Vitamin B6 Prevents IL-1β Protein Production by Inhibiting NLRP3 Inflammasome Activation*

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Vitamin B6 includes six water-soluble vitamers: pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and their phosphorylated forms. Pyridoxal 5’-phosphate (PLP) is an important cofactor for many metabolic enzymes. Several lines of evidence demonstrate that blood levels of PLP are significantly lower in patients with inflammation than in control subjects and that vitamin B6 has anti-inflammatory effects, with therapeutic potential for a variety of inflammatory diseases. Although one of our group previously demonstrated that PL inhibits the NF-κB pathway, the molecular mechanism by which vitamin B6 suppresses inflammation is not well understood. Here, we showed that both PL and PLP suppressed the expression of cytokine genes in macrophages by inhibiting Toll-like receptor (TLR)-mediated TAK1 phosphorylation and the subsequent NF-κB and JNK activation. Furthermore, PL and PLP abolished NLRP3-dependent caspase-1 processing and the subsequent secretion of mature IL-1β and IL-18 in LPS-primed macrophages. In contrast, PM and PN had little effect on IL-1β production. PLP, but not PL, markedly reduced the production of mitochondrial reactive oxygen species (ROS) in peritoneal macrophages. Importantly, PL and PLP reduced IL-1β production induced by LPS and ATP, or by LPS alone, in mice. Moreover, PL and PLP protected mice from lethal endotoxic shock. Collectively, these findings reveal novel anti-inflammatory activities for vitamin B6 and suggest its potential for preventing inflammatory diseases driven by the NLRP3 inflammasome.

Vitamin B6 is ingested from a variety of foods and can also be taken as a dietary supplement or clinical drug. The B6 vitamer pyridoxal 5’-phosphate (PLP) is an essential cofactor for many enzymes involved in amino acid metabolism. There is growing evidence that vitamin B6 has anti-inflammatory activity. Epidemiological evidence indicates that patients with inflammation have significantly lower blood levels of PLP than control subjects (1, 2). In patients with rheumatoid arthritis, high-dose vitamin B6 supplementation (100 mg/day) suppresses plasma IL-6 and TNF-α levels (3). Both human and animal studies have demonstrated an inverse relationship between vitamin B6 and colon cancer (4, 5). Recent clinical trials found that in Alzheimer’s patients, B-vitamin supplementation (folic acid, vitamin B6, and vitamin B12) slowed the shrinkage of the whole brain and decreased atrophy in specific regions of the brain (6). A low vitamin B6 intake is associated with an increased risk of Parkinson’s disease (7). Because inflammatory mechanisms are implicated in these diseases, vitamin B6 may be useful in preventing inflammatory diseases. Notably, B6 vitamer pyridoxal (PL) inhibits the LPS-induced activation of NF-κB, which is an important transcription factor for many inflammation-related genes in mouse macrophage RAW264.7 cells (8). However, the anti-inflammatory mechanisms of vitamin B6 are poorly understood.

The pleiotropic inflammatory cytokine IL-1β, which is primarily produced by myeloid cells such as monocytes and macrophages, induces the proliferation and/or production of other inflammatory cytokines, leukocyte adhesion molecules, or acute-phase proteins in leukocytes, myeloid cells, endothelial cells, hepatocytes, and so forth. (9). IL-1β is synthesized as a precursor form (pro-IL-1β) that has to be proteolytically processed into a mature form to gain biological activity (10). The former step (pro-IL-1β synthesis) is mainly regulated by a cytoplasmic signaling pathway that activates NF-κB (signal 1), which is triggered, for example, by TLRs. The latter step (IL-1β processing) can be catalyzed by several proteases; of these, caspase 1 is the most important, as shown by the severe defects in mature IL-1β production in caspase-1-deficient mice (11, 12).

