteers (Tang et al., 2011). All procedures were in accordance with HIC protocol (protocol # 1005006865). All volunteers consented to donate their blood. In brief, 3.2% citrate buffer was used to prevent coagulation. Blood was centrifuged at 250 $\times$ 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 and resuspended in Tyrode's buffer. 100 million platelets/ml were used for activation. House dust mite extract (50 μ g) was 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 at indicated ratio for 1 hr. Platelets were resuspended in Tyrode's buffer. Supernatant was collected for human Dkk-1 ELISA. For details, see [Supplemental Experimental Procedures](#).

Statistical Significance

Statistically significant differences were determined by one-way ANOVA with Bonferroni's post hoc test, Dunnett's post hoc test, or Student's t test with Graph Pad Prism software (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.01.008>.

AUTHOR CONTRIBUTIONS

W.-J.C., A.K.E., D.M.-P., and K.G.-P. performed *L. major* experiments and analyzed results. W.-J.C. and P.Y.C. performed HDM asthma experiments and analyzed results. W.-J.C. and A.M.T. performed mouse platelet experiments. L.H. scored histology samples. W.H.T., J.H., D.S.K., and A.M.T. contributed experimental advice for human platelet experiments. P.S. and P.J.L. advised and helped with the Flexivent assay. J.H.S. performed immunohistochemistry. S.-T.K. and J.-H.P. assisted with in vitro experiments. S.E.M. gave technical help. C.V.R. provided experimental materials and advised the writing of the manuscript. D.M.-P. conceived the *Leishmania* study, provided experimental materials, and wrote the paper. O.H. analyzed results and provided experimental advice with discussion. W.-J.C. and A.L.M.B. conceived all experimental studies and wrote the paper.

