Control of the innate immune response by the mevalonate pathway

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Deficiency in mevalonate kinase (MVK) causes systemic inflammation. However, the molecular mechanisms linking the mevalonate pathway to inflammation remain obscure. Geranylgeranyl pyrophosphate, a non-sterol intermediate of the mevalonate pathway, is the substrate for protein geranylgeranylation, a protein post-translational modification that is 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) result in autoinflammatory familial Mediterranean fever syndrome. We found that protein geranylgeranylation enabled Toll-like receptor (TLR)-induced activation of phosphatidylinositol-3-OH kinase (PI(3)K) by promoting the interaction between the small GTPase Kras and the PI(3)K catalytic subunit p110 δ . Macrophages that were deficient in GGTase I or p110 δ exhibited constitutive release of interleukin 1 β that was dependent on *MEFV* but independent of the NLRP3, AIM2 and NLRC4 inflammasomes. In the absence of protein geranylgeranylation, compromised PI(3)K activity allows 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, which are inhibitors of the 3-hydroxyl-3-methylglutaryl co-enzyme A reductase (HMGCR), in the treatment of hypercholesterolemia. MVK catalyzes the phosphorylation of mevalonate, a crucial step in the mevalonate pathway¹. Patients carrying loss-of-function mutations in *MVK* develop inflammatory phenotypes, including recurrent fever, lymphadenopathy, arthritis and elevated inflammatory cytokines in serum. There are two types of MVK deficiency, the less-severe hyperimmunoglobulin D and periodic fever syndrome (HIDS) and the more severe mevalonate aciduria (MA)². The autoinflammatory phenotypes in patients with mevalonate-kinase deficiency (MKD) suggest that the mevalonate pathway critically regulates the inflammatory response, but the underlying molecular mechanisms remain unknown.

Unlike the conventional inflammatory cytokines, interleukin 1 β (IL-1 β) and the closely related interleukin-18 (IL-18) are atypical in that they are synthesized as inactive precursors in the cytosol³. Maturation and release of these cytokines depends on inflammasomes, large protein complexes that consist of a receptor, the adaptor

protein ASC and pro-inflammatory caspases⁴. Dysregulation of inflammasome activity and hyper-production of IL-1 β are associated with autoinflammatory diseases⁵. One such disease, familial Mediterranean fever (FMF), is caused by mutations in *MEFV*, which encodes human PYRIN, an innate immune sensor that forms a caspase-1-activating inflammasome in response to bacterial toxins⁶. These FMF-associated mutant forms of PYRIN confer a heightened sensitivity to PYRIN inflammasome, leading to hyper-production of IL-1 β and autoinflammation.

The PI(3)Ks also have important roles in the regulation of the innate immune response^{7,8}. Kinases downstream of PI(3)Ks, such as Akt1 (ref. 9), glycogen synthase kinase-3 β (Gsk3 β)¹⁰ and the mechanistic Target of Rapamycin (mTOR)¹¹, inhibit TLR-induced pro-inflammatory cytokine production through post-translational modification of transcription factors such as NF- κ B, AP-1, CREB and STAT3 (refs. 12,13). PI(3)K activity is regulated by multiple factors, including Ras and Rho family small GTPases¹⁴.

In addition to cholesterol, the mevalonate pathway also synthesizes other intermediates, such as the non-sterol isoprenoid geranylgeranyl pyrophosphate (GGPP). GGPP can be used as substrate for

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protein post-translational modifications; namely, protein geranylgeranylation¹⁵. Protein geranylgeranylation is catalyzed by GGTase I. Macrophages that are deficient in the β -subunit of GGTase I (protein geranylgeranyl transferase-1 β subunit; encoded by *Pggt1b*) are hyper-activated by lipopolysaccharide (LPS). Mice with conditional deficiency in *Pggt1b* in myeloid cells also develop inflammatory arthritis spontaneously¹⁶. These observations suggest that protein geranylgeranylation controls the innate immune response; however, the underlying molecular mechanisms remain unclear.

