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Accessibility
Control of the innate immune response by the mevalonate pathway

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Abstract

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Competing Financial Interests
The authors declare no competing interests.

Author Contributions
D.W. conceived and designed the study, conducted most of the experiments and drafted the manuscript. A.S.L., C.E.F, M.K.A., M.S., Z.J., C.V.R, J.C.K., S.C., R.M.G. and S.D.F. conducted experiments and/or provided technical support. D.M. conducted experiments and provided lab management. G.G. provided animal colony maintenance and performed experiments. K.G. provided Pik3cd−/− mouse femurs and critically read the manuscript. J.J.C. and D.K. provided lab space at NIH and help with human studies. R.H. contributed to helpful discussion. N.S, E.M.G. and K.A.F. reviewed primary data and critically read the manuscript. D.T.G. provided lab space, reviewed primary data and contributed to helpful discussions. M.O.B. provided the Pge1tfl/fl Lys2-Cre mouse strain, reviewed primary data and contributed to the preparation of the manuscript.
Deficiency of mevalonate kinase (MVK) causes systemic inflammation. However, the molecular mechanisms linking the mevalonate pathway to inflammation remain obscure. Geranylgeranyl pyrophosphate (GGPP), a non-sterol intermediate of the mevalonate pathway, is the substrate for protein geranylgeranylation, protein post-translational modification catalyzed by protein geranylgeranyl transferase I (GGTase I). Pyrin is an innate immune sensor that forms an active inflammasome in response to bacterial toxins. Mutations in MEFV (encoding human PYRIN) cause autoinflammatory Familial Mediterranean Fever (FMF) syndrome. Here, we show that protein geranylgeranylation enables Toll-like receptor (TLR)-induced phosphatidylinositol-3-OH kinase PI(3)K) activation by promoting the interaction between the small GTPase Kras and the PI(3)K catalytic subunit p110δ. Macrophages deficient for GGTase I or p110δ exhibited constitutive interleukin-1β release that was MEFV-dependent, but NLRP3-, AIM2- and NLRC4-inflammasome independent. In the absence of protein geranylgeranylation, compromised PI(3)K activity allows for an unchecked TLR-induced inflammatory responses and constitutive activation of the Pyrin inflammasome.

The mevalonate pathway is a fundamental metabolic pathway responsible for cholesterol biosynthesis that has been successfully targeted by statins, inhibitors of the 3-hydroxy-3-methylglutaryl Co-enzyme A Reductase (HMGCR), for treating hypercholesterolemia. Mevalonate Kinase (MVK) catalyzes the phosphorylation of mevalonate—a crucial step in the mevalonate pathway. Patients carrying loss-of-function mutations of MVK develop inflammatory phenotypes including recurrent fever, lymphadenopathy, arthritis and elevated inflammatory cytokines in serum. MVK deficiency can be categorized into the less severe Hyperimmunoglobuline D and periodic fever syndrome (HIDs) and the more severe Mevalonate Aciduria (MA). The autoinflammatory phenotypes in MKD patients suggest that the mevalonate pathway critically regulates the inflammatory response however the underlying molecular mechanisms remain unknown.

Unlike the conventional inflammatory cytokines, IL-1β and the closely related IL-18 are atypical in that they are synthesized as inactive precursors in the cytosol. Maturation and release of these cytokines depends on inflammasomes, large protein complexes that consist of a receptor, the adaptor protein ASC, and proinflammatory caspases. Dysregulation of inflammasome activity and hyper-production of IL-1β is associated with disease conditions termed autoinflammatory diseases. One such disease, Familial Mediterranean Fever (FMF), is caused by mutations in MEFV gene encoding human PYRIN, an innate immune sensor that forms a caspase-1-activating inflammasome in response to bacterial toxins. These FMF associated mutant forms of PYRIN conferred a heightened sensitivity to PYRIN inflammasome, leading to hyper-production of IL-1β and autoinflammation.

The phosphatidylinositol-3-OH kinases PI(3)Ks also have important roles in the regulation of the innate immune response. Kinases downstream of PI(3)Ks such as Akt, glycogen synthase kinase-3β (Gsk3β) and the mechanistic Target of Rapamycin (mTOR) inhibits TLR-induced proinflammatory cytokine production through post-translational modification of transcription factors such as NF-κB, AP-1, CREB and STAT3. PI(3)Ks activity is regulated by multiple factors including Ras and Rho family small GTPases.

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Besides cholesterols, the mevalonate pathway also synthesizes other intermediates such as the non-sterol isoprenoids-geranylgeranyl pyrophosphate (GGPP). GGPP can be used as substrate for protein post-translational modifications, namely protein geranylgeranylation. Protein geranylgeranylation is catalyzed by protein geranylgeranyl transferase-I (GGTase I). Macrophages deficient for the β-subunit of GGTase I (protein geranylgeranyl transferase-I β subunit, Pggt1b), are hyper-activated by LPS. Mice with conditional deficiency of Pggt1b in myeloid cells also develop inflammatory arthritis spontaneously. These observations suggested that protein geranylgeranylation controls the innate immune response; however, the underlying molecular mechanisms remain unclear.