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Supplemental Information

The Wnt Antagonist Dickkopf-1 Promotes

Pathological Type 2 Cell-Mediated Inflammation

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Figure S1

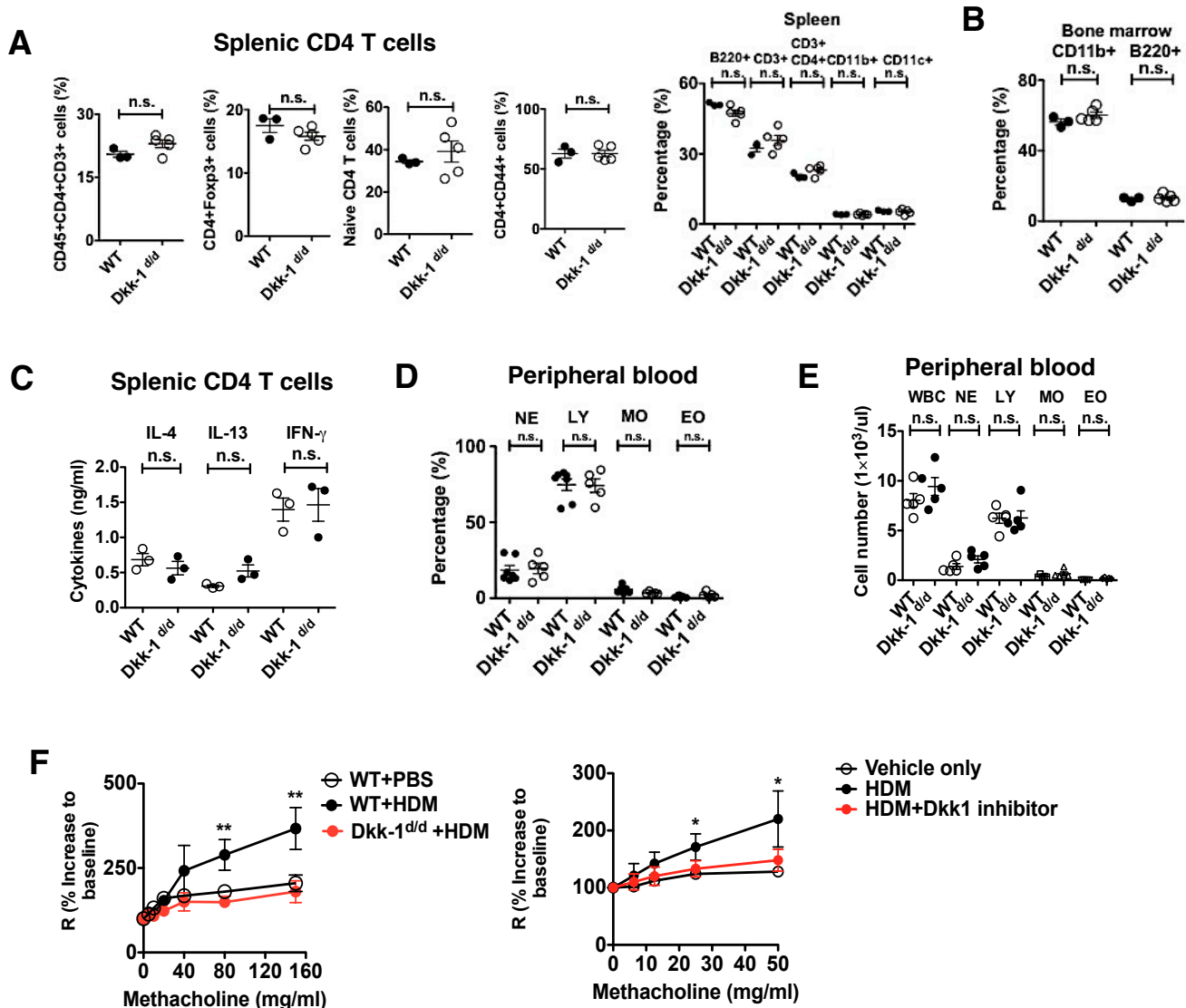


Figure S1, related to Figure 1. Characterization of Dkk-1^{d/d} mice (A, B) Six to ten week-old Dkk-1^{d/d} mice (n=5) and their wildtype littermate controls (n=3) were used. Single cell suspensions from spleen and bone marrow (B) were prepared and analyzed by flow cytometry. (C) Splenic CD4 T cells were isolated by MACS and stimulated with α CD3 and α CD28 mAb (2 μ g/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 μ g 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 ($\pm$ s.e.m.). *, p< 0.05