We uncovered an unanticipated link between the mevalonate pathway and TLR-induced PI(3)K activation. Through protein geranylgeranylation, the mevalonate pathway enabled TLR-induced PI(3)K activation by promoting the interaction between the Ras family small GTPase Kras and the PI(3)K catalytic subunit p110 δ . Inhibition of the mevalonate pathway decreased the production of GGPP and limited protein geranylgeranylation, thereby impairing PI(3)K activation. Impaired PI(3)K activation resulted in hyper-inflammatory conditions, including hyper-production of pro-inflammatory 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 used enzyme-linked immunosorbent assay (ELISA) to assess the response of *Pggt1b*-deficient bone-marrow-derived macrophages (BMDMs) to stimulation by ligands of TLRs. Stimulation of *Pggt1b*-deficient macrophages with ligands to TLR4 (LPS), TLR9 (CpG), TLR2 (Pam3CSK4) and TLR7 (R848) led to potent upregulation of pro-inflammatory cytokines such as IL-1 β , TNF, IL-6 and IL-12 in comparison with 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, we also observed enhanced production of granulocyte growth factor G-CSF and neutrophil chemotactic factor

KC(Cxcl1), as well as dampened production of chemokines MCP-1, MIP1 β and MIP1 α , in a multiplex-cytokine assay (Supplementary Fig. 1b). *Pggt1b*^{fl/fl}*Lyz2-Cre* mice had elevated frequencies of granulocytes in their circulation (Fig. 2a,b). To further investigate the physiological relevance of altered cytokine production caused by *Pggt1b* deficiency *in vivo*, we challenged mice with LPS at a lethal dose of 30 mg per kg of body weight. Within 12 h of LPS challenge, 80% of *Pggt1b*^{fl/fl}*Lyz2-Cre* mice died, whereas none of the *Pggt1b*^{fl/+}*Lyz2-Cre* mice died (Fig. 2c). The increased susceptibility of mice to endotoxic shock correlated with higher LPS-induced serum concentrations of the pro-inflammatory cytokines IL-1 β , TNF and IL-6 (Fig. 2d–f). Together these data suggest that *Pggt1b* differentially regulate TLR-induced inflammatory cytokine production.

IKK and MAPK activation are not affected by *Pggt1b* deficiency

The altered cytokine production in *Pggt1b*-deficient macrophages prompted us to evaluate the activation of the IKK, MAPK and TBK1 signaling pathways downstream of TLRs¹⁷. We stimulated wild-type and *Pggt1b*-deficient macrophages with a low dose of LPS (10 ng/ml) and evaluated the activation of IKK β , MAPK and TBK1 by immunoblot analysis with phosphorylation-specific antibodies. Activation of IKK β , JNK, p38, Erk and TBK1 was not enhanced or suppressed in *Pggt1b*-deficient macrophages compared with their activation in wild-type control cells (Supplementary Fig. 2a,b). Endocytosis of TLR4 induced by LPS stimulation was not impaired or enhanced in *Pggt1b*-deficient macrophages either (Supplementary Fig. 2c), suggesting that there is no TLR trafficking defect¹⁸. We conclude that the altered cytokine profile in *Pggt1b*-deficient macrophages is not a result of altered activation of IKK, MAPK, TBK1 or TLR endocytosis.

Compromised PI(3)K activation in the absence of *Pggt1b*

The cytokine production profile that we observed in *Pggt1b*-deficient macrophages is markedly similar to that in myeloid cells deficient in PI(3)K-Akt signaling components, such as p85 α ^{19,20}, p110 δ ¹⁸, mTOR¹¹ and Akt1 (ref. 9), or treated with Gsk3 β inhibitors¹⁰. We speculated that protein geranylgeranylation regulates TLR-induced cytokine production by controlling PI(3)K-Akt activation. *Pggt1b*-deficient macrophages displayed substantially lower constitutive Akt phosphorylation. When *Pggt1b*-deficient BMDMs were stimulated with LPS, phosphorylation of Akt at both Ser473 and Thr308 was severely compromised compared with wild-type control cells (Fig. 3a). Consistent with compromised Akt activation, phosphorylation of Gsk3 β at Ser9 was also severely diminished in *Pggt1b*-deficient cells

Figure 1 *Pggt1b* deficiency augments pro-inflammatory cytokine production while suppressing IFN- β and IL-10 production. (a–g) ELISA of IL-1 β (a), TNF (b), IL-6 (c), IL-12 (d), IFN- β (e), IL-10 (f) and RANTES (g) in the supernatant of BMDMs after 8 h of stimulation with medium or TLR ligands (horizontal axes). Data are representative of three independent experiments with similar results (mean $\pm$ s.d.).