Here our data revealed an unanticipated link between the mevalonate pathway and TLR-induced PI(3)Ks activation. Through protein geranylgeranylation, the mevalonate pathway enables TLR-induced PI(3)K activation by promoting the interaction between Ras family small GTPase-Kras and PI(3)K catalytic subunits p110δ. Inhibition of the mevalonate pathway decreases the production of GGPP, limits protein geranylgeranylation, and therefore impairs PI(3)Ks activation. Impaired PI(3)Ks activation causes hyper-inflammatory conditions including hyper-production of proinflammatory cytokines, elevated expression of Pyrin protein and spontaneous activation of the Pyrin inflammasome.

**Results**

**Differential regulation of cytokine production by Pggt1b**

To define the role of protein geranylgeranylation in the regulation of TLR-induced production of cytokines, we assessed the response of Pggt1b-deficient bone marrow derived macrophages (BMDMs) to stimulation with ligands of Toll-like receptors by ELISA. Stimulation of Pggt1b-deficient macrophages with ligands to TLR4 (LPS), TLR9 (CpG), TLR2 (Pam3CSK4) and TLR7 (R848) led to potent up-regulation of proinflammatory cytokines such as IL-1β, TNF, IL-6, and IL-12 in comparison to wild-type control BMDMs (Fig. 1a–d). Concurrently, the production of anti-inflammatory IL-10 as well as type I interferon and the chemokine RANTES was suppressed in Pggt1b deficient macrophages (Fig. 1e–g). Notably, Pggt1b deficient BMDMs exhibited decreased production of both pro- and anti-inflammatory cytokines following stimulation with the TLR3 ligand poly I:C. Quantitative RT-PCR revealed that the altered cytokine production in Pggt1b deficient cells was caused by altered levels of transcription (Supplementary Fig. 1a), suggesting that protein geranylgeranylation differentially regulates cytokine gene expression at the transcriptional level. In addition to the commonly studied cytokines, enhanced production of granulocyte growth factor G-CSF and neutrophil chemotactic factor KC(Cxcl1) as well as dampened production of chemokines MCP-1, MIP1β and MIP1α, were also observed in a multiplex-cytokine assay (Supplementary Fig. 1b). Pggt1bfl/flLyz2-Cre mice had elevated frequencies of granulocytes in their circulation (Fig. 2a and b). To further investigate the physiological relevance of altered cytokine production caused by Pggt1b deficiency in vivo, we challenged mice with lipopolysaccharide (LPS) at a lethal dose of 30mg/kg of body weight. Within 12 hours after LPS challenge, 80% of Pggt1bfl/flLyz2-Cre mice died compared to no death of Pggt1bfl/+Lyz2-Cre mice (Fig. 2c). The increased susceptibility of mice to endotoxic shock correlated with higher LPS-induced serum concentrations of the

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proinflammatory cytokines IL-1β, TNF and IL-6 (Fig. 2d–f). Together, these data suggest that Pgg1t1b differentially regulate TLR-induced inflammatory cytokine production.

**IKK and MAPKs activation not affected by Pgg1t1b deficiency**

The altered cytokine production in Pgg1t1b deficient macrophages prompted us to evaluate the activation of the IKK, the MAPKs and the TBK1 signaling pathways downstream of TLR. Wild-type and Pgg1t1b deficient macrophages were stimulated with a low dose of LPS (10ng/ml) and the activation of IKKβ, MAPKs and TBK1 was evaluated with phospho-specific antibodies by immunoblot. The activation of IKKβ, JNK, p38, Erk and TBK1 was not enhanced or suppressed in Pgg1t1b deficient macrophages compared to wild-type controls (Supplementary Fig. 2a and 2b). Endocytosis of TLR4 induced by LPS stimulation was not impaired or enhanced in Pgg1t1b deficient macrophages (Supplementary Fig. 2c), either, suggesting there is no TLR trafficking defect. We concluded that the altered cytokine profile in Pgg1t1b deficient macrophages was not caused by altered activation of IKK, the MAPKs, TBK1 or TLR endocytosis.

