IMMUNOLOGY

PDLIM2 Inhibits T Helper 17 Cell Development and Granulomatous Inflammation Through Degradation of STAT3

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Granuloma formation is an important host defense mechanism against intracellular bacteria; however, uncontrolled granulomatous inflammation is pathologic. T helper 17 (T_H17) cells are thought to have a pathogenic role in autoimmune and inflammatory diseases, including in granulomas. Here, we report that the PDZ-LIM domain protein PDLIM2 inhibited T_H17 cell development and granulomatous responses by acting as a nuclear ubiquitin E3 ligase that targeted signal transducer and activator of transcription 3 (STAT3), a transcription factor critical for the commitment of naïve $CD4^+$ T cells to the T_H17 lineage. PDLIM2 promoted the polyubiquitination and proteasomal degradation of STAT3, thereby disrupting STAT3-mediated gene activation. Deficiency in PDLIM2 resulted in the accumulation of STAT3 in the nucleus, enhanced the extent of T_H17 cell differentiation, and exacerbated granuloma formation. This study delineates an essential role for PDLIM2 in inhibiting T_H17 cell–mediated inflammatory responses by suppressing STAT3 signaling and provides a potential therapeutic target for the treatment of autoimmune diseases.

INTRODUCTION

A granuloma serves to compartmentalize intracellular bacteria, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, and limit their infection within a restricted area (*1*). However, exaggerated granulomatous responses lead to tissue damage and they impair normal organ function. For example, in tuberculosis, excessive granulomatous lesions in the lung may lead to respiratory failure. Moreover, some human autoimmune disorders, such as Crohn's disease, Wegener's granulomatosis, and sarcoidosis, manifest pathologic granulomatous inflammation in various organs. It is speculated that these human granulomatous diseases are caused by dysregulated host immune responses to as yet unidentified antigens or microorganisms. Indeed, *Propionibacterium acnes*, a Gram-positive intracellular bacterium, might be related to be the cause of human sarcoidosis (*2*, *3*), which manifests granulomas in the lung, liver, and eye.

T helper 17 (T_H17) cells are a newly identified helper $CD4^+$ T cell subset that is distinct from T_H1 and T_H2 cells as defined by cytokine profile (*4*, *5*). Whereas T_H1 and T_H2 cells produce the cytokines interferon-γ (IFN-γ) and interleukin-4 (IL-4), respectively, T_H17 cells secrete IL-17, IL-21, and IL-22 as effector cytokines. T_H17 cells can induce a massive inflammatory response and have essential roles in eliminating microbial pathogens. However, T_H17 cells are considered to be highly pathogenic, because excessive and prolonged activation of T_H17 cells may cause human autoimmune and inflammatory diseases, including rheumatoid arthritis and inflammatory bowel diseases. Studies have also implicated T_H17 cells as being critically involved in the development of granulomatous inflammation (*6*, *7*), which suggests that controlling the development of T_H17 cells will be an important strategy to prevent and treat granulomatous diseases.

The mechanisms by which T_H17 cell differentiation is promoted have been extensively studied. Several cytokines mediate the development of T_H17 cells. Transforming growth factor–β (TGF-β) and IL-6 together induce the differentiation of naïve $CD4^+$ T cells into T_H17 cells, whereas IL-21 acts as an autocrine growth factor for T_H17 cells and IL-23 stabilizes the T_H17 phenotype (*4*, *5*). Moreover, that transcription factor signal transducer and activator of transcription 3 (STAT3) is required for the commitment of naïve $CD4^+$ T cells to the T_H17 lineage, a situation analogous to the requirements for STAT4 and STAT6 in the development of T_H1 and T_H2 cells, respectively (*8*, *9*). However, the molecular mechanisms that inhibit the differentiation of T_H17 cells are not fully understood.

PDLIM2 (also known as SLIM or mystique) is a nuclear protein that contains both PDZ (postsynaptic density 95–discs large–zonula occludens 1) and LIM (abnormal cell lineage 11–islet 1–mechanosensory abnormal 3) domains (*10*, *11*). Here, we identified PDLIM2 as an E3 ubiquitin ligase for STAT3 that inhibited T_H17 cell differentiation. PDLIM2-deficient mice showed exacerbated liver granulomatous inflammation in response to *P. acnes*, a process in which T_H17 cells are thought to have a pathogenic role. In *Pdlim2*$^{-/-}$ $CD4^+$ T cells, the development of T_H17 cells, as well as the expression of RORγt, a STAT3-dependent master transcription factor required for the differentiation of T_H17 cells (*12*), was augmented. We further showed that PDLIM2 promoted the polyubiquitination and proteasome-dependent degradation of STAT3 protein, thereby terminating STAT3 signaling. Consistently, deficiency in PDLIM2 resulted in increased amounts of nuclear STAT3 protein and STAT3-mediated transactivation of gene expression. Our work demonstrates that PDLIM2 acts as an endogenous inhibitor for STAT3 activation and T_H17 cell–mediated inflammatory responses.

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RESULTS

Pdlim2$^{-/-}$ mice exhibit enhanced T_H17 cell–dependent responses

We previously reported that T_H1 cell development and inflammatory responses in response to *L. monocytogenes* are enhanced in mice lacking PDLIM2 (*10*). Infection by such intracellular bacteria, which was previously thought to promote T_H1-type immune responses, is now known to induce T_H17 cell differentiation as well (*7*, *13*, *14*). Moreover, PDLIM2 is found in the nucleus in not only T_H1 cells but also other T cell subsets, including T_H17 cells (fig. S1). These findings led us to examine whether PDLIM2 also controlled the development of T_H17 cells. For this purpose, we used the established model of *P. acnes*–induced liver granuloma formation (*15*). Because IL-6 is essential for the generation of T_H17 cells (*16*, *17*), we first examined IL-6–deficient mice (*18*) to clarify the contribution of T_H17 cells to *P. acnes*–mediated development of liver granulomas. We observed massive granuloma formation in the livers of *Il-6*$^{+/+}$ mice on day 7 after injection of heat-killed *P. acnes* (Fig. 1A). By contrast, the number of granulomas in *Il-6*$^{-/-}$ mice was significantly reduced (the number of granulomas per 3.8 mm^2 of tissue was 29.25 compared to 8.0, $P = 0.029$) (Fig. 1A and fig. S2A). Moreover, $CD4^+$ T cells isolated from *Il-6*$^{-/-}$ mice injected with *P. acnes* produced lower amounts of the T_H17 effector cytokines IL-17, IL-21, and IL-22 than did *Il-6*$^{+/+}$ mice, whereas production of the T_H1 effector cytokine IFN-γ was comparable in comparison to that produced by *Il-6*$^{+/+}$ mice (Fig. 1C), suggesting that the ability to mount a T_H17 cell–dependent response was specifically impaired in *Il-6*$^{-/-}$ mice. These data indicated that *P. acnes* induced T_H17 cell–dependent granuloma formation.