(Fig. 3b). Gsk3 β is phospho-inactivated by Akt at Ser9 (ref. 21). Thus, decreased Ser9 phosphorylation correlates with higher Gsk3 β kinase activity. Indeed, immunoblot analysis revealed that phosphorylation of the c-Jun Thr239 and RelA Ser536, two well-established Gsk3 β substrates²¹ (Supplementary Fig. 3a), was enhanced in *Pggt1b*-deficient BMDMs compared with wild-type control cells (Fig. 3b). Phosphorylation of Thr389 on p70S6K kinase, a direct substrate of mTORC1, was greatly decreased in *Pggt1b*-deficient cells (Fig. 3c). Accordingly, pre-treatment of *Pggt1b*-deficient macrophages with a Gsk3 β inhibitor (SB216763) partially reversed the phenotypes (Supplementary Fig. 3b–f). On the basis of these results, we conclude

that *Pggt1b* controls the PI(3)K-Akt-Gsk3 β and mTOR kinase cascade downstream of TLR in macrophages.

Pggt1b deficiency is a phenocopy of *Pik3cd*^{-/-} BMDMs

Class IA PI(3)Ks are heterodimers composed of a regulatory subunit (p85 α or p85 β) and a catalytic subunit (p110 α , p110 β or p110 δ). Although p110 α and p110 β are ubiquitously expressed, p110 δ expression is enriched in hematopoietic cells and appears to be more important in immune cell signaling²². It has been reported that p110 δ is involved in balancing the production of pro- versus anti-inflammatory cytokines and protects mice from endotoxic shock¹⁸. We found that LPS stimulation alone induced robust IL-1 β release in p110 δ -deficient (*Pik3cd*^{-/-}) BMDMs (Fig. 4a). *Pik3cd*^{-/-} BMDMs also displayed enhanced production of IL-12, TNF and IL-6 and suppressed production of IFN- β (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 probably through p110 δ of the class IA PI(3)K family.

Constitutively active p110 δ ‘rescues’ *Pggt1b* deficiency

To further confirm that protein geranylgeranylation controls cytokine production by regulating p110 δ activation, we infected wild-type or *Pggt1b*-deficient BMDMs with retrovirus harboring cDNAs encoding wild-type p110 δ , *Pggt1b* or a constitutively active form of p110 δ (E1121K)²³. Expression of E1121K, but not wild-type p110 δ , completely abrogated LPS-induced IL-1 β release and restored the production of

Figure 4 Protein geranylgeranylation controls TLR-induced cytokine production and inflammasome activation through the PI(3)K-p110δ. (a–e) ELISA of IL-1β (a), IL-12(p40) (b), TNF (c), IL-6 (d) and IFN-β (e) in wild-type and *Pik3cd*^{-/-} (p110δ) BMDMs stimulated with LPS. (f) ELISA of IL-1β, TNF, IL-12(p40) or IL-10 in supernatants of wild-type (WT) or *Pggt1b*-deficient BMDMs reconstituted with empty vector (EV) or harboring cDNAs encoding wild-type p110δ, wild-type *Pggt1b* or a constitutively active form of p110δ (E1121K). NS, not significant ($P > 0.05$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-way ANOVA). Data are representative of three independent experiments with similar results (mean $\pm$ s.d.).