**Compromised PI(3)K activation in the absence of Pgg1t1b**

The cytokine production profile observed in Pgg1t1b deficient macrophages is strikingly similar to that in myeloid cells deficient for PI(3)K-Akt signaling components such as p85α, p110δ, mTOR and Akt1, or treated with Gsk3β inhibitors. We speculated that protein geranylgeranylation regulates TLR-induced cytokine production by controlling PI(3)K-Akt activation. Pgg1t1b deficient macrophages displayed substantially lower constitutive Akt phosphorylation. When Pgg1t1b deficient BMDMs were stimulated with LPS, phosphorylation of Akt at both Ser473 and Thr308 was severely compromised compared to wild-type control cells (Fig. 3a). Consistent with compromised Akt activation, phosphorylation of Gsk3β at Ser9 was also severely diminished in Pgg1t1b deficient cells (Fig. 3b). Gsk3β is phospho-inactivated by Akt at Ser 9. Therefore, decreased Ser 9 phosphorylation correlates with higher Gsk3β kinase activity. Indeed, western blot revealed that phosphorylation of the c-Jun Thr239 and RelA Ser536, two well-established Gsk3β substrates (Supplementary Fig. 3a) was enhanced in Pgg1t1b deficient BMDMs compared to wild-type control cells (Fig. 3b). Phosphorylation of Thr389 of p70S6K kinase, a direct substrate of mTORC1 was greatly decreased in Pgg1t1b deficient cells (Fig. 3c). Accordingly, pre-treatment of Pgg1t1b deficient macrophages with a Gsk3β inhibitor (SB216763) partially reversed the phenotypes (Supplementary Fig. 3b–f). Based on these results, we concluded that Pgg1t1b controls the PI(3)K-Akt-Gsk3β and mTOR kinase cascade downstream of TLR in macrophages.

**Pgg1t1b deficiency phenocopies Pik3cd−/− BMDMs**

Class IA PI(3)Ks are heterodimers composed of a regulatory subunit (p85α or p85β) and a catalytic subunit (p110α, p110β, or p110δ). Although p110α and p110β are ubiquitously expressed, p110δ expression is enriched in hematopoietic cells and appears to play a more important role in immune cell signaling. It was reported that p110δ plays an important role in balancing the production of pro- vs. anti-inflammatory cytokines and protects mice from endotoxic shock. We found LPS-stimulation alone induced robust IL-1β release in p110δ-deficient (Pik3cd−/−) BMDMs (Fig. 4a). Pik3cd−/− BMDMs also displayed enhanced
production of IL-12, TNF, IL-6 and suppressed IFN-β production (Fig. 4b–e). These phenotypes exactly mirrored those observed in Pggt1b deficient BMDMs (Fig. 1a). Together, these results suggest that protein geranylgeranylation regulates TLR signaling most likely through p110δ of the class IA PI(3)K family.

**Constitutively active p110δ rescues Pggt1b deficiency**

To further confirm that protein geranylgeranylation controls cytokine production through regulating p110δ activation, wild type or Pggt1b deficient BMDMs were infected with retrovirus harboring cDNAs encoding wild type p110δ, Pggt1b or a constitutively active form of p110δ (E1121K)\(^{23}\). Expression of the E1121K but not the wild type p110δ completely abrogated LPS-induced IL-1β release and restored the production of TNF, IL-12 and IL-10 to the level observed in wild type BMDMs, an effect also observed in Pggt1b deficient BMDMs reconstituted with wild type Pggt1b (Fig. 4f). These results further confirmed that Pggt1b controls p110δ activity downstream of TLR

**Pggt1b controls the interaction between Kras and p110δ**

To investigate how protein geranylgeranylation regulates PI(3)K activity, we focused on proteins that can be geranylgeranylated. None of the PI(3)K family members carries a CAAX motif essential for prenylation. However, the catalytic subunits of the class IA PI(3)Ks have a Ras-binding domain (RBD). Ras proteins directly bind to RBD of the PI(3)K catalytic subunits and activate class I PI(3)K\(^{24}\). The Ras family small GTPases is composed of three members, namely Hras, Kras and Nras. Computational analysis of farnesylation versus geranylgeranylation potentials using the Prenylation Prediction Suite\(^{25}\) showed that murine Kras and Nras tend to be geranylgeranylated, while Hras is unlikely to be modified by geranylgeranylation (Table 1). This is consistent with a previous report indicating that human KRAS can be geranylgeranylated in the presence of farnesylation inhibitors while HRAS can only be farnesylated\(^{26}\). *In vivo* labeling of primary BMDMs using \(^{3}\)H-geranylgeranyl pyrophosphate demonstrated that murine Ras proteins can be geranylgeranylated in macrophages under physiological conditions and this process depends on Pggt1b (Fig. 5a).

To evaluate which murine Ras proteins mediate PI(3)K activation, we knocked down the expression of Kras, Nras, or Hras in primary BMDMs using siRNA. Only knocking down the expression of Kras, but not Hras or Nras in primary BMDMs dramatically decreased Akt phosphorylation upon LPS stimulation (Fig. 5b–d). We therefore concluded that Kras, but not H or Nras is essential for TLR-induced PI(3)K activation.

In the carboxyl terminal CAAX motifs of prenylated proteins, C is cysteine, A is aliphatic amino acid and X can be any amino acid. The carboxyl terminal (X) directs either farnesylation or geranylgeranylation. GGTase I prefers substrates with a carboxyl terminal leucine (CAAL)\(^{27}\). To investigate how prenylation regulates Ras GTPases interaction with p110δ, we constructed wild type and CAAL mutant forms of Ras-encoding cDNAs (Table 2) into retroviral vectors. These CAAL mutant Ras GTPases are predicted to be more likely to be geranylgeranylated using the Prenylation Suite (data not shown). Stable cell lines were established by transducing immortalized wild type BMDMs with retroviral vectors.
Immunoprecipitation of endogenous p110δ revealed that interaction of Kras-CVIL (the CAAL (geranylgeranylated) form) with p110δ is dramatically enhanced in comparison to the wild type Kras (CTVM) protein. In the same setting of the experiments, the interaction between Nras or Hras with p110δ was adversely affected by the increased potential of geranylgeranylation (Fig 5e). This result suggests that protein geranylgeranylation specifically increases the interaction between Kras and p110δ.