We then administered heat-killed *P. acnes* to *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice. The granuloma formation that we observed in the livers of *Pdlim2*$^{-/-}$ mice was significantly enhanced compared to that in the livers of *Pdlim2*$^{+/+}$ mice (the number of granulomas per 3.8 mm^2 of tissue was 2.9 compared to 31.8, $P = 0.001$) (Fig. 1B and fig. S2B). Moreover, *P. acnes*–primed *Pdlim2*$^{-/-}$ $CD4^+$ T cells produced higher amounts of IL-17, IL-21, and IL-22 than did similarly primed *Pdlim2*$^{+/+}$ cells (Fig. 1D). Consistent with our previous report (*10*), the amount of IFN-γ produced by *P. acnes*–primed *Pdlim2*$^{-/-}$ $CD4^+$ T cells was also augmented compared to that produced by *P. acnes*–primed *Pdlim2*$^{+/+}$ cells. In contrast, the amount of the T_H2 cytokine IL-4 was not affected by PDLIM2 deficiency. These data demonstrated that PDLIM2 inhibited the development not only of T_H1 cells but also of T_H17 cells, as well as inhibiting granuloma formation.

Pdlim2$^{-/-}$ cells show enhanced T_H17 cell differentiation in vitro

To clarify how PDLIM2 was involved in the regulation of T_H17 cell differentiation, we isolated total spleen cells from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice, cultured them with heat-killed *P. acnes*, and evaluated T_H17 cell differentiation. *Pdlim2*$^{-/-}$ $CD4^+$ T cells produced two- to fivefold more

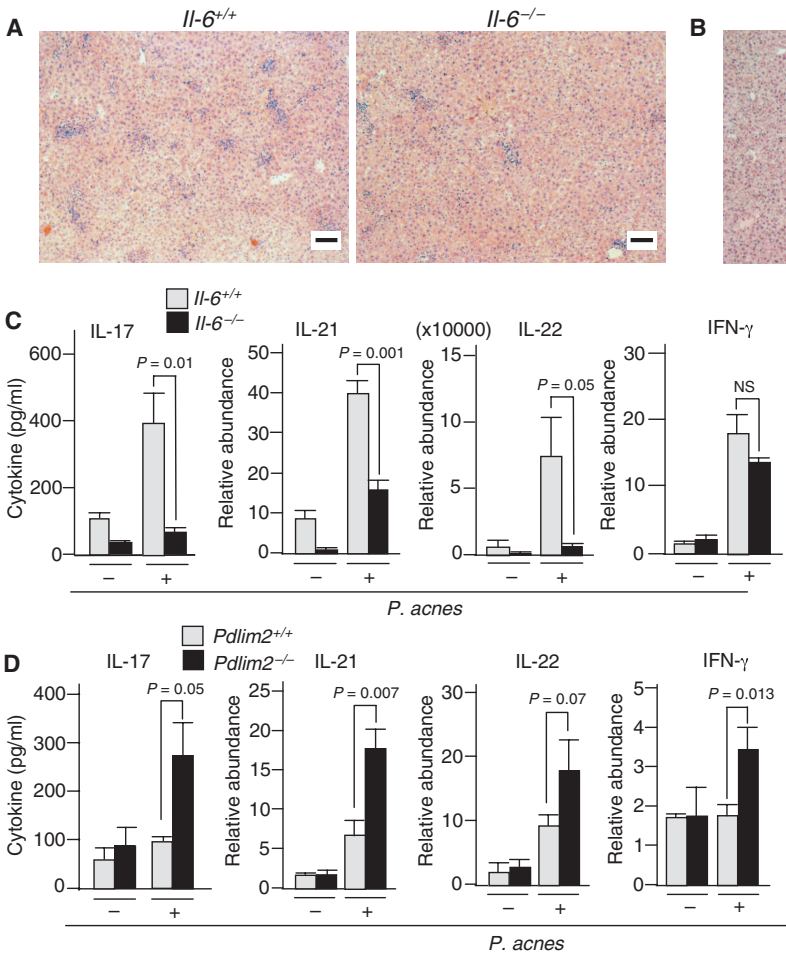

Fig. 1. Enhanced liver granuloma formation and T_H17 cell differentiation in *Pdlim2*$^{-/-}$ mice. (**A** and **B**) Representative H&E-stained liver sections on day 7 after intraperitoneal injection of heat-killed *P. acnes* (0.3 mg). Scale bars, 100 μm. (**C** and **D**) ELISA analysis of IL-17 production and real-time RT-PCR analysis of the abundances of mRNAs of IL-21, IL-22, IFN-γ, and IL-4 in $CD4^+$ T cells from mice that were untreated (–) or were treated (+) with heat-killed *P. acnes* (1 mg for IL-17 experiments and 0.3 mg for IL-21, IL-22, IFN-γ, and IL-4 experiments). Three to seven mice per group were analyzed. Shown are the mean values ± SEM. NS, not significant.

IL-17, IL-21, and IL-22 than did *Pdlim2*$^{+/+}$ cells (Fig. 2A). Moreover, *P. acnes*–induced IFN-γ production was enhanced in *Pdlim2*$^{-/-}$ CD4$^+$ T cells compared to that in *Pdlim2*$^{+/+}$ cells. *Pdlim2*$^{-/-}$ CD4$^+$ T cells also produced increased amounts of IL-4 compared to that produced by *Pdlim2*$^{+/+}$ cells, but the increase was not as prominent as for other cytokines.

In response to *P. acnes*, dendritic cells produce inflammatory cytokines, including IL-6, which then act on CD4$^+$ T cells and promote their differentiation into T_H17 cells (*5*). PDLIM2 is found in dendritic cells and CD4$^+$ T cells (*10*, *11*). We therefore isolated CD4$^+$ T cells and CD11c$^+$ dendritic cells from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice and cocultured them in various combinations in the presence of heat-killed *P. acnes* to determine which cell type was affected by a deficiency in PDLIM2. The amount of IL-17 produced by *Pdlim2*$^{-/-}$ CD4$^+$ T cells was significantly enhanced compared to that by *Pdlim2*$^{+/+}$ CD4$^+$ T cells regardless of the origin of the cocultured dendritic cells. In addition, although to a lesser extent, coculture with *Pdlim2*$^{-/-}$ dendritic cells also increased the extent of T_H17 cell development of both *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ CD4$^+$ T cells (Fig. 2B), a result consistent with our finding that *Pdlim2*$^{-/-}$ dendritic cells produced higher amounts of IL-6 in response to *P. acnes* than did wild-type dendritic cells (fig. S3). These data suggested that the CD4$^+$ T cells in *Pdlim2*$^{-/-}$ mice were the major contributors to the augmented T_H17 cell development, although the dendritic cells also had some role. To demonstrate that PDLIM2 regulated T_H17 cell differentiation in a T cell–intrinsic manner, we cultured purified naïve CD4$^+$ T cells under T_H17-skewing conditions with TGF-β and IL-6. *Pdlim2*$^{-/-}$ CD4$^+$ T cells showed a marked increase in the amount of IL-17A that they produced compared to that produced by *Pdlim2*$^{+/+}$ cells; however, the percentage of *Pdlim2*$^{-/-}$ CD4$^+$ T cells that produced IL-17A (that is, cells that were IL-17A$^+$) was only marginally higher than that of *Pdlim2*$^{+/+}$ CD4$^+$ T cells (Fig. 2C). Moreover, IL-6–induced production of IL-21 by *Pdlim2*$^{-/-}$ T cells was also enhanced compared to that produced by *Pdlim2*$^{+/=}$ T cells (Fig. 2D). Additionally, IL-6–induced production of RORγt, a STAT3-dependent master transcription factor that is required for the differentiation of T_H17 cells (*12*), was increased in *Pdlim2*$^{-/-}$ cells compared to that in *Pdlim2*$^{+/+}$ CD4$^+$ T cells (Fig. 2E). Together, these data suggest that PDLIM2 inhibits the ability of naïve CD4$^+$ T cells to differentiate into T_H17 cells.