Caspase 1 is also synthesized as an inactive precursor and is fully activated by autoprocessing. The cytoplasmic signaling pathway that activates caspase 1 (signal 2) has been extensively studied recently, revealing that the inflammasome, which is a cytoplasmic multiprotein complex consisting of sensor proteins (such as NLRP3, NLRC4, or AIM2), adaptor proteins (ASC), and caspase 1, forms a platform to activate caspase 1 (10, 13). Of the sensor proteins, NLRP3 has been studied most...
intensively because it responds (directly or indirectly) not only to pathogen-associated molecules (such as bacterial ionophores, pore-forming toxins, and viral RNA), but also to a variety of environmental and endogenous inflammatory substances (including asbestos, silica, ATP, urate crystals, β-amylloids, cholesterol crystals, and even fatty acids) (14–22). Accordingly, NLRP3 has been implicated in a variety of inflammatory diseases, including inflammatory bowel disease, gout, Alzheimer’s disease, arteriosclerosis, and diabetes (19, 21, 23–28). In addition, NLRP3 mutations are known to cause autoinflammatory syndromes that are collectively called cryopyrin-associated periodic syndrome (29).

In this study, we demonstrate a novel role of vitamin B6 in suppressing IL-1β production by inhibiting the activation of the NLRP3 inflammasome. Furthermore, we show that vitamin B6 prevented LPS-induced endotoxic shock in vivo, suggesting that the NLRP3 inflammasome is an important target for vitamin B6 anti-inflammatory activity.

Results

**Vitamin B6 Inhibited LPS-induced NF-κB and JNK Activation and Gene Expression**—We initially investigated the overall effect of vitamin B6 on IL-1β secretion from macrophages stimulated with LPS plus ATP. To this end, thiglycollate-induced peritoneal macrophages were cultured for 24 h with or without B6 vitamer supplementation (500 μM), and then sequentially stimulated with LPS (TLR4 agonist) and ATP (NLRP3 activator) to induce IL-1β secretion. Under these conditions, IL-1β secretion was strongly inhibited by PL or PLP but not by pyridoxamine (PM) or pyridoxine (PN) (Fig. 1A). Titration experiments indicated that as little as 50 μM PL or PLP significantly suppressed IL-1β secretion (Fig. 1B). The LPS-induced intracellular accumulation of pro–IL-1β and secretion of IL-6 and TNF-α were also inhibited by PL or PLP, but not by PM or PN (Fig. 1, C–E). Furthermore, the LPS-induced expression of Il1b, Il6, Ptgs2, and Ccl2 mRNAs in peritoneal macrophages was inhibited by PL or PLP, but not by PM or PN (Fig. 1, F–J). PL and PLP, as well as PM and PN (although to a lesser extent), suppressed Nlrp3 mRNA expression (Fig. 1F). These results were consistent with the previous findings that PL inhibited LPS-induced NF-κB activation and expression of NF-κB target genes (Nos2 and Ptgs2) in the Raw264.7 mouse macrophage cell line (8). Actually, Western blotting analyses indicated that events upstream of LPS-induced NF-κB activation, including the phosphorylation of TAK1 and IkB kinases and the degradation of IkBa, were severely suppressed by PL or PLP (Fig. 1K). PL and PLP also inhibited the LPS-induced JNK phosphorylation (Fig. 1K), which occurs downstream of TAK1 and contributes to IL-1β gene expression (30, 31).

In addition, PL and PLP inhibited IL-6 and TNF-α production induced by other TLR ligands, including the TLR3 ligand poly(I:C), the TLR2 ligand Pam3CSK4, the TLR7 ligand imiquimod (R837), and CpG oligodeoxynucleotides, which are TLR9 ligands (Fig. 2). Taken together, our results demonstrate that both PL and PLP negatively regulate the TLR-mediated activation of NF-κB and MAPK pathways by inhibiting TAK1 phosphorylation, and thereby inhibit the expression of cytokine genes including Il1b in primary macrophages.

**Vitamin B6 Suppressed NLRP3 Inflammasome Activation**—To further investigate the effect of vitamin B6 on signal 2, which leads to the proteolytic maturation of IL-1β, peritoneal macrophages were cultured with LPS for 16 h to fully induce intracellular pro–IL-1β accumulation, and then treated with B6 vitamers and stimulated with ATP for 6 h to activate the NLRP3 inflammasome. Under these conditions, IL-1β secretion was again inhibited by as little as 50 μM PL or PLP, but not by 500 μM PM or PN (Fig. 3, A and B). IL-1β secretion induced by other NLRP3 activators such as nigericin, R837, and monosodium urate (MSU) crystals was also inhibited by PL and PLP (Fig. 3C). The secretion of IL-1β, which, like IL-1β undergoes proteolytic maturation catalyzed by caspase-1, was also suppressed by PL and PLP (Fig. 3D). In contrast, neither PL nor PLP added after LPS treatment affected the secretion of IL-6, an NF-κB-dependent but caspase-1-independent cytokine (Fig. 3E). In addition, ATP treatment after LPS priming induced no more phosphorylation of TAK1 and IkB kinases and degradation of IkBa, and PL and PLP did not affect these events (Fig. 3F). These data indicated that the suppression of IL-1β secretion by PL and PLP under these conditions was not due to the inhibition of NF-κB.