Since Ras-PI(3)K interaction is essential for optimal PI(3)K activation, we, therefore, hypothesized that protein geranylgeranylation regulates Kras-p110δ interaction. Immunoprecipitation of p110δ from cell lysate of LPS-stimulated BMDMs revealed that Kras proteins were constitutively associated with p110δ in resting wild-type cells. This is consistent with the higher basal level of Akt phosphorylation in wild type cells (Fig. 3a). Upon LPS stimulation, Ras proteins dissociated from p110δ (Fig. 5f). However, in Pggt1b deficient BMDMs, this constitutive association was dramatically decreased. Although LPS stimulation could induce transient interaction between Kras proteins and p110δ, this interaction was much weaker and was not sustained for more than 10 minutes (Fig. 5f). We did not see altered interaction between Kras and other class IA catalytic subunit p110α and p110β. These observations suggested that the interaction between Kras and p110δ is regulated by protein geranylgeranylation.

**Pyrin inflammasome mediates IL-1β release in Pggt1b−/− BMDMs**

The success of treating Mevalonate Kinase Deficient (MKD) patients with anti-interleukin-1 agents such as the recombinant IL-1 receptor antagonist-anakinra suggests that IL-1 and inflammasomes play an important role in the pathogenesis of MKD. Stimulation of Pggt1b deficient macrophages with LPS alone caused robust IL-1β maturation and release, whereas the same stimulation did not induce robust IL-1β production in wild-type cells (Fig. 1a and Fig. 6a). The LPS-induced processing and release of IL-1β in Pggt1b deficient BMDMs began two hours after LPS stimulation and peaked at six hours (Supplementary Fig. 4a). In addition to LPS, CpG (TLR9), Pam3CSK4 (TLR2) and R848 (TLR7), but not poly I:C, induced spontaneous IL-1β maturation and release in Pggt1b deficient BMDMs (Supplementary Fig. 4b), suggesting that TLR3 signaling is differentially regulated by protein geranylgeranylation from other TLRs. Re-expression of Pggt1b in Pggt1b deficient BMDMs completely reversed the spontaneous IL-1β maturation upon LPS stimulation (Supplementary Fig. 4c and 4d).

Further analysis using BMDMs from compound transgenic strains of mice showed that IL-1β maturation and release was abrogated in Pggt1b−/− Casp1−/− or Pggt1b−/− Pycard−/− (Pycard encodes ASC) BMDMs stimulated with LPS, but not in Pggt1b−/− Nlrp3−/− cells (Supplementary Fig. 5a–c). These results indicated that TLR-induced spontaneous IL-1β maturation in Pggt1b deficient BMDMs is mediated by caspase-1 and ASC, but not NLRP3. Since Caspase-11 mediated IL-1β processing also depends on NLRP3 (Ref. 29), a role of caspase-11 was ruled out.

Pyrin has been defined as an innate immune sensor that forms a caspase-1-activating inflammasome in response to bacteria toxins. Mutations in the human PYRIN-encoding
MEFV gene cause Familial Mediterranean Fever. Using siRNA approach, we discovered that only knocking down the expression of the Pyrin-encoding Mefv, but not Aim2 or Nlrc4 caused a dramatic inhibition of IL-1β release and caspase-1 processing in Pggt1b deficient BMDMs (Fig. 6b, c), suggesting that the Pyrin inflammasome mediates LPS-induced IL-1β secretion in Pggt1b deficient BMDMs. Similar to Pggt1b deficient cells, stimulation of Pik3cd−/− BMDMs with LPS alone also induced robust IL-1β production (Fig. 4a and Fig. 6d). The maturation of IL-1β in Pik3cd−/− BMDMs was accompanied by release of the caspase-1 p20 subunit, an indication of the activation of capase-1 inflammasomes (Fig. 6d). More importantly, re-expression of a constitutively active form of p110δ (E1121K) completely rescued the IL-1β-producing phenotype in Pggt1b deficient BMDMs (Fig. 6e and Fig. 4f). Furthermore, an siRNA approach showed that only knocking down the expression of Mefv severely inhibits LPS-induced inflammasome activation in Pik3cd−/− BMDMs (Fig. 6f). Taken together, these data indicate that GGTase I controls Pyrin inflammasome activation through the PI(3)K-p110δ signaling pathway.