PDLIM2 mediates suppression of STAT3 signaling

We previously demonstrated that PDLIM2 is a nuclear E3 ubiquitin ligase for STAT4, suppressing its activation and the differentiation of T_H1 cells. Here, we found that IL-6–induced expression of T_H17 cell–related genes was increased in *Pdlim2*$^{-/-}$ CD4$^+$ T cells compared to that in *Pdlim2*$^{+/+}$ cells (Fig. 2, C and D). Although IL-6 is reported to activate both STAT1 and STAT3 (*19*), we found that IL-6 specifically induced tyrosine phosphorylation of STAT3 in CD4$^+$ T cells (fig. S4). We therefore investigated whether PDLIM2 regulated the activity of STAT3. We first analyzed the effect of PDLIM2 on STAT3-mediated transcriptional activation in experiments with a luciferase reporter driven by the α*2-macroglobulin* promoter, which contains STAT3-binding sites. The stimulation of the cells transfected with this reporter plasmid by leukemia inhibitory factor (LIF) led to a marked increase in luciferase reporter activity, whereas cotransfection of cells with a plasmid encoding PDLIM2 markedly impaired STAT3-mediated transactivation of the reporter in a dose-dependent

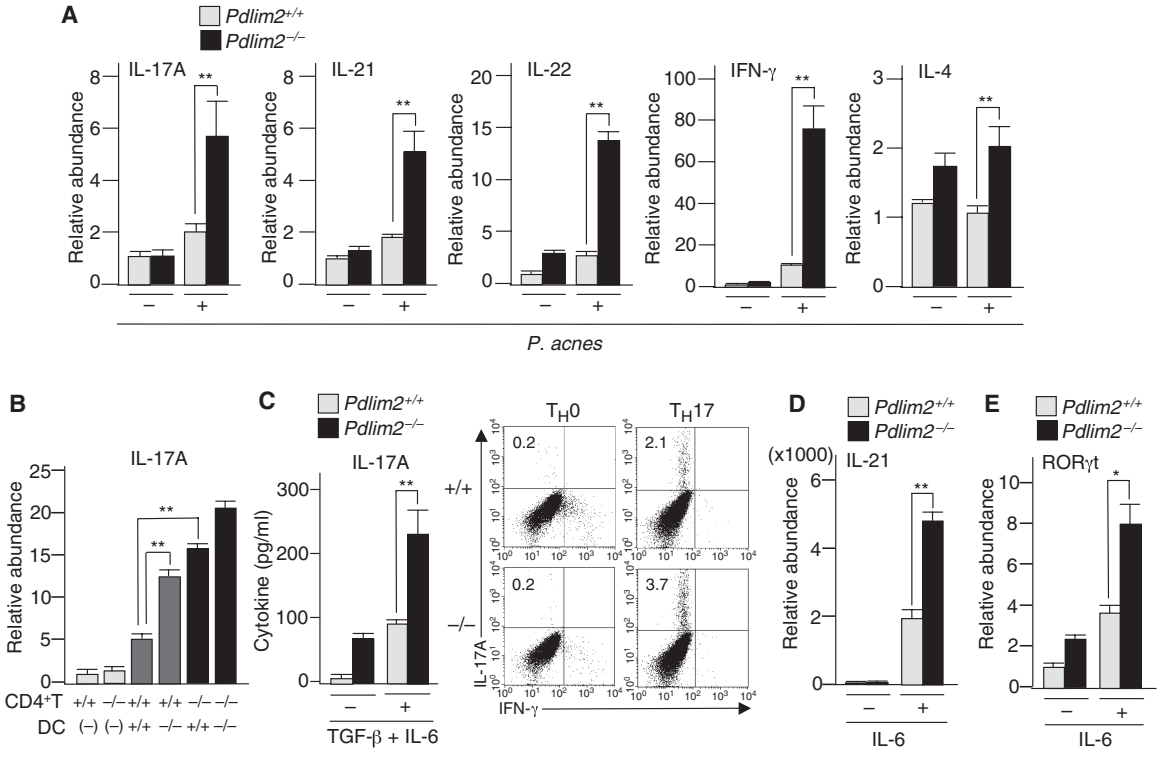

Fig. 2. Enhanced in vitro T_H17 differentiation of cells from *Pdlim2*$^{-/-}$ mice. (**A**) Real-time RT-PCR analysis of genes expressing T_H17-type cytokines by *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ CD4$^+$ T cells in *P. acnes*–induced in vitro T_H17 cell differentiation experiments. (**B**) Heat-killed *P. acnes*–induced expression of the gene encoding IL-17A by CD4$^+$ T cells cocultured with CD11c$^+$ dendritic cells (DC) isolated from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice in the presence of heat-killed *P. acnes* was analyzed by real-time RT-PCR assay. (**C**) ELISA analysis of IL-17A production (left) and flow cytometric analysis of intracellular IL-17A and IFN-γ (right) in naïve CD4$^+$ T cells from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice. Numbers in the flow cytometry dot plots indicate the percentages of IL-17A$^+$ cells. (**D**) Real-time RT-PCR analysis of the expression of the genes encoding IL-17A and IL-21 in naïve CD4$^+$ T cells from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice. (**E**) Real-time RT-PCR analysis of IL-6–induced expression of the gene encoding RORγt in CD4$^+$ T cells from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice. Data are representative of at least three independent experiments and are shown as means ± SD. **P* < 0.05; ***P* < 0.01.

manner (Fig. 3A). PDLIM2 also suppressed transactivation induced by STAT3-C, a cytokine-independent, constitutively active form of STAT3 (Fig. 3B), suggesting that PDLIM2 acted directly on STAT3 to suppress STAT3-mediated gene activation.

PDLIM2 physically interacts with STAT3

We next tested whether PDLIM2 physically associated with STAT3. We coimmunoprecipitated PDLIM2 with STAT3 from unstimulated human embryonic kidney (HEK) 293T cells, and this association was substantially enhanced in cells stimulated with LIF (Fig. 3C). Consistent with this finding, PDLIM2 interacted with tyrosine-phosphorylated STAT3 (Fig. 3D). Furthermore, STAT3 Y705F, a mutant STAT3 in which the tyrosine residue essential for STAT3 activation was mutated and unable to be phosphorylated, also associated with PDLIM2 (Fig. 3D), suggesting that PDLIM2 bound to both unphosphorylated and phosphorylated forms of STAT3. We next delineated the domains of STAT3 that mediated the interaction with PDLIM2 in experiments with a series of STAT3 deletion mutants. We found that the DNA binding and the N-terminal domains of STAT3 interacted with PDLIM2 (Fig. 3E), suggesting that STAT3 contains multiple binding sites for PDLIM2. In a reciprocal analysis, we determined which