TNF, IL-12 and IL-10 to the levels observed in wild-type BMDMs; we found the same result in *Pggt1b*-deficient BMDMs reconstituted with wild-type *Pggt1b* (Fig. 4f). These findings further confirm 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 carry a CAAX motif, which is 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 the RBD of the PI(3)K catalytic subunits and activate class I PI(3)K²⁴. The Ras family small GTPases is composed of three members: Hras, Kras and Nras. Computational analysis of farnesylation versus geranylgeranylation potentials using the Prenylation Prediction Suite²⁵ revealed that murine Kras and Nras tended to be geranylgeranylated, whereas Hras was unlikely to be modified by geranylgeranylation (GGTase I: Hras, -7.02; Nras, 1.415; Kras, 2.638; farnesyl transferase (FTase) I: Hras,

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) Real-time RT-PCR analysis of *Hras*, *Kras* and *Nras* transcripts (key) in mouse BMDMs 48 h after transfection with control siRNA or siRNA directed against *Hras*, *Kras* or *Nras* (horizontal axis); results are presented relative to those of the control gene *Gapdh*. (c) Immunoblot analysis of Akt phosphorylated at Ser473 and total (pan-) Akt in BMDMs transfected with siRNA as in b and 48 h later, stimulated for various times (below lanes) with LPS. (d) Quantitation of Akt phosphorylated at S473 in the LPS-stimulated samples in c, calculated as the ratio of phosphorylated Akt to total Akt and presented relative to the ratio in cells treated with control siRNA (Scramble), set as 100%. (e) Immunoblot analysis of lysates (Input) or anti-p110δ immunoprecipitates (IP) 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 analysis of anti-p110α, anti-p110β and anti-p110δ immunoprecipitate or lysates of wild-type or *Pggt1b*-deficient BMDMs stimulated with LPS. Data are representatives of two (a) or three (b–f) independent experiments with similar results.

Figure 6 Spontaneous secretion of IL-1 β from *Pggt1b* and *Pik3cd*^{-/-} macrophages is mediated by the Pyrin inflammasome. (a) Immunoblot analysis of culture supernatants or lysates of wild-type or *Pggt1b*-deficient BMDMs stimulated with various concentrations (above lanes) of LPS alone or with LPS plus nigericin. (b) Real-time RT-PCR analysis of *Nlr4*, *Aim2* and *Mefv* transcripts in primary BMDMs 48 h after transfection of control nontargeting siRNA (NT) or siRNA directed against *Aim2*, *Nlr4* or *Mefv*; results are presented relative to those of the control gene *Gapdh*. (c) Immunoblot analysis of supernatants or lysates of *Pggt1b*-deficient BMDMs 'pre-transfected' with siRNA as in b and stimulated with LPS. (d) Immunoblot analysis of supernatants or lysates of wild-type, *Akt1*^{-/-} and *Pik3cd*^{-/-} BMDMs stimulated with LPS. (e) Immunoblot analysis of supernatants or lysates of *Pggt1b*-deficient BMDMs reconstituted with EV or harboring cDNAs encoding wild-type p110 δ , wild-type *Pggt1b* or the constitutively active form of p110 δ (E1211K). (f) Immunoblot analysis of *Pik3cd*^{-/-} BMDMs 'pre-transfected' with siRNA as in b stimulated with LPS. (g,h) Real-time RT-PCR analysis of *Mefv* transcripts in *Pggt1b*-deficient (g) or *Pik3cd*^{-/-} (h) BMDMs stimulated with LPS; results presented as in b. (i) Immunoblot analysis of Pyrin in lysates of LPS-stimulated *Pggt1b*-deficient and control BMDMs. Data are representative of three independent experiments with similar results (mean and s.d. in b,g,h).

1.305; Nras, 0.003; Kras, 1.816). This is consistent with a published report indicating that human KRAS can be geranylgeranylated in the presence of farnesylation inhibitors, whereas HRAS can only be farnesylated²⁶. *In vivo* labeling of primary BMDMs using ³H-geranylgeranyl pyrophosphate revealed that murine Ras proteins can be geranylgeranylated in macrophages under physiological conditions and that 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 small interfering RNA (siRNA). Knocking down the expression of Kras, but not Hras or Nras, in primary BMDMs markedly decreased Akt phosphorylation following LPS stimulation (Fig. 5b–d). We therefore conclude that Kras, but not Hras or Nras, is essential for TLR-induced PI(3)K activation.