The Pggt1b and p110δ controls MeFv expression

Our data showed that Mefv mRNA expression is barely detectable in wild type BMDMs without stimulation, and was induced by LPS treatment. In the Pggt1b or Pik3cd deficient BMDMs, the basal level of Mefv mRNA expression was higher compared to wild type controls. More strikingly, Mefv expression was hyper-induced in both Pggt1b and Pik3cd KO cells (Fig. 6g and 6h). Consistently, Pyrin protein level was much higher in Pggt1b deficient BMDMs (Fig. 6i). It is known that expression of Mefv is driven by NF-κB Rel A30 and that Gsk3β promotes the transactivation activity of Rel A through phosphorylation of RelA.10 We have shown the rampant Gsk3β activity and enhanced phosphorylation of Rel A in Pggt1b deficient BMDMs (Fig. 3b). Taken together, our results support the notion that enhanced NF-κB Rel A transactivational activity drives hyper-expression of Mefv that may contribute to TLR-induced IL-1β release in Pggt1b or p110δ deficient BMDMs.

Enhanced production of IL-1β in PBMCs from HID patients

To mimic human disease conditions of HID patients, we used healthy human peripheral blood mononuclear cells (PBMCs) treated with simvastatin, an inhibitor of HMGCR to inhibit the mevalonate pathway. When healthy PBMCs were treated with LPS, only minimal amounts of IL-1β maturation were detected in the supernatant. Robust IL-1β production only occurred when PBMCs from healthy donors were pre-treated with simvastatin to inhibit the mevalonate pathway (Fig. 7a). However, when PBMCs from HID patients were stimulated with LPS alone, a fraction of the patients showed hyper-production of IL-1β without inhibition of the mevalonate pathway with simvastatin (patients #1, #2, #3), in a few other cases, there was spontaneous production of IL-1β without any stimulation, potentially caused by pathogen infection (patient #4, #5, #6) (Fig. 7a). Gene expression profiling revealed that healthy PBMCs, when treated with LPS and simvastatin, displayed enhanced production of proinflammatory cytokines in comparison to cells stimulated with LPS alone (Fig. 7b). Accordingly, when healthy PBMCs were treated with simvastatin and LPS, phosphorylation of Akt Ser473 was severely compromised (Fig. 7c). These results corroborated our findings in the mouse model of protein geranylgeranylation deficiency and highlighted how the mevalonate pathway controls the innate immune response through the
control of PI(3)K-Akt signaling by protein geranylgeranylation in both mouse and human cells (Supplementary Fig. 6).

Discussion

The crucial role of the PI(3)K-Akt-Gsk3β and mTOR kinase cascade in the regulation of TLR-induced cytokine production has emerged as an important aspect of innate immune signal transduction,12,21. However, the underlying molecular mechanisms remain not completely understood. B-Cell Adaptor for PI(3)K (BCAP) is required for PI(3)K activation induced by MyD88-dependent TLR4, TLR7 and TLR9.18,31. It is not clear whether endosomal TLR3-induced PI(3)K activation also depends on BCAP. TLR3 activates PI(3)K by recruiting PI(3)K complex directly to its cytoplasmic tails.32 Here we showed decreased production of IFN-β in Pggt1b deficient BMDMs stimulated with LPS and poly I:C. Pggt1b deficient BMDMs displayed attenuated PI(3)K activation upon LPS and poly I:C (data not shown) stimulation. This is consistent with published data that PI(3)K positively regulates IFN-α production through inactivation of Gsk3β33,34,35. It is also of note that unlike ligands to TLR2, 4, 7 and 9, TLR3 ligand poly I:C stimulation induced TNF and IL-6 were decreased in Pggt1b deficient BMDMs. Poly I:C also failed to activate inflammasome in the absence of Pggt1b. These results indicate that protein geranylgeranylation differentially regulates MyD88-dependent and independent signaling pathways, the precise underlying mechanism merits further investigation.

Previous studies have shown that binding of Ras proteins to class IA PI(3)K RBD is essential for both physiological and pathological functions of PI(3)Ks24,36. However, how the interaction between Ras and PI(3)K contributes to the TLR-induced innate immune response is not completely understood. In the early 90s, the finding that farnesylation of KRAS is essential for its oncogenic function had galvanized tremendous enthusiasm in developing farnesyl transferase (FTase) inhibitors to treat human cancers with KRAS mutations. Unfortunately, the FTase inhibitors showed poor efficacy in clinical trials because KRAS can also be geranylgeranylated when FTase I is inhibited.38,39,40 FTase I and GGTase I are both hetero-dimers that share an α subunit and have distinct but highly homologous β subunits.15 Our data showed that murine Ras proteins are geranylgeranylated by GGTase I in wild type mouse BMDMs. In earlier studies, knockout of the β subunit of FTase I (Fntb) was shown to block farnesylation of proteins but had no impact on Kras signaling, membrane targeting or function and had no impact on cytokine production in myeloid cells.16 Moreover, the absence of Fntb in macrophages did not induce any inflammatory phenotypes in vivo. Mice deficient for both Pggt1b and Fntb in macrophages display phenotypes that are indistinguishable from that of the Pggt1b single knockout.16 Taken together, our data suggested that protein geranylgeranylation, but not farnesylation, positively regulates Kras and p110δ interaction that is essential for the activation of p110δ and its downstream kinase cascades.