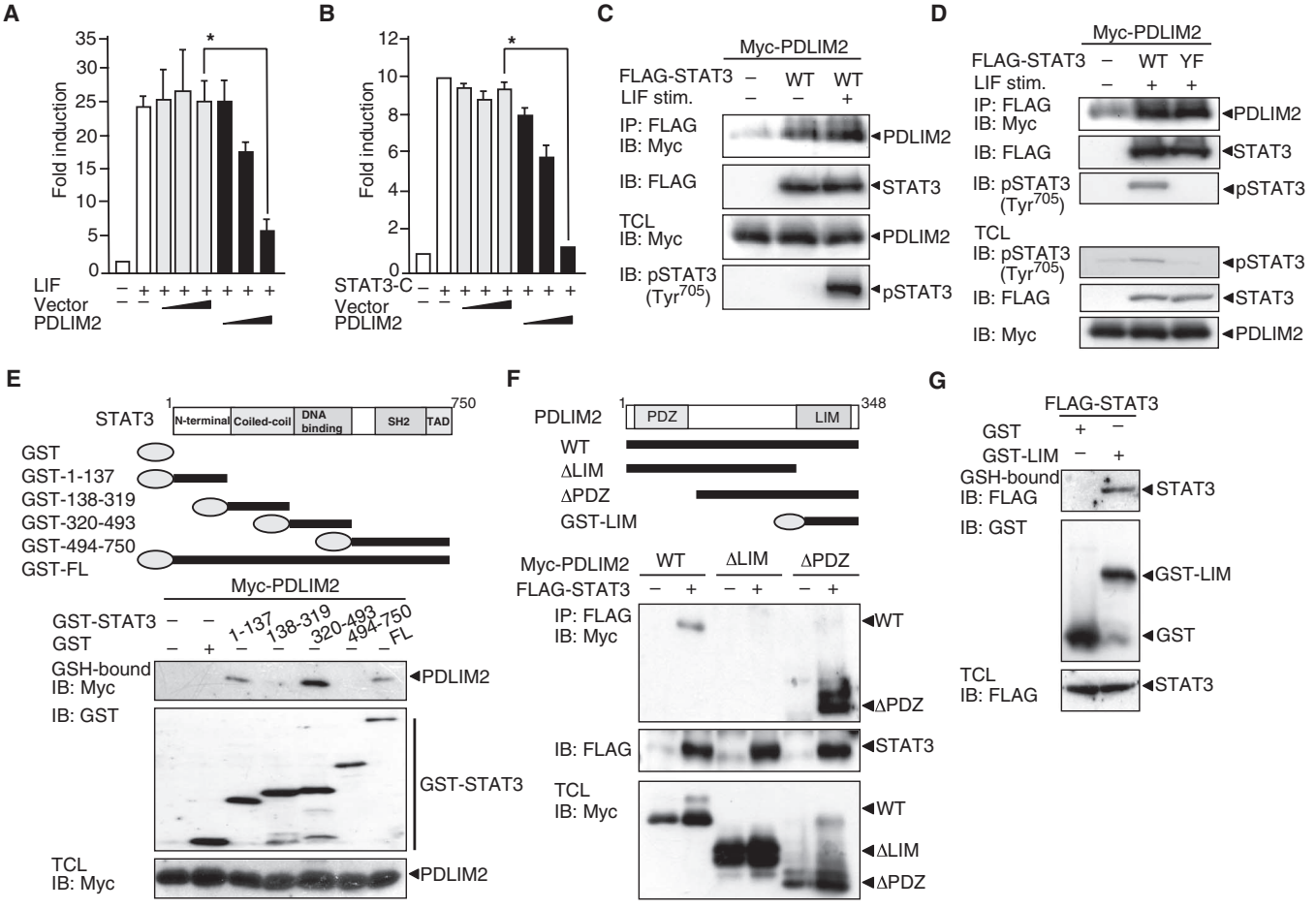

Fig. 3. PDLIM2 physically associates with STAT3 and suppresses STAT3 signaling. (**A** and **B**) Luciferase activity in (A) HEK 293T cells cotransfected with an *α2-macroglobulin* luciferase reporter and either control or PDLIM2 expression plasmids that were stimulated by LIF (100 ng/ml) for 8 hours, or in (B) HEK 293T cells cotransfected with plasmid encoding STAT3-C. (**C** and **D**) Interaction between PDLIM2 and (C) wild-type (WT) STAT3 or (D) STAT3 Y705F mutant (YF) in the lysates of HEK 293T cells that had been transfected with the indicated plasmids and then were stimulated without or with LIF (100 ng/ml) for 30 min. Samples were subjected to immunoprecipitation (IP) with antibody against FLAG followed by Western blotting analysis (IB) with antibody against the c-Myc tag. TCL, total cell lysates. (**E**) Critical region of STAT3 for interaction with PDLIM2. Western blotting analysis of the lysates of HEK 293T cells transfected with plasmids encoding PDLIM2 and GST-fused STAT3 deletion mutants, which were pulled down with GSH-Sepharose (GSH-bound). The constructs of the STAT3 deletion mutants are shown on the top. (**F**) Critical region of PDLIM2 that is required for its association with STAT3. Western blotting analysis of lysates from HEK 293T cells transfected with plasmids encoding FLAG-STAT3 and the indicated PDLIM2 mutants, which were subjected to immunoprecipitation with antibody against the FLAG tag. The constructs of the PDLIM2 deletion mutants are shown on the top. (**G**) In vitro binding of the LIM domain of PDLIM2 to STAT3. Western blotting analysis of lysates from HEK 293T cells that were transfected with plasmid encoding FLAG-STAT3, incubated in vitro with GST-LIM, and subjected to pull-down assay with GSH-Sepharose. Data are representative of at least three independent experiments and are shown as means ± SD. $^{**}P < 0.01$.

domain in PDLIM2 was involved in the interaction with STAT3. A PDLIM2 mutant lacking the LIM domain (PDLIM2-ΔLIM) failed to interact with STAT3, whereas a PDLIM2 mutant lacking the PDZ domain (PDLIM2-ΔPDZ) was still functional in this assay (Fig. 3F). In addition, the purified recombinant LIM domain of PDLIM2 could also interact in vitro with purified STAT3 (Fig. 3G). These data suggested that the LIM domain of PDLIM2 was required for its interaction with STAT3.

PDLIM2 mediates the ubiquitination and degradation of STAT3

Because PDLIM2 is an E3 ubiquitin ligase, we next determined whether STAT3 was a target of PDLIM2-mediated ubiquitination. We found that STAT3 was polyubiquitinated in HEK 293T cells only when it was coexpressed with PDLIM2 (Fig. 4A). Consistent with our previous finding that PDLIM2 functions as an E3 ubiquitin ligase through its LIM domain (*10*), which is similar to a really interesting new gene (RING) finger domain, PDLIM2-ΔLIM was impaired in its ability to mediate the polyubiquitination of either STAT3 or PDLIM2 itself (Fig. 4, B and C). Furthermore, the purified recombinant LIM domain from PDLIM2 supported the polyubiquitination of STAT3 in vitro (Fig. 4D). Ubiquitinated proteins are degraded through a proteasomal pathway; thus, we assessed whether PDLIM2 regulated the steady-state abundance of STAT3 protein. Transfection of HEK 293T cells with plasmid encoding PDLIM2 resulted in a marked reduction in the amount of endogenous STAT3 protein compared to that in cells transfected with empty vector (Fig. 4E), an effect that was reversed when the cells were treated with MG132, an inhibitor of the proteasome-dependent degradation pathway (Fig. 4E). We also tested which domain in PDLIM2 was important for the degradation of STAT3 in experiments with PDLIM2 deletion mutants. Consistent with the requirement for the LIM domain for ubiquitination of STAT3, we found that PDLIM2-ΔLIM failed to result in decreased amounts of endogenous STAT3, whereas PDLIM2-ΔPDZ reduced the amount of STAT3 as efficiently as did wild-type PDLIM2 (Fig. 4F). Together, these data suggested that PDLIM2 promoted the polyubiquitination and proteasomal degradation of STAT3 through its LIM domain.