In the carboxy-terminal CAAX motifs of prenylated proteins, C represents cysteine, A represents aliphatic amino acid and X can be any amino acid. The carboxyl terminal (X) directs either farnesylation or geranylgeranylation. GGase I prefers substrates with a carboxyl terminal leucine (CAAL)²⁷. To investigate how prenylation regulates Ras GTPases interaction with p110 δ , we constructed wild-type and CAAL mutant forms of Ras-encoding cDNAs (wild type: Hras, CVLS; Nras, CVLM; Kras, CTVM; CAAL mutant: Hras, CVLL; Nras, CVIL;

Kras, CVIL) into retroviral vectors. The Prenylation Suite predicted these CAAL mutant Ras GTPases to be more likely to be geranylgeranylated (data not shown). Stable cell lines were established by transduction of immortalized wild-type BMDMs with retroviral vectors harboring cDNAs encoding either wild-type or the CAAL mutant form of Ras proteins. Immunoprecipitation of endogenous p110 δ revealed that interaction of Kras-CVIL (the CAAL (geranylgeranylated) form) with p110 δ was markedly enhanced in comparison with the wild-type Kras (CTVM) protein. In the same setting of the experiments, the interaction between Nras or Hras with p110 δ was adversely affected by the increased potential of geranylgeranylation (Fig. 5e). This result suggests that protein geranylgeranylation specifically increases the interaction between Kras and p110 δ .

Given that Ras-PI(3)K interaction is essential for optimal PI(3)K activation¹⁴, we hypothesized that protein geranylgeranylation regulates the Kras-p110 δ interaction. Immunoprecipitation of p110 δ from cell lysate of LPS-stimulated BMDMs revealed that Kras proteins were constitutively associated with p110 δ in resting wild-type cells. This was consistent with the higher basal level of Akt phosphorylation in wild-type cells (Fig. 3a). Following LPS stimulation, Ras proteins dissociated from p110 δ (Fig. 5f). However, in *Pggt1b*-deficient BMDMs, this constitutive association was markedly decreased.

Figure 7 Enhanced cytokine production and inflammasome activation in PBMCs obtained from patients with HIDS and treated with LPS and simvastatin. (a) Immunoblot analysis of uncleaved (pro-) and cleaved IL-1 β in supernatants of PBMCs obtained from healthy control subjects or patients with HIDS (1–6) and stimulated with LPS alone or LPS plus simvastatin. (b) Expression of genes encoding pro-inflammatory cytokines (left margin) in PBMCs obtained from healthy control subjects and stimulated with LPS alone or LPS plus simvastatin. (c) Immunoblot analysis of Akt phosphorylated at Ser473 and total Akt in lysates of human PBMCs pre-treated overnight with the vehicle DMSO or simvastatin (5 μ M) and then stimulated with LPS. Data are representative of two experiments with similar results.

Although LPS stimulation could induce transient interaction between Kras proteins and p110 δ , this interaction was much weaker and was not sustained for more than 10 min (Fig. 5f). We did not see altered interaction between Kras and the class IA catalytic subunits p110 α and p110 β . These observations suggest that the interaction between Kras and p110 δ is regulated by protein geranylgeranylation.

Pyrin inflammasome mediates IL-1 β release in *Pggt1b*^{-/-} BMDMs

The success of treating patients with MKD with anti-interleukin-1 agents such as the recombinant IL-1 receptor antagonist anakinra²⁸ suggests that IL-1 and inflammasomes are important in the pathogenesis of MKD. Stimulation of *Pggt1b*-deficient macrophages with LPS alone resulted in robust IL-1 β maturation and release, whereas the same stimulation did not induce robust IL-1 β production in wild-type cells (Figs. 1a and 6a). The LPS-induced processing and release of IL-1 β in *Pggt1b*-deficient BMDMs began 2 h after LPS stimulation and peaked at 6 h (Supplementary Fig. 4a). In addition to LPS, CpG (TLR9), Pam3CSK4 (TLR2) and R848 (TLR7), but not poly(I:C), induced spontaneous IL-1 β maturation and release in *Pggt1b*-deficient BMDMs (Supplementary Fig. 4b), suggesting that TLR3 signaling is differentially regulated by protein geranylgeranylation than other TLRs. Re-expression of *Pggt1b* in *Pggt1b*-deficient BMDMs completely reversed the spontaneous IL-1 β maturation following LPS stimulation (Supplementary Fig. 4c,d).