Western blotting analyses indicated that PL and PLP inhibited the generation of mature IL-1β (p17) and of the p10 fragment of mature caspase-1 when these vitamers were added after LPS treatment and before ATP stimulation (Fig. 3G). However, the LPS-induced NLRP3 and pro–IL-1β expression and the constitutive caspase-1 expression at both mRNA and protein levels were not inhibited by PL or PLP (data not shown and Fig. 3G), consistent with the notion that NF-κB-dependent gene expression was not inhibited under these conditions.

Inflammasome activation induces pyroptosis, a caspase-1-dependent programmed cell death. Pyroptotic cells rupture rapidly, releasing lactate dehydrogenase (LDH) and other cytoplasmic contents. Pyroptosis would also facilitate the IL-1β release from macrophages. PL and PLP inhibited LDH release from macrophages at 1, 2, and 3 h, but not 6 h after ATP stimulation (Fig. 3I). These results indicate that PL and PLP delayed pyroptosis; however, their suppression of IL-1β secretion at 6 h was not due to the inhibition of pyroptosis (Fig. 3H). Taken together, these results indicate that PL and PLP can inhibit the signal 2 mediated by the NLRP3 inflammasome.

**Vitamin B6 Did Not Affect the Signal 2 Mediated by the NLRC4 and AIM2 Inflammasomes**—The NLRP3, NLRC4, and AIM2 inflammasomes can be specifically activated by different bacterial species under certain conditions. For example, *Staphylococcus aureus* and *Salmonella typhimurium* at the logarithmic growth phase activate mainly the NLRP3 and NLRC4 inflammasome, respectively (32–34). In contrast, the infection of unprimed macrophages with *Listeria monocytogenes* followed by penicillin G treatment, which causes intracellular releases of bacterial DNA, induces the AIM2-dependent secretion of IL-1β (35).

To investigate whether PL and PLP inhibit IL-1β production induced by *S. aureus* and *S. typhimurium*, unprimed macrophages and LPS-primed macrophages were treated with PL or PLP and then infected with the bacteria. In unprimed macrophages, the signal 1 for pro–IL-1β production depended on the bacterial infection. Under such conditions, PL and PLP inhibi-
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PL and PLP Suppress Signal 1

A and B, peritoneal macrophages were treated with the indicated B6 vitamers (500 μM in A, and the indicated concentrations in B) for 24 h, stimulated with LPS for 16 h, and finally exposed to 5 mM ATP for 6 h. The IL-1β concentration in culture supernatants was determined by ELISA. C–E, peritoneal macrophages were treated with B6 vitamers for 24 h, and then stimulated with LPS for 16 h. Pro-IL-1β and GAPDH (loading control) were detected by Western blotting (C). The IL-6 and TNF-α concentrations in culture supernatants were determined by ELISA (D and E). F–J, peritoneal macrophages were treated with B6 vitamers for 24 h, and then stimulated with LPS for 16 h, after which the Il1b, Il6, Ptgs2, Ccl2, and Nlrp3 mRNAs were quantified by real-time PCR. K, peritoneal macrophages were treated with PL or PLP for 2 h, and then stimulated with LPS for the indicated times. The total and/or phosphorylated (p-) forms of TAK1, IKKs, IκBα, and JNKs were detected by Western blotting. A, B, and D–J, data show mean ± S.D.; n = 3. Asterisks indicate significant differences (**, p < 0.01) from the control group (c). All experiments were repeated at least three times, and representative data are shown.
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![Graphs and images showing effects of Vitamin B6 on IL-1β and TNF-α secretion](image)

Under the same conditions, pyroptosis is induced by caspase-11-dependent but NLRP3 inflammasome-independent manner (37). Consistently, ASC−/− macrophages released LDH to a similar extent as wild-type macrophages (Fig. 4G). Importantly, PL and PLP reduced LDH release, suggesting that PL and PLP could inhibit caspase-11-dependent pyroptosis (Fig. 4G).