PDLIM2 deficiency results in enhanced STAT3 activation

Finally, we investigated the role of endogenous PDLIM2 in regulating STAT3 activation. We first evaluated the stability of STAT3 protein in mouse embryonic fibroblasts (MEFs) derived from *Pdlim2*$^{+/+}$ and *Pdlim2*$^{-/-}$ mice and treated with cycloheximide and found that STAT3 protein was more stable in the PDLIM2-deficient cells than in the wild-type cells (Fig. 5A). We next assessed the activity of STAT3 in *Pdlim2*$^{-/-}$ cells. LIF-induced, STAT3-dependent gene expression in *Pdlim2*$^{-/-}$ MEFs was substantially increased compared to that in *Pdlim2*$^{+/+}$ cells as measured by luciferase assay (Fig. 5B). Moreover, specific knockdown of PDLIM2 by small interfering RNA (siRNA), in which PDLIM2 mRNA abundance was reduced to about 35% of that in control cells (fig. S5), also resulted in a substantial increase in the amount of STAT3 protein (Fig. 5C) and an enhancement in IL-6–induced STAT3 transactivation (Fig. 5D). These data indicated that PDLIM2 inhibited both the amount of STAT3 protein and the extent of STAT3 activation.

We then examined whether endogenous STAT3 in CD4^{+} T cells could be regulated by a proteasome-dependent degradation pathway. MG132 increased the amount of nuclear, but not cytoplasmic, STAT3 in IL-6–stimulated cells (Fig. 5E), indicating that the amount of nuclear STAT3, which

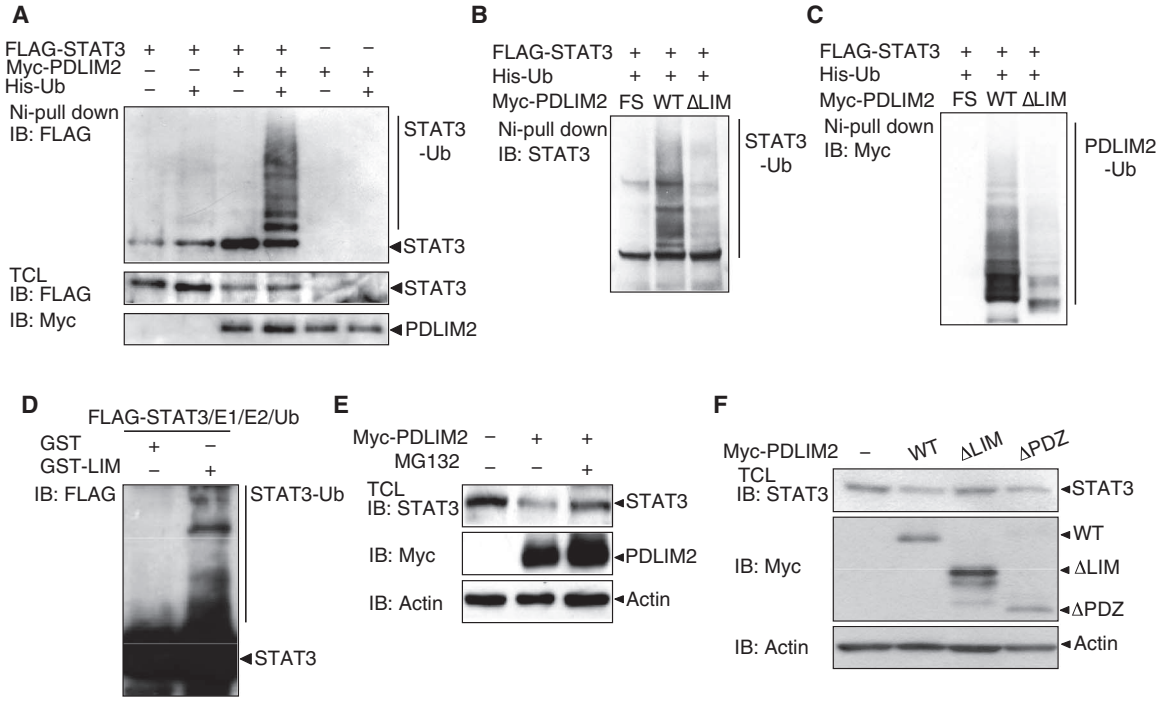

Fig. 4. PDLIM2 promotes the ubiquitination and degradation of STAT3. (**A** to **C**) Ubiquitination assay for STAT3 in HEK 293T cells cotransfected with plasmid encoding histidine-tagged ubiquitin (His-Ub) and the indicated plasmids. His-tagged proteins were purified by Ni-NTA resin (Ni-pull down). Polyubiquitination of STAT3 (A and B) and autoubiquitination of PDLIM2 (C) were detected with the indicated antibodies. (**D**) In vitro polyubiquitination of STAT3 by the LIM domain of PDLIM2 (GST-LIM). The lysates from HEK 293T cells cotransfected with plasmids encoding FLAG-STAT3, E1 enzyme, and E2 enzyme were incubated in vitro with GST-LIM and ubiquitin and were then subjected to Western blotting analysis with antibody against the FLAG tag to detect ubiquitinated STAT3. (**E**) Western blotting analysis for STAT3 in HEK 293T cells transfected with plasmid encoding c-Myc–PDLIM2, which were left untreated or were treated with MG132 (20 μM) for 3 hours. (**F**) Western blotting analysis for STAT3 in HEK 293T cells transfected with plasmids encoding the indicated PDLIM2 mutants. Data are representative of at least three independent experiments.

corresponds to the transcriptionally active population of STAT3 molecules, could be controlled by proteasomal degradation. Furthermore, there was more nuclear, but not cytoplasmic, STAT3 in IL-6–stimulated *Pdlim2*$^{-/-}$ CD4^{+} T cells than in similarly treated *Pdlim2*$^{+/+}$ cells (Fig. 5F). Together, these data demonstrate that PDLIM2 functions as a nuclear ubiquitin E3 ubiquitin ligase for STAT3, which inhibits STAT3-mediated signaling by ubiquitin- and proteasome-dependent degradation. The resulting increase in STAT3 activity then contributed to the enhanced T_H17 cell development seen in *Pdlim2*$^{-/-}$ mice.

DISCUSSION

STAT3 is involved in the pathogenesis of various human diseases, including autoimmune and inflammatory disorders. The activation of STAT3 is therefore tightly regulated at multiple levels to prevent these pathological conditions. Several factors are known to associate with STAT3 and suppress its activity. These include protein tyrosine phosphatases (PTPs), such as PTP1B (*20*), TC-PTP (*21*), and PTP-RT (*22*), which directly dephosphorylate activated STAT3, and the protein inhibitor of activated STAT3 (PIAS3), which blocks STAT3–DNA binding interactions (*23*). The ubiquitin- and proteasome-dependent degradation of STAT3 also inhibits its activity (*24*, *25*); however, the endogenous ubiquitin E3 ligase responsible for STAT3 degradation has remained elusive. Here, we have shown that PDLIM2 is a nuclear ubiquitin E3 ligase that targets STAT3 and terminates STAT3-mediated signaling.