Further analysis using BMDMs from compound transgenic strains of mice revealed that IL-1 β 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 indicate that TLR-induced spontaneous IL-1 β maturation in *Pggt1b*-deficient BMDMs is mediated by caspase-1 and ASC, but not by NLRP3. Given that caspase-11-mediated IL-1 β processing also depends on NLRP3 (ref. 29), we ruled out the possibility of a role for caspase-11.

Pyrin has been defined as an innate immune sensor that forms a caspase-1-activating inflammasome in response to bacteria toxins⁶. Mutations in the human PYRIN-encoding *MEFV* gene result in FMF⁵. Using an siRNA approach, we found that knocking down the expression of *Mefv*, but not *Aim2* or *Nlrp4*, resulted in a marked inhibition of IL-1 β release and caspase-1 processing in *Pggt1b*-deficient BMDMs (Fig. 6b,c), suggesting that the Pyrin inflammasome mediates LPS-induced IL-1 β secretion in *Pggt1b*-deficient BMDMs. Similar to *Pggt1b*-deficient cells, stimulation of *Pik3cd*^{-/-} BMDMs with LPS alone also induced robust IL-1 β production (Figs. 4a and 6d). The maturation of IL-1 β in *Pik3cd*^{-/-} BMDMs was accompanied by the release of the caspase-1 p20 subunit, an indication of the activation of caspase-1 inflammasomes (Fig. 6d). Notably, re-expression of a constitutively active form of p110 δ (E1121K) completely rescued the IL-1 β -producing phenotype in *Pggt1b*-deficient BMDMs (Figs. 4f and 6e). Furthermore, an siRNA approach revealed that knocking down just the expression of *Mefv* severely inhibited 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-p110 δ signaling pathway.

Pggt1b and p110 δ control *Mefv* expression

We found that *Mefv* mRNA expression was barely detectable in wild-type BMDMs without stimulation, and its expression was induced by LPS treatment. In the *Pggt1b*- or p110 δ -deficient BMDMs, the basal level of *Mefv* mRNA expression was higher than that of wild-type controls. Notably, *Mefv* expression was hyper-induced in both *Pggt1b*-deficient BMDMs and p110 δ -deficient cells (Fig. 6g,h). Consistent with that, Pyrin protein expression was much higher in *Pggt1b*-deficient BMDMs (Fig. 6i). It is known that expression of *Mefv* is driven by the NF- κ B subunit RelA³⁰ and that the kinase Gsk3 β promotes the transactivation activity of RelA via phosphorylation of RelA¹⁰. We found rampant Gsk3 β activity and enhanced phosphorylation of RelA in *Pggt1b*-deficient BMDMs (Fig. 3b). Taken together, our results support the notion that enhanced NF- κ B RelA transactivational activity drives hyper-expression of *Mefv* that may contribute to TLR-induced IL-1 β release in *Pggt1b*- or p110 δ -deficient BMDMs.

Enhanced production of IL-1 β in PBMCs from patients with HIDS

To mimic the disease conditions of patients with HIDS, we used healthy human peripheral blood mononuclear cells (PBMCs) treated with simvastatin, an inhibitor of HMGCR, to inhibit the mevalonate pathway¹. When we treated healthy PBMCs with LPS, we detected only minimal amounts of IL-1 β maturation in the supernatant. Robust IL-1 β production only occurred when PBMCs from healthy donors were pre-treated with simvastatin to inhibit the mevalonate pathway (Fig. 7a). However, when PBMCs from patients with HIDS were stimulated with LPS alone, a fraction of the patients showed