Figure S2

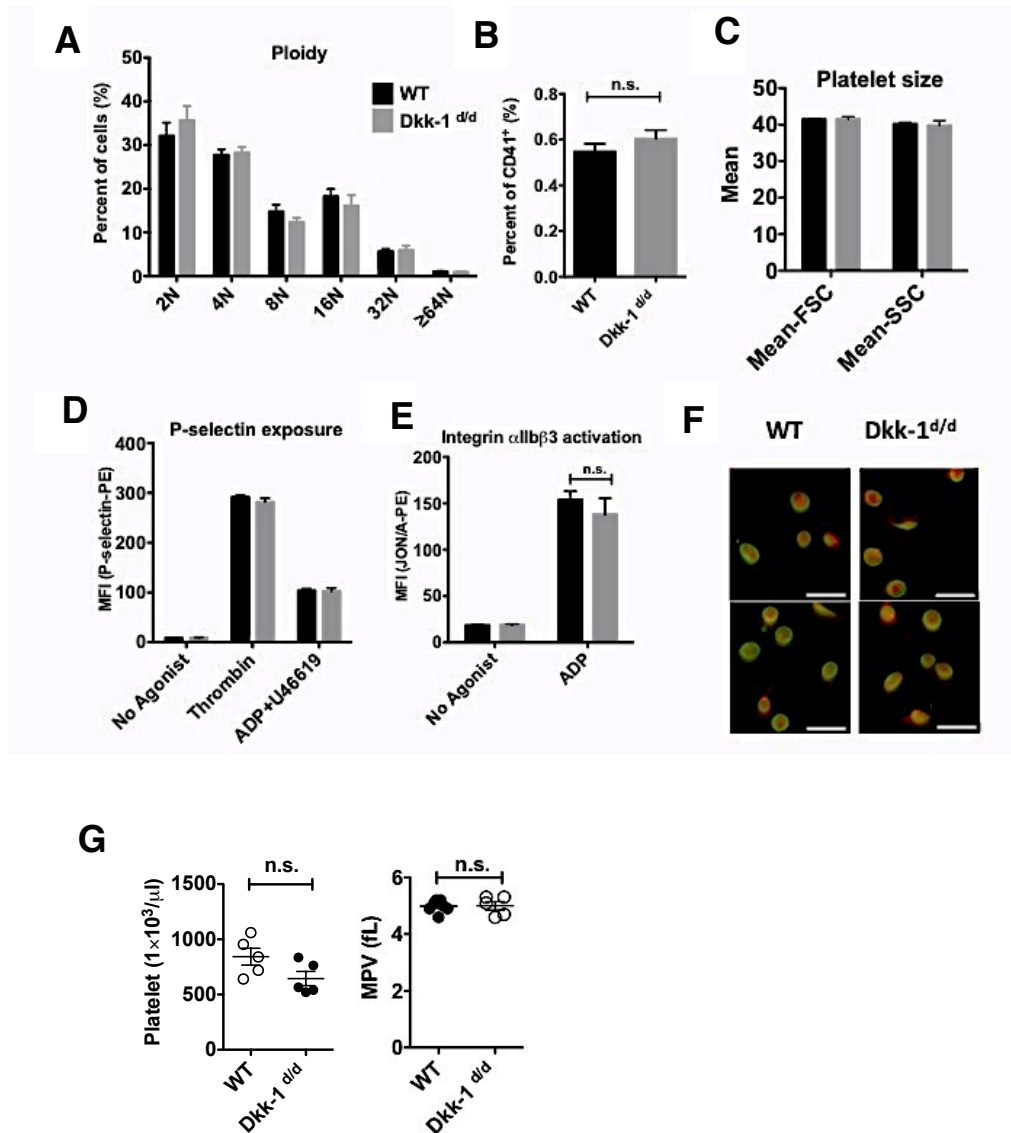


Figure S2, related to Figure 1. Dkk-1^{d/d} mice have normal megakaryocyte ploidy, platelet size and platelet activation Six to ten week-old Dkk-1^{d/d} mice (n=3) and their wildtype littermate controls (n=3) were used. **(A)** Ploidy profile of CD41⁺ bone marrow cells assessed by propidium iodide staining (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 α IIb β 3 receptor in response to ADP **(E)** at comparable levels to WT (n=3 per genotype). **(F)** Platelet cytoskeletal architecture assessed by b-tubulin and Phalloidin (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 ($\pm$ s.e.m.). A representative of two independent experiments are shown.