PDLIM2 promoted the polyubiquitination and proteasomal degradation of STAT3 by means of its LIM domain. Consistent with this, a deficiency in PDLIM2, through either targeted gene disruption or siRNA-mediated knockdown, resulted in the accumulation of STAT3 in the nucleus and enhanced STAT3-mediated gene activation, possibly because of insufficient degradation of STAT3 protein. We also demonstrated that the LIM domain of PDLIM2 was essential for its binding to STAT3. It is possible that the inability of PDLIM2-ΔLIM to polyubiquitinate STAT3 might be ascribed to the impaired binding of this mutant to STAT3; however, this is unlikely, because in addition to losing the ability to polyubiquitinate STAT3, the PDLIM2-ΔLIM mutant also lost the ability to undergo autoubiquitination. Protein ubiquitination requires three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). RING-type E3 ligases promote the transfer of the polyubiquitin chain from the E2 enzyme to the substrates by binding to E2 through their RING finger domain and interacting with substrate proteins generally through other regions. We speculate that, in the case of PDLIM2, the LIM domain is required for the binding to both the E2 enzyme and the substrate STAT3. This is consistent with the finding that the LIM domain of PDLIM2 was sufficient to lead to the polyubiquitination of STAT3 in vitro.

We also demonstrated that PDLIM2 bound to not only the phosphorylated but also the unphosphorylated form of STAT3, although PDLIM2 binds to phosphorylated but not unphosphorylated STAT4 only when cells were activated (*10*). We propose the following possibility to account for this difference in the binding ability of PDLIM2. In addition

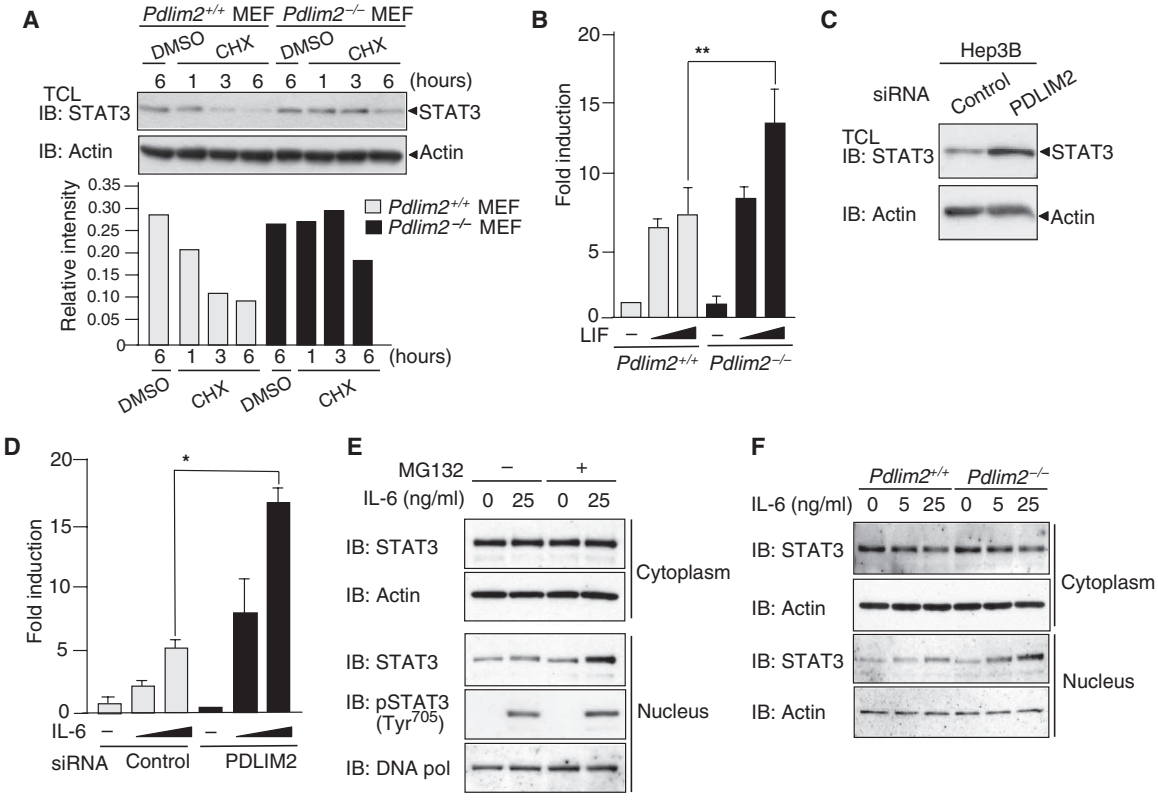

Fig. 5. Enhanced STAT3 activation in the context of PDLIM2 deficiency. (**A**) STAT3 protein amounts in *Pdlim2*$^{+/+}$ or *Pdlim2*$^{-/-}$ MEFs treated with cycloheximide (CHX) (50 ng/ml). Bottom shows the densitometric analysis of the intensities of the bands corresponding to STAT3 protein relative to those of actin. (**B**) Luciferase activity in *Pdlim2*$^{+/+}$ or *Pdlim2*$^{-/-}$ MEFs transfected with plasmid encoding the α*2-macroglobulin* promoter–driven luciferase reporter, which were stimulated without or with LIF (30 or 100 ng/ml) for 8 hours. (**C**) STAT3 protein abundance in Hep3B cells transfected with control siRNA or PDLIM2-specific siRNA. (**D**) Luciferase activity in Hep3B cells first transfected with control or PDLIM2-specific siRNA and then with plasmid encoding the α*2-macroglobulin* promoter–driven luciferase reporter, which were then stimulated without or with IL-6 (10 or 30 ng/ml) for 8 hours. Data in (C) and (D) represent the means ± SD. *$P < 0.05$; **$P < 0.01$. (**E** and **F**) Cytoplasmic and nuclear STAT3 protein amounts in WT CD4^{+} T cells pretreated without or with MG132 (20 μM) for 1.5 hours and stimulated with IL-6 for 1 hour (E) or, in the case of *Pdlim2*$^{+/+}$ or *Pdlim2*$^{-/-}$ CD4^{+} T cells, stimulated with IL-6 for 1 hour (F). Data are representative of at least three independent experiments.

to undergoing cytokine-induced nuclear translocation, STAT3 continuously shuttles between the cytoplasm and the nucleus even in unstimulated cells, a process that is independent of the tyrosine phosphorylation state of STAT3. This unphosphorylated form of STAT3 can associate with other transcription factors, such as nuclear factor κB (NF-κB), to bind to DNA and drive gene expression by mechanisms distinct from those used by phosphorylated STAT3 (*26*). Because PDLIM2 is a nuclear protein, PDLIM2 may bind to unphosphorylated STAT3 that is part of a transcription factor complex in the nucleus.