hyper-production of IL-1 β without inhibition of the mevalonate pathway by simvastatin (patients 1, 2 and 3); in a few other cases, there was spontaneous production of IL-1 β without any stimulation, which may have been caused by pathogen infection (patient 4, 5 and 6) (Fig. 7a). Gene-expression profiling revealed that healthy PBMCs, when treated with LPS and simvastatin, displayed enhanced production of pro-inflammatory cytokines compared with 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 corroborate our findings in the mouse model of protein geranylgeranylation deficiency and highlight how the mevalonate pathway controls the innate immune response by controlling 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-Gsk3 β and mTOR kinase cascade in the regulation of TLR-induced cytokine production has emerged as an important aspect of innate immune signal transduction^{8,12,21}. However, the underlying molecular mechanisms are 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 (refs. 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 tails³². We found decreased production of IFN- β in Pgg1b-deficient BMDMs stimulated with LPS and poly(I:C). Pgg1b-deficient BMDMs displayed attenuated PI(3)K activation following LPS and poly(I:C) (data not shown) stimulation. This is consistent with published data that PI(3)K positively regulates IFN- β production through inactivation of Gsk3 β ^{33–35}. 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 Pgg1b-deficient BMDMs. Poly(I:C) also failed to activate inflammatory in the absence of Pgg1b. These results indicate that protein geranylgeranylation differentially regulates MyD88-dependent and MyD88-independent signaling pathways; the precise underlying mechanism merits further investigation.

Published 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)Ks^{24,36}. However, the manner in which the interaction between Ras and PI(3)K contributes to the TLR-induced innate immune response is not completely understood. In the early 1990s, the finding that farnesylation of KRAS is essential for its oncogenic function galvanized tremendous enthusiasm in developing FTase inhibitors to treat human cancers with KRAS mutations. Unfortunately, the FTase inhibitors showed poor efficacy in clinical trials³⁷, as KRAS can also be geranylgeranylated when FTase I is inhibited^{38–40}. FTase I and GGTase I are both hetero-dimers that share an α subunit and have distinct, but highly homologous, β subunits¹⁵. Our data revealed 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 effect on Kras signaling, membrane targeting or function, and had no effect on cytokine production in myeloid cells¹⁶. Moreover, the absence of *Fntb* in macrophages did not induce any inflammatory phenotypes *in vivo*. Mice deficient in both *Pgg1b* and *Fntb* in macrophages display phenotypes that are indistinguishable from that of the *Pgg1b* single-knockout mouse¹⁶. Taken together, our data suggested that protein geranylgeranylation, but not farnesylation, positively regulates a Kras and p110 δ interaction that is essential for the activation of p110 δ and its downstream kinase cascades.

It has been shown that deficiency in p110 δ in dendritic cells causes a defect in intracellular trafficking of TLR4 following LPS stimulation¹⁸. However, we found no such defect in Pgg1b-deficient BMDMs. Such discrepancies can be attributed to either differences in the cell types examined (macrophage versus DCs) or in the genetic backgrounds of the mouse strains used. Although we saw enhanced production of pro-inflammatory cytokines in healthy PBMCs treated with LPS and simvastatin, the production of INF- β and IL-10 was not consistent with that observed in Pgg1b-deficient mouse BMDMs. We believe the discrepancy is caused by the difference of the experimental settings. The phenotype observed in Pgg1b-deficient mouse BMDMs was caused solely by the deficiency in protein geranylgeranylation, whereas simvastatin inhibition of the mevalonate pathway results in the shortage of not only GGPP, but also a variety of other intermediates downstream of HMGCR in the metabolic pathway. The effect 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 a markedly increased basal level of I κ B α in Pgg1b-deficient cells. I κ B α itself is a target of NF- κ B. Rampant Gsk3 β kinase activity caused an increased level of NF- κ B/RelA phosphorylation as a result of Pgg1b deficiency leading to an inhibited constitutive level of Akt activity. This in turn increased the transactivational activity of RelA to drive the expression of I κ B α gene in resting Pgg1b-deficient cells.