Figure S3

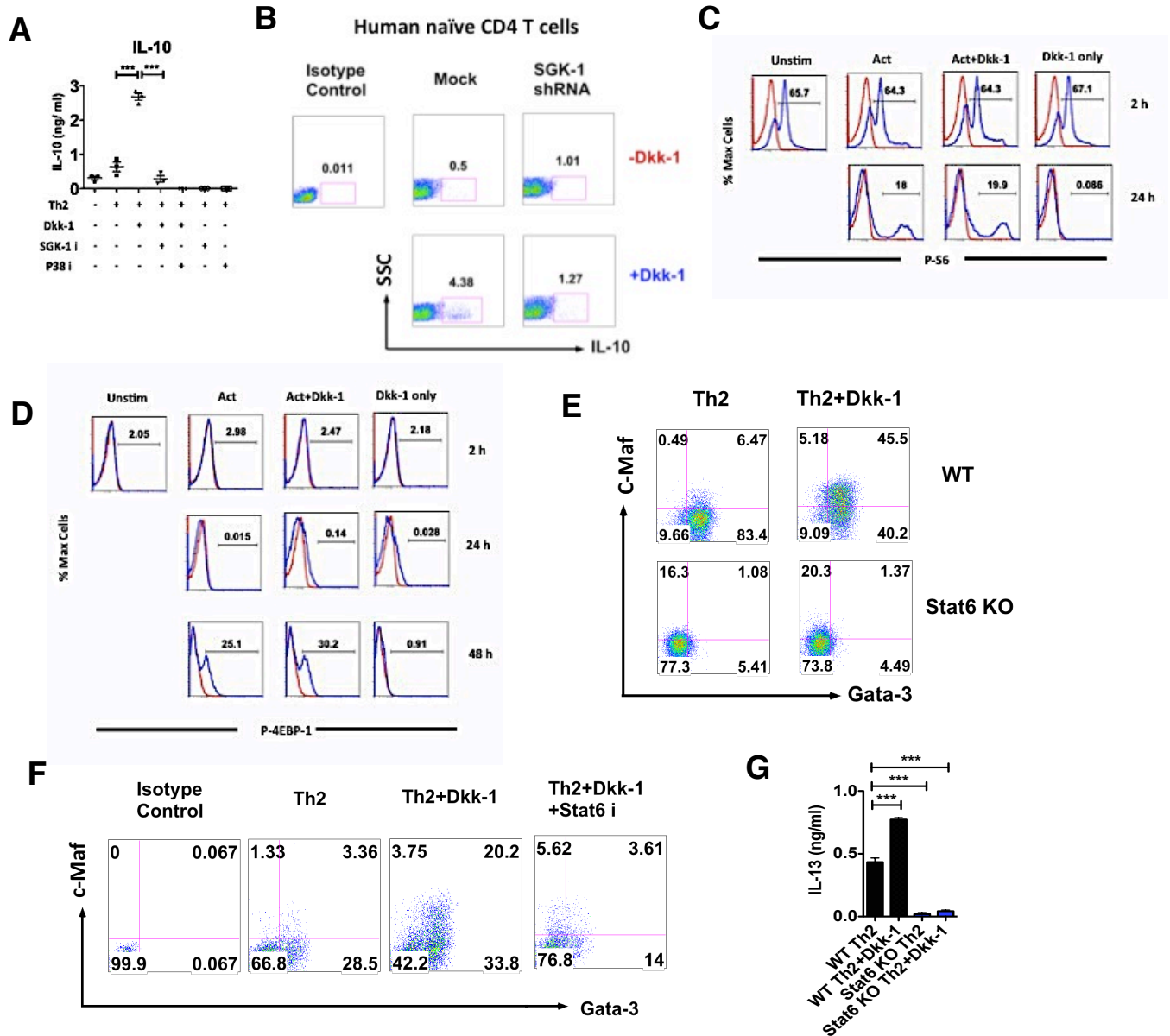


Figure S3, related to Figure 5. Dkk-1 induces IL-10 in CD4 T cells via SGK-1 and p38 MAPK

(A) Naïve CD4 T cells were differentiated into Th₂ cells with or without Dkk-1 (30 ng/ml), SGK-1 inhibitor (2 μ M) or (B) p38 inhibitor (10 μ M) 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 μ g/ml) for 48 hr. The selected cells were then stimulated with α CD3 mAb (OKT3) and α CD28 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 ($\pm$ s.e.m.). (C, D) Splenic CD4+CD25- cells from 6 week-old C57BL/6 mice were isolated and activated with α CD3 and α CD28 mAb (2 μ g/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 Dunnett's post-hoc test was performed. ***, p<0.0005. A representative of two independent experiments is shown.

Figure S4

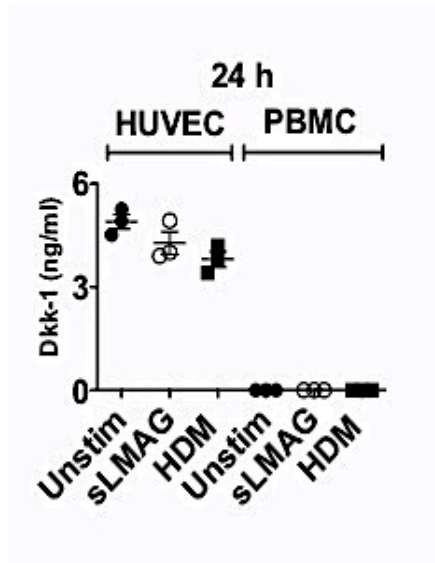


Figure S4, related to Figure 6. Dkk-1 is not induced by sLMAG or HDM in HUVECs or PBMCs.

HUVECs (2×10^5 /ml) (n=2) or human PBMCs (n=3) (2×10^6 /ml) were stimulated with either HDM extract (15 μ g/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