We previously demonstrated that PDLIM2 not only promoted the polyubiquitination of STAT4 but also suppressed the tyrosine phosphorylation of STAT4 independently of proteasomal degradation (*10*), although the precise mechanism involved remains unknown. We therefore tested whether PDLIM2 had an effect on the tyrosine phosphorylation status of STAT3. However, we found that PDLIM2 could not inhibit the tyrosine phosphorylation of STAT3, which suggested that PDLIM2 regulated STAT3 activation through a mechanism different from that used to regulate STAT4.

STAT3 is a master regulator of the development of the highly proinflammatory T_H17 subset of T cells. Because T_H17 cells play a key role in the pathogenesis of autoimmune diseases, the molecular mechanisms that stimulate or enhance the differentiation of T_H17 cells have been intensively studied. For example, in addition to STAT3, RORγt (*12*), RORx (*27*), IRF4 (*28*), Runx1 (*29*), Batf (*30*), and IκBα (*31*) have been identified as key transcription factors that promote T_H17 cell development. In contrast, we have only limited information on how the differentiation of T_H17 cells is terminated. Several cytokines, including IL-2, IL-4, IFN-γ, and IL-27, and the transcription factor Ets-1 inhibit T_H17 cell differentiation (*4*, *32*). However, the molecular mechanisms by which these factors suppress T_H17 cell development remain unclear. Here, we demonstrated that PDLIM2 inhibited T_H17 cell differentiation thorough the attenuation of the IL-6– and STAT3-mediated signaling pathway in $CD4^+$ T cells.

We have shown that *Pdlim2*$^{-/-}$ mice exhibited exaggerated *P. acnes*–induced granulomatous inflammation, which we demonstrated to be a T_H17 cell–mediated response. Certain human autoimmune and inflammatory disorders are characterized by excessive granulomatous lesions, which are often pathogenic. It is speculated that these diseases might be caused by uncontrolled immune responses, possibly because of the impaired inhibitory system of the host. Our data suggest that the PDLIM2-mediated pathway could be a candidate for this regulatory system preventing immunopathology. In future, *Pdlim2*$^{-/-}$ mice may be useful models for clarifying the pathogenesis of human granulomatous disorders.

Studies have shown that T_H17 cells are critically involved in the development of several autoimmune diseases that were previously considered to be mediated by T_H1 cells. However, other studies suggested that these T cell subsets are not mutually exclusive but cooperatively induce the inflammatory responses. For example, upon infection with *M. tuberculosis*, T_H17 cells produce the chemokines that are required to recruit IFN-γ–producing T_H1 cells to the sites of inflammation (*13*). Moreover, certain microorganisms, including *P. acnes*, can induce the development of IFN-γ– and IL-17–producing cells, which are suggested to be more potent effector cells (*14*, *33*). PDLIM2 may therefore regulate the development of both T_H17 and T_H1 cells depending on the stages of a disease or the types of infecting pathogens. Thus, the PDLIM2 pathway represents a useful new molecular target for the treatment of human autoimmune and inflammatory diseases mediated by T_H17 cells, T_H1 cells, or both.

MATERIALS AND METHODS

Reagents, antibodies, and plasmids

IL-6 was a gift from Ajinomoto Co. LIF was from Intergen. MG132 was obtained from the Peptide Institute. Cycloheximide and human TGF-β were purchased from Wako Pure Chemical Industries. Murine IL-12, IL-4, and IFN-γ were from R&D Systems. Expression vectors for STAT3-C were provided by J. F. Bromberg (Rockefeller University, New York, NY) (*34*), and the STAT3 luciferase reporter was provided by T. Hirano (Osaka University Medical School) (*35*). FLAG-tagged wild-type STAT3 and the Y705F mutant STAT3 were previously described (*36*). c-Myc–tagged PDLIM2 and its mutants were previously described (*10*, *11*). To prepare recombinant PDLIM2 protein, we expressed the glutathione *S*-transferase (GST)–tagged LIM domain of PDLIM2 (GST-LIM) in *Escherichia coli* and subsequently purified it on a GST column. Antibodies against c-Myc, GST, actin, the DNA polymerase-δ catalytic subunit, protein kinase C, and Sp1 were all obtained from Santa Cruz Biotechnology. Antibody against STAT3 was from Santa Cruz Biotechnology (for the experiments shown in Figs. 4, E and F, and 5, A and C) or from BD Transduction Laboratories (for the experiments shown in Fig. 5, E and F). Antibody against phosphorylated STAT3 (pSTAT3, Tyr^{705}), STAT1, and pSTAT1 (Tyr^{701}) were obtained from Cell Signaling. Mouse monoclonal and rabbit polyclonal antibodies against the FLAG tag were purchased from Sigma-Aldrich. Antiserum against PDLIM2 was previously described (*10*). Antibodies against CD3, CD28, and IL-4 were obtained from BD Biosciences. Antibody against IFN-γ was from eBioscience. For intracellular cytokine staining, allophycocyanin (APC)–conjugated antibody against CD4 (BD Biosciences), phycoerythrin (PE)–conjugated antibody against IL-17A (BD Biosciences), and fluorescein isothiocyanate (FITC)–conjugated antibody against IFN-γ (eBioscience) were used. Heat-killed *P. acnes* was provided by H. Tsutsui (Hyogo College of Medicine).

Cells and transfections

HEK 293T, Hep3B cells, and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). MEFs were prepared from embryos on day 13.5 after conception. $CD4^+$ T cells and dendritic cells purified from spleens were cultured in RPMI 1640 medium supplemented with 10% FCS. Bone marrow–derived dendritic cells (BMDCs) were obtained by culture of bone marrow cells for 7 days with human ligand for the receptor tyrosine kinase Flt3 (10 ng/ml). The methods used for transient transfection were as follows: the standard calcium precipitation protocol was used for HEK 293T cells; jetPEI (PolyPlus-transfection) was used for Hep3B cells; and Lipofectamine 2000 (Invitrogen) was used to transfect MEFs.

Small interfering RNA

Lipofectamine RNAiMAX (Invitrogen) was used for the transfection of cells with siRNAs. The sequence of siRNA targeting human PDLIM2 was as follows: 5′-GUACCAGCAUCGCGAACCATT-3′. The sequence of the control siRNA was as follows: 5′-UUCUCCGAACGUGUCACGUTT-3′.

Reporter assays

For luciferase reporter assays in HEK 293T cells, cells were transfected with plasmids encoding an *α2-macroglobulin* promoter–driven luciferase reporter and β-galactosidase together with empty vector or a plasmid encoding PDLIM2. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega). Luciferase activities were normalized to those of β-galactosidase. For luciferase assays in MEFs and Hep3B cells, we used the Dual-Luciferase Reporter Assay System (Promega).

Coimmunoprecipitations, GST pull downs, and Western blotting analysis

Total cell lysates were prepared as previously described (*21*). For coimmunoprecipitation experiments, total cell lysates were incubated with

antibody against the FLAG tag as well as protein G–Sepharose, washed four times, and analyzed by Western blotting. For GST pull-down experiments, total cell lysates were incubated with glutathione (GSH)–Sepharose and subjected to Western blotting analysis. To detect in vitro binding of the LIM domain of PDLIM2 to STAT3, we incubated lysates from HEK 293T cells that had been transfected with plasmid encoding FLAG-tagged STAT3 in vitro with the GST-tagged LIM domain of PDLIM2 and pulled them down with GSH-Sepharose. Cytoplasmic and nuclear extracts were prepared as previously described (*10*).