The PI(3)K-Akt-Gsk3 β and mTOR pathway have pivotal roles in controlling a variety of essential cellular functions such as cell growth, survival, differentiation and metabolism⁴¹. Dysregulation of PI(3)K activity is implicated in a myriad of human diseases, particularly cancer and diabetes. Our data implicate protein geranylgeranylation in the regulation of TLR-induced p110 δ 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. Thus, our findings may provide clues for manipulating the mevalonate pathway for the intervention of diseases such as cancer, diabetes and aging.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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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., X.Z. 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.L.K. provided lab space at the US National Institutes of Health 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 *Pggt1b^{fl/fl}Lyz2-Cre* mouse strain, reviewed primary data and contributed to the preparation of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6J mice from The Jackson Laboratory were bred at the University of Massachusetts Medical School. *Pggt1b^{fl/fl}Lyz2-Cre* mice were generated as described⁴². *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 K. Gritsman; the mouse strain was originally generated in the laboratory of J. Ihle⁴³ and had been backcrossed to C57BL6 background in the Gritsman laboratory. Bones from C57BL6J mice were used as controls for the *in vitro* experiments depicted in **Figures 4a–e** and **6d** and **Supplementary Figure 5**. Caspase-1 and Asc-deficient⁴⁴ mice have been described previously. Mouse strains were maintained in specific pathogen-free conditions in the University of Massachusetts 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 the University of Massachusetts 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 the University of Massachusetts Medical School, the Duke University and the National Human Genome Research Institute. A total of three healthy control (two male, one female) and six HIDS patients (two males, four females) were used in the experiments depicted in **Figure 7**. PBMCs were collected by density centrifugation according to the manufacturer's instructions (GE Healthcare).

BMDM culture and stimulation. Bone marrow cells from wild-type and various knockout mice were cultured in DMEM with 10% FBS and 20% L929 supernatants. For immunoblot analysis, BMDMs were primed with 200 ng/ml LPS unless otherwise indicated for 6 h and then stimulated with nigericin or the vehicle for an additional hour. For controls in **Supplementary Figure 6**, BMDMs were transfected with poly(dA:dT) (1 μg/10⁶ cells, Sigma-Aldrich) 2 h after LPS priming and were left for an additional 6 h before harvesting. For ELISAs, BMDMs were stimulated with TLR ligands for 8 h 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 min before 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 2 μg/ml puromycin for 3 consecutive days starting on day 6. Viable cells were used for subsequent experiments described in **Figures 4d** and **6e**.

siRNA knockdown of gene expression in BMDMs. Day 7 culture of BMDMs were transfected with siGenome smart pool (GE Healthcare Dharmacon) of non-targeting control (D-001206-13-05) or siGenome 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),

Nlr4 (M-055000-01-0005). 48 h after siRNA transfection, total RNA were extracted and cDNAs were synthesized. Transcript abundance of murine *Hras*, *Kras*, *Nras*, *Mefv*, *Nlr4* and *Aim2* were determined by quantitative PCR. Primers sequences are listed in **Supplementary Table 1**. The relative abundance of the transcripts was normalized against that of GAPDH. siRNA treated cells were also stimulated with or without LPS for 30 min, cell lysates were prepared and subjected to immunoblot analysis using anti-phosphor-Akt S473 antibody. Quantitation of Akt phosphorylation in **Figure 5d** was carried out using ImageJ 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 quantitative RT-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 described⁴⁵. 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 1**, or with the NanoString nCounter Analysis System (**Fig. 7b**).

Immunoblot analysis. Proteins from the cell culture supernatants were precipitated by methanol-chloroform extraction. Cells were lysed with 1% NP40 lysis buffer. Antibodies used for immunoblot analysis and immunoprecipitation are listed in **Supplementary Table 2**. p110δ immunoprecipitation was performed using a previously described protocol⁴⁶. The phosphorylation of Akt (**Fig. 5d**) was quantified through the use of ImageJ software.

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 intraperitoneal with LPS (Sigma-Aldrich) at a lethal dose of 30 mg/kg bodyweight and returned to the cage and closely observed at 1-h intervals. Blood samples were taken again at 2 h 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.

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