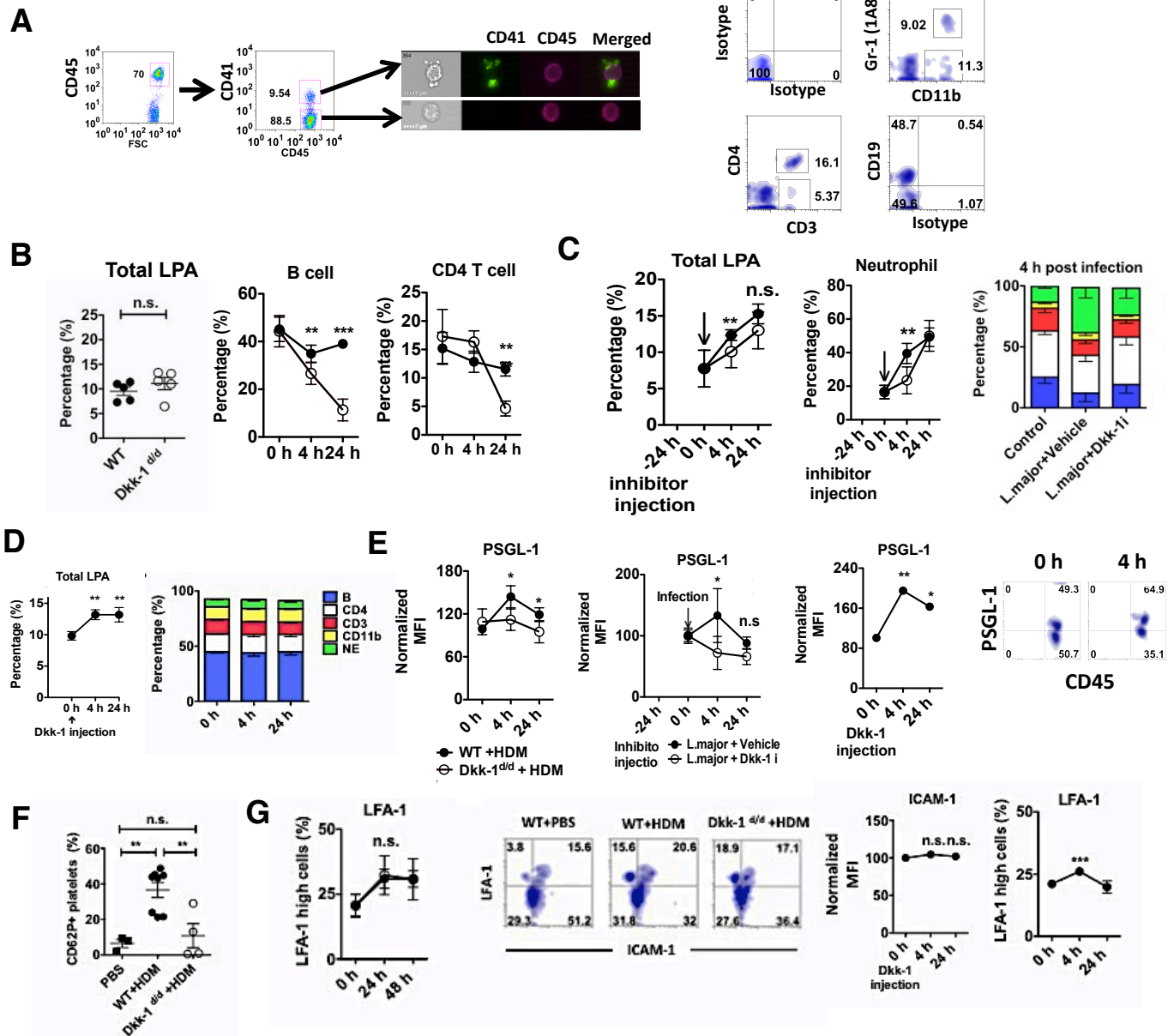


Figure S5, related to Figure 7. Regulation of LPA formation by Dkk-1 (A) Peripheral blood from 6-week old C57BL/6 male mice was analyzed by AMNIS. (B) (Left panel) The basal LPA population (CD45⁺CD41⁺) was measured in Dkk-1^{d/d} mice (n=5) and their wildtype littermate controls (n=5). (Right panels) 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