It has been shown that deficiency of p110δ in dendritic cells caused a defect in intracellular trafficking of TLR4 upon LPS stimulation.41 However, we did not found such defect in Pggt1b deficient BMDMs. Such discrepancies can be attributed to either the cell type difference (macrophage vs DCs) or the difference of genetic backgrounds of the mouse.
strains used. Although we saw enhanced production of proinflammatory cytokines in healthy PBMCs treated with LPS and simvastatin, the production of INF-β and IL-10 was not consistent with that observed in Pggt1b deficient mouse BMDMs. We believe the discrepancy is caused by the difference of the experimental settings. The phenotype observed in Pggt1b deficient mouse BMDMs was caused solely by the deficiency of protein geranylgeranylation, whereas simvastatin inhibition of the mevalonate pathway causes the shortage of not only GGPP but also a variety of other intermediates downstream of HMGCR in the metabolic pathway. The impact of the shortage of those intermediates on inflammatory responses remains to be investigated, but could explain why simvastatin inhibition does not completely recapitulate the phenotypes caused by protein geranylgeranylation deficiency.

We observed dramatically increased basal level of IκBα in Pggt1b deficient cells. IκBα itself is a target of NFκB. Rampant Gsk3β kinase activity due to inhibited constitutive level of Akt activity as a result of Pggt1b deficiency causes increased level of NFκB/RelA phosphorylation. This in turn increased the transactivational activity of RelA to drive the expression of IκBα gene in resting Pggt1b deficient cells.

The PI(3)K-Akt-Gsk3β and mTOR pathway plays pivotal roles in controlling a variety of essential cellular functions such as cell growth, survival, differentiation and metabolism. Dysregulation of PI(3)K activity is implicated in a myriad of human diseases, particularly cancer and diabetes. The data presented here implicate protein geranylgeranylation in the regulation of TLR-induced p110δ activation in myeloid cells. It is possible that the same mechanisms exist in other tissues and cell types to control class IA PI(3)Ks downstream of growth factor receptors. Therefore, our studies may provide clues for manipulating the mevalonate pathway for the intervention of diseases such as cancer, diabetes and aging.

Online Methods

Mice

C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) were bred at UMass Medical School. Pggt1b fl/fl, Lyz2-Cre mice were generated as described. Pggt1b fl/fl, Lyz2-Cre and littermate control Pggt1b fl/+ , Lyz2-Cre or Pggt1b +/+ ,Lyz2-Cre were generated by first crossing the Pggt1b fl/fl , Lyz2-Cre to C57BL6/J, then intercrossing of the resultant Pggt1b fl/+ ,Lyz2-Cre mice. Littermate controls were used in the experiments. Mouse bones from Pik3cd−/− (encoding p110δ) mice were provided by Kira Gritsman, the mouse strain was originally generated in James Ihle’s lab and had been backcrossed to C57BL6 back ground in the Gritsman Lab. Bones from C57BL6J mice were used as controls for the in vitro experiments depicted in Fig.4a–e and Fig. 6d and Supplementary Fig. 5. Caspase-1 and Asc KO mice have been described previously. Mouse strains were maintained in specific pathogen-free conditions in UMass Medical School or the animal facility at Duke University, and the animal protocols were carried out in accordance with the guidelines set forth by the Institutional Animal Care and Use Committees of UMass Medical School or Duke University.
Patient Blood Samples

HIDs patients and healthy controls were included in the study after informed consent under protocols approved by the Institutional Review Board of UMass Medical School, the Duke University and the National Human Genome Research Institute. A total of three healthy control (two male one female) and 6 HIDs patients (2 males, 4 females) were used in the experiments depicted in Fig. 7. PBMCs were collected by density centrifugation according to the manufacturer’s instructions (GE Healthcare).

BMDM Culture and Stimulations

Bone marrow cells from wild-type and various knockout mice were cultured in DMEM with 10% fetal bovine serum and 20% L929 supernatants. For Western blots, BMDMs were primed with 200ng/ml LPS unless otherwise indicated for six hours and then stimulated with nigericin or the vehicle for one additional hour. For controls in Supplementary Fig. 6, BMDMs were transfected with poly dA:dT (1μg/10^6 cells, Sigma-Aldrich) two hours after LPS priming and were left for an additional six hours before harvesting. For ELISAs, BMDMs were stimulated with TLR ligands for 8 hours before harvested for ELISA. TLR ligands LPS, poly I:C, Pam3CSK4, R848 and CpG ODN1826 were purchased from Invivogen. In some cases, Gsk3β inhibitors (SB216763, Abcam and XXVII, Millipore) were added 30 minutes prior to TLR stimulation. For reconstitution experiments, BMDM cultures were infected with retrovirus (pMSCV Clontech) harboring cDNAs encoding wild type or constitutively active form of p110δ or wild type Pggt1b on day 4. The BMDM culture was selected in BMDM medium with 2μg/ml puromycin for 3 consecutive days starting on day 6. Viable cells were used for subsequent experiments described in Fig. 4d and 6e.