Ubiquitination assays

For ubiquitination assays in cells, we transfected HEK 293T cells with plasmids encoding histidine-tagged ubiquitin, FLAG-STAT3, and c-Myc–PDLIM2. Thirty-six hours later, we treated the cells with MG132 (20 μM) for 1 hour. We purified His-tagged proteins with Ni-NTA resin (Clontech), as previously described (*37*). For in vitro ubiquitination assays, lysates from HEK 293T cells transfected with plasmids encoding FLAG-tagged STAT3, UBEIL (E1), and UbcH5c (E2) (Biomol) were incubated in vitro for 2 hours with recombinant GST or GST-LIM, which were expressed in *E. coli*, together with ubiquitin (Biomol) in ubiquitination buffer [50 mM tris-HCl (pH 8.0), 50 mM NaCl, 1 mM adenosine triphosphate, and 1 mM dithiothreitol], and then samples were analyzed by Western blotting with antibody against FLAG.

Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was prepared with an RNeasy micro kit (Qiagen), and complementary DNA (cDNA) was generated with the PrimeScript RT reagent kit (Takara Bio Inc.). Quantitative real-time polymerase chain reaction (PCR) analyses were performed by real-time PCR with an ABI Prism 7000 sequence detection system (Applied Biosystems). The primer sets and probes for mouse *Il-17a* (Mm00439619), *Il-21* (Mm00517640), *Il-22* (Mm00444241), *Ifn-γ* (Mm00801778), *Il-4* (Mm00445259), *Pdlim2* (Mm00524320), and 18*S* ribosomal RNA (rRNA) from the TaqMan Gene Expression Assay (Applied Biosystems) were used for the reactions. To analyze RORγt expression, we used the SYBR Premix Ex Taq II kit (Takara Bio Inc.) and the primer pairs 5′-AGCTTTGTGCAGATCTAAGG-3′ and 5′-TGTCCTCCTCAGTAGGGTAG-3′ (Hokkaido System Science). Data were normalized to the amount of 18*S* rRNA.

Mice

Pdlim2$^{-/-}$ mice on a BALB/c background were previously described (*10*). *Il-6*$^{-/-}$ mice on a C57BL/6 background (*18*) were provided by M. Kopf (Max Planck Institute of Immunobiology, Freiburg, Germany). Mice were maintained under specific pathogen–free conditions. All experiments were performed in accordance with guidelines approved by the RIKEN Yokohama Institute Animal Use Committee.

In vivo challenge with heat-killed *P. acnes*

Mice were injected intraperitoneally with heat-killed *P. acnes*. On day 7, livers were removed, fixed with Bouin's fixative solution, sectioned, and stained with hematoxylin and eosin (H&E). To analyze the production of T_H17- and T_H1-type cytokines, we purified splenic CD4$^+$ T cells by MACS columns (Miltenyi Biotec) on the same day. Cells were then stimulated in vitro with plate-bound antibody against CD3 (10 μg/ml) for 24 hours, and the amounts of IL-17 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA, R&D Systems). Alternatively, total RNA was extracted from purified CD4$^+$ T cells without additional culture and subjected to real-time reverse transcription–PCR (RT-PCR) analysis for the expression of the genes encoding IL-21, IL-22, IFN-γ, and IL-4.

In vitro T_H17 differentiation experiments

Naïve and total CD4$^+$ T cells were purified from spleens with MACS columns. To measure the production of IL-17, we cultured the cells with antibody against IL-4 (5 μg/ml), antibody against IFN-γ (5 μg/ml), TGF-β (2.5 ng/ml), and IL-6 (25 ng/ml) together with plate-bound antibody against CD3 (1 μg/ml) and soluble antibody against CD28 (1 μg/ml) for 3 days, and restimulated the cells with antibody against CD3 (1 μg/ml) for 20 hours. IL-17A production in the culture supernatants was measured by ELISA (eBioscience). To detect the production of IL-21 and RORγt, we cultured cells with IL-6 (1 ng/ml for IL-21 experiments or 5 ng/ml for RORγt experiments) together with plate-bound antibody against CD3 (1 μg/ml) and soluble antibody against CD28 (1 μg/ml) for 2 days. Total RNA was then extracted and subjected to real-time RT-PCR analysis. For intracellular cytokine staining, cells were cultured with antibody against IL-4 (5 μg/ml), antibody against IFN-γ (5 μg/ml), TGF-β (5 ng/ml), and IL-6 (25 ng/ml) together with plate-bound antibody against CD3 (2 μg/ml) and soluble antibody against CD28 (1 μg/ml) for 3 days, and then cells were restimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (0.5 μg/ml) in the presence of GolgiPlug (BD Biosciences) for 5 hours. Cells were then fixed and permeabilized by BD Cytofix/Cytoperm kit (BD Biosciences), incubated with APC-conjugated antibody against CD4, PE-conjugated antibody against IL-17A, and FITC-conjugated antibody against IFN-γ, and analyzed with a FACSCalibur flow cytometer (Becton Dickinson). To detect PDLIM2, we cultured CD4$^+$ T cells for 4 days with antibody against CD3 and antibody against CD28 alone to generate T_H0 cells, or together with IL-12 (10 ng/ml) to generate T_H1 cells, or IL-4 (10 ng/ml) to generate T_H2 cells, or with TGF-β (2.5 ng/ml) and IL-6 (10 ng/ml) to generate T_H17 cells. For *P. acnes*–induced T_H17 differentiation experiments in vitro, we cultured total spleen cells with heat-killed *P. acnes* (2 μg/ml) for 2 days. CD4$^+$ T cells were then purified by MACS columns (Miltenyi Biotec) and subjected to real-time RT-PCR analysis. For dendritic cell–T cell coculture experiments, splenic CD11c$^+$ dendritic cells were purified by MACS columns. Splenic CD4$^+$ T cells were cocultured with dendritic cells in the presence of heat-killed *P. acnes* (2 μg/ml) for 2 days and were then subjected to real-time RT-PCR analysis.

SUPPLEMENTARY MATERIALS

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Fig. S1. PDLIM2 in T helper cell subsets.
Fig. S2. *P. acnes*–induced granuloma formation in *Il-6*– and *Pdlim2*-deficient mice.
Fig. S3. Enhanced *P. acnes*–induced IL-6 production by *Pdlim2*$^{-/-}$ dendritic cells.
Fig. S4. IL-6 induces tyrosine phosphorylation of STAT3, but not STAT1.
Fig. S5. Reduction in *Pdlim2* mRNA abundance by PDLIM2-specific siRNA.

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PDLIM2 Inhibits T Helper 17 Cell Development and Granulomatous Inflammation Through Degradation of STAT3

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LIMiting Inflammation by T Cells

Although much is known about the development of T helper 17 (T_H17) cells, a proinflammatory T cell type that is important for the immune response to pathogens, comparatively little is known about how these cells are inhibited to prevent chronic inflammation and autoimmune diseases. Tanaka *et al*. have identified the E3 ubiquitin ligase PDLIM2 as an endogenous inhibitor of T_H17 development by targeting the essential transcription factor STAT3 (signal transducer and activator of transcription 3) for destruction. Mice lacking PDLIM2 had worse inflammatory disease than did control mice, suggesting that PDLIM2 might be a therapeutic target to prevent T_H17 cell–mediated inflammatory diseases.