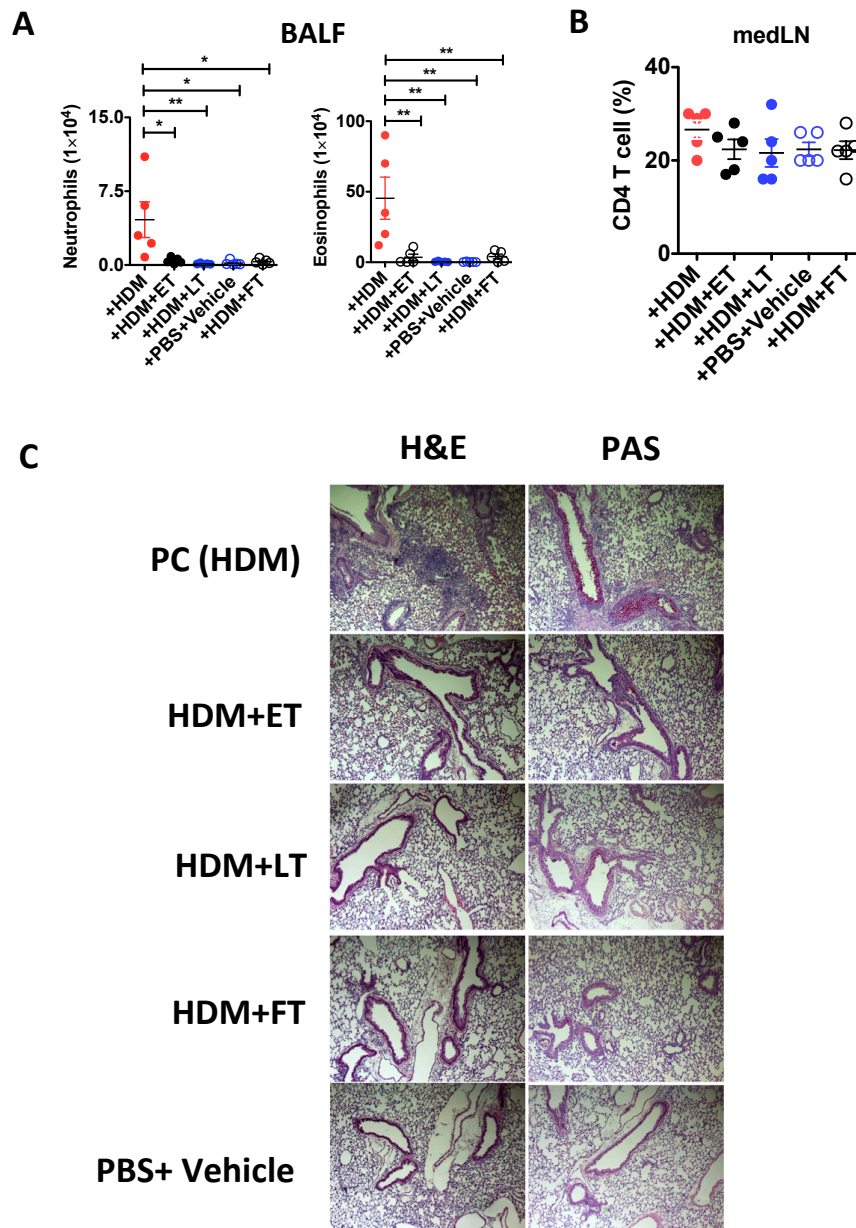


Figure S6, related to Figure 7. Pharmacological antagonism of Dkk-1 (A) Neutrophils and eosinophils in BALF were counted for each group (n=5) as described in Figure 7E. **(B)** CD4 T cell percentages in med LNs were measured by flow cytometry. **(C)** Representative images H&E and PAS staining were shown. 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 ($\pm$ 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 CD8 β (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-SR α expression vector (Slanetz et al., 1991) Briefly, CHO (Chinese Hamster Ovary) cells were transfected with Dkk-1-pFRSV-SR α and then Dkk-1 expression was amplified by methotrexate (MTX) treatment. Before harvest, MTX was removed and cells were washed. As a control, pFRSV-SR α 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-3 β 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% H₂O₂ 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-rich plasma was washed (140mM NaCl, 5mM KCl, 12mM Na₃C₆H₅O₇, 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 MgCl₂, 5mM NaHCO₃, 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 MgCl₂, 5 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 5.5 mM dextrose, 0.42 mM Na₂HPO₄, 12 mM NaHCO₃ [pH 7.4]), spinning at 2800rpm to discard the supernatant. For staining, washed blood was resuspended in Tyrodes-HEPES with 2 mM CaCl₂. 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 (DynaBeads). 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, 2×10⁶ frozen PBMCs were thawed and stimulated with soluble leishmania antigen (equivalent to 5×10⁶ 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, 2×10⁴ 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 and resuspended

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 *doublerridge* 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 (sLMaAg) was employed. Single cell suspension of draining and non-draining lymph node cells was incubated with sLMaAg (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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