siRNA Knockdown of Gene Expression in BMDMs

Day 7 culture of BMDMs were transfected with siGenome smart pool (GE Healthcare Dharmacon, Inc, Chicago, IL) of non-targeting control (D-001206-13-05) or siGeneome Smart Pool against mouse Hras (M-046324-01-0005), Kras (M-043846-02-0005), Nras (M-044216-01-0005), Mefv (M-048693-00-0005), Aim2 (M-044968-01-005), Nlrc4 (M-055000-01-0005). 48 hours after siRNA transfection, total RNA were extracted and cDNAs were synthesized. Transcript abundance of murine Hras, Kras, Nras, Mefv, Nlrc4, Aim2 were determined by quantitative PCR. Primers sequences are listed in Supplementary Table S1. The relative abundance of the transcripts was normalized against that of GAPDH. siRNA treated cells were also stimulated with or without LPS for 30 minutes, cell lysates were prepared and subjected to Western blot analysis using anti-phosphor-Akt S473 antibody. Quantitation of Akt phosphorylation in Fig. 5d was carried out using Image J and normalized against pan-Akt bands in the corresponding lane. Phosphorylation of Akt in control, non-targeting siGenome smart pool were used as 100% and compared with other samples.

ELISA, Multiplex Assays and QRT-PCR

Cell culture supernatants or sera were analyzed by ELISA for IL-1β, TNF, IL-6, IL-12p40, IL-10 (eBiosciences) and Rantes (R&D systems). A sandwich ELISA for mouse IFN-β was
performed as described. Cytokines from cell culture supernatants were quantitated using the Bio-Rad Bio-Plex™ Mouse Cytokine Assay kit. Transcript abundance of cytokines were determined by quantitative PCR using primers listed in supplementary table S1.

**Immunoblot**

Proteins from the cell culture supernatants were precipitated by methanol-chloroform extraction. Cells were lysed with 1% NP40 lysis buffer. Antibodies used for Western blot and immunoprecipitation are listed in Supplement Table S2. p110δ immunoprecipitation was performed using a previously described protocol.

**Endotoxic Shock**

Prior to injection, blood samples were taken from mice. A total of 48 mice, 24 male and 24 female, were used in three independent experiments. Mice were then injected i.p. with LPS (Sigma-Aldrich) at a lethal dose of 30mg/kg bodyweight and returned to the cage and closely observed at one hour intervals. Blood samples were taken again at 2 hours post LPS injection. Survival time of mice was recorded, and survival curves were generated using GraphPad Prism. Plasma was separated from blood samples and inflammatory cytokines were analyzed using ELISA (eBiosciences).

**Statistical Analysis**

Two-way ANOVA multiple factor comparison was performed using Prism (Graphpad) software.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Pgt1b deficiency augments proinflammatory cytokine production while suppressing IFN-β and IL-10 production

Quantitation of IL-1β (a), TNF (b), IL-6 (c), IL-12 (d), IFN-β (e), IL-10 (f) and RANTES (g) in the supernatant of BMDMs by ELISA eight hours after stimulation with TLR ligands. Data (mean±SD) are representative of three independent experiments with similar results.
Figure 2. Neutrophilia and enhanced susceptibility to endotoxic shock in \(Pgt1b^{fl/fl}\)Lyz2-Cre mice
(a) Representative contour-plot of CD11b\(^{+}\), Ly6G\(^{+}\) granulocytes in the peripheral blood of \(Pgt1b^{fl/+}\)Lyz2-Cre and \(Pgt1b^{fl/fl}\)Lyz2-Cre mice. (b) Granulocyte percentage from sex- and age-matched control \(Pgt1b^{fl/+}\)Lyz2-Cre (n=7) and \(Pgt1b^{fl/fl}\)Lyz2-Cre (n=7) mice. (c) Survival of \(Pgt1b^{fl/+}\)Lyz2-Cre (dotted line) (n=8) and \(Pgt1b^{fl/fl}\)Lyz2-Cre (solid line) (n=8) mice injected with a lethal dose of LPS. ELISA quantitation of IL-1\(\beta\) (d), TNF (e) and IL-6 (f) in the serum from \(Pgt1b^{fl/+}\)Lyz2-Cre and \(Pgt1b^{fl/fl}\)Lyz2-Cre mice two hours post LPS injection. \(*P=0.011, **P=0.0061, ***P=0.0006, ****P<0.0001\) (Two Way ANOVA). Data are representative of two (a, b) or three (c–f) independent experiments.
Figure 3. Compromised LPS-induced Akt, GSK3β and mTOR Phosphorylation in the Absence of Pgg1b
(a) Immunoblots of phospho-Ser473 and Thr308 of Akt in the cell lysate of BMDMs stimulated with LPS at 10ng/ml. (b) Immunoblots of phospho-Gsk3β Ser9, phospho-RelA Ser536 and phospho-c-Jun Thr239 in the cell lysate of BMDMs stimulated with LPS at 10ng/ml. (c) Immunoblots of phospho-p70S6K Thr389 in the cell lysate of -BMDMs stimulated with LPS at 10ng/ml. Data in (a), (b) and (c) are from the same experiment which is representative of three independent experiments with similar results.
**Figure 4.** Protein Geranylgeranylation controls TLR-induced cytokine production and inflammasome activation through the PI(3)Kinase-p110δ

Production of IL-1β (a), IL-12(p40) (b), TNF(c), IL-6 (d) and IFN-β (e) by wild-type and Pik3cd<sup>−/−</sup> (p110δ) BMDMs stimulated with LPS; (f) Production of IL-1β, TNF, IL-12(p40) or IL-10 in supernatants of wild type (WT) or Pgg1b deficient BMDMs reconstituted with empty vector (EV) or that harboring cDNAs encoding wild type p110δ, wild type Pgg1b or a constitutively active form of p110δ (E1211K). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Two Way ANOVA). Data (mean±SD) are representative of 3 independent experiments with similar results.
Figure 5. Protein geranylgeranylation controls the Kras-p110δ interaction that licenses TLR-induced p110δ activation

(a) Autoradiography of in vivo labeling of Ras proteins immunoprecipitated from wild type or Pggt1b deficient BMDMs. (b) Transcript abundance of Hras, Kras, and Nras in mouse BMDMs 48 hours after transfection with control or siRNA against H, K, Nras genes; (c) Immunoblot of phospho-Akt S473 after LPS stimulation of BMDMs 48 hours after transfection with control or siRNA against Hras, Kras, or Nras as described in (b); (d) Image J quantitation of phospho-Akt S473 in LPS-stimulated samples described in (c), the ratios between the phospho-Akt S473 band and the corresponding pan-Akt band were calculated. The ratio in siRNA scramble control was set as 100%. (e) Immunoblot of lysate or anti-
p110δ immunoprecipitates of wild type immortalized macrophages stably infected with EV or pMSCV constructs containing cDNAs encoding wild type or the geranylgeranylated form (CAAL) of Hras, Kras, and Nras proteins; (f) Immunoblot of anti-p110α, β and δ immunoprecipitate or lysate of wild type or Ptgt1b deficient BMDMs stimulated with LPS. Data are representatives of two (a) or three (b–f) independent experiments with similar results.
Figure 6. Spontaneous IL-1β secretion in Pggt1b and Pik3cd−/− macrophages is mediated by pyrin inflammasome

(a) Immunoblots of the culture supernatant or the cell lysate of LPS or LPS plus nigericin-stimulated wild-type and Pggt1b deficient BMDMs; (b) Relative abundance of transcripts of Nlrc4, Aim2 and Mefv in primary BMDMs 48 hours after transfection with control or siRNA against Aim2, Nlrc4 or Mefv; (c) Immunoblot of supernatant or cell lysate of Pggt1b deficient BMDMs pre-transfected with siRNA as described in (b) and stimulated with LPS; (d) Immunoblot of supernatants or cell lysate of wild type, Akt1−/− and Pik3cd−/− BMDMs stimulated with LPS; (e) Immunoblot of supernatant or cell lysate of of Pggt1b deficient BMDMs reconstituted with EV or that harboring cDNAs encoding wild type p1106, wild
type Pggt1b or a constitutively active form of p110δ (E1211K); (f) Immunoblot of Pik3cd−/−BMDMs stimulated with LPS that was pre-transfected with siRNA against Aim2, Nlrc4 and Mefv (g) & (h) Relative transcript abundance of Mefv in Pggt1b deficient (g) or Pik3cd−/− (h) BMDMs upon LPS stimulation; (i) Immunoblot of Pyrin in cell lysate of LPS-stimulated Pggt1b deficient and control BMDMs. Data are representative of 3 independent experiments with similar results.
Figure 7. Enhanced cytokine production and inflammasome activation in PBMCs from HIDs patients treated with LPS and simvastatin
(a) Immunoblot of cleaved IL-1β from supernatant of LPS or LPS plus simvastatin stimulated PBMCs from healthy control or HIDs patients. (b) NanoString analysis of gene expression of proinflammatory cytokines from healthy control PBMCs stimulated with LPS or LPS plus simvastatin. (c) Immunoblot of pan-Akt and phospho-Akt-Ser473 of cell lysates of human PBMCs stimulated with LPS pre-treated overnight with vehicle or simvastatin (5μM). Data are representative of two experiments on the same patient samples with similar results.
### Table 1

GGTase I vs. GTaseI Scores

|          | FTase I | GGTase I |
|----------|---------|----------|
| Hras     | 1.305   | -7.02    |
| Nras     | 0.003   | 1.415    |
| Kras     | 1.816   | 2.638    |
Table 2
Wild Type vs L-mutant CAAX Motif

|       | WT  | CAAL |
|-------|-----|------|
| Hras  | CVLS| CVLL |
| Nras  | CVLM| CVIL |
| Kras  | CTVM| CVIL |