Vitamin B6 Inhibited Signal 1 and Signal 2 for IL-1β Production in Human Cells—To test the inhibitory effects of vitamin B6 on IL-1β secretion requiring signal 1 and signal 2 and on TNF-α secretion that requires only signal 1 in human cells, we used macrophagic cells differentiated from the THP-1 human monocytic cell line by PMA treatment (THP-1 macrophages). The THP-1 macrophages were treated with PL or PLP before or after LPS treatment, and then stimulated with nigericin to activate the NLRP3 inflammasome. As expected, potent IL-1β secretion was observed after sequential stimulation with LPS and nigericin, whereas TNF-α secretion was fully induced by LPS stimulation alone; nigericin did not affect TNF-α production (Fig. 5, A–D). When the THP-1 macrophages were treated with PL or PLP before LPS treatment, the secretion of both IL-1β and TNF-α was inhibited (Fig. 5, A and B). In contrast, when PL or PLP were added after LPS treatment, only IL-1β secretion was inhibited; TNF-α secretion was unaffected (Fig. 5, C and D). These results, which were consistent with our results using mouse peritoneal macrophages, suggest that PL and PLP inhibit both signal 1 and signal 2 in human cells.

Vitamin B6 Suppressed ASC Speck Formation and Oligomerization—ASC forms large aggregates called “specks” when inflammasomes are activated. ASC speck formation is suggested to be involved in efficient caspase-1 activation and IL-1β processing. Therefore, we investigated whether PL and PLP inhibited ASC speck formation. ASC specks formed when LPS-primed peritoneal macrophages were stimulated with nigericin (Fig. 6A, upper panels, and 6B). However, treating LPS-primed macrophages with PL or PLP prior to nigericin stimulation strongly inhibited the ASC speck formation (Fig. 6A, middle and lower panels, and 6B). Consistently, PL and PLP inhibited ASC oligomerization under the same conditions (Fig. 6C). Taken together with our observation that PL and PLP selectively inhibit the NLRP3 inflammasome, these results suggest that PL and PLP inhibit signal 2 by targeting NLRP3 or upstream events that induce the NLRP3 inflammasome.

Mitochondrial Reactive Oxygen Species (ROS) Production Was Inhibited by PLP but Not by PL—Because the NLRP3 inflammasome is activated by structurally diverse molecules, it has been postulated that different activators induce common intracellular events that eventually cause NLRP3 inflammasomes to form. The efflux of K+ and the resulting decrease in intracellular K+ concentration has been proposed as a common upstream event in NLRP3 inflammasome formation (10, 38). Therefore, we measured cellular potassium levels using inductively coupled plasma mass spectrometry. Consistent with previous findings (38), treating LPS-primed macrophages with ATP or nigericin decreased the cellular potassium level. This event was not affected by PL or PLP treatment (Fig. 6D).

Mitochondrial ROS generation has also been proposed as a common upstream event in NLRP3 activation (39). Consistently, treating LPS-primed macrophages with ATP enhanced the MitoSOX Red fluorescence, indicating elevated mitochondrial ROS generation (Fig. 6E). Furthermore, Mito-TEMPO, a mitochondria-targeted antioxidant that inhibited ATP-induced mitochondrial ROS elevation, suppressed IL-1β production in LPS-primed macrophages (Fig. 6, E and F). Mito-TEMPO also inhibited IL-1β production induced by other NLRP3 activators (Fig. 6F). Because vitamin B6 also acts as an antioxidant (40), we examined whether PL and PLP could inhibit mitochondrial ROS generation. Interestingly, this event was markedly inhibited by PLP but not by PL (Fig. 6G). These results indicate that PLP has a potential to suppress mitochondrial ROS generation, which can, at least in part, explain the ability of PLP to inhibit NLRP3-dependent IL-1β production.
Vitamin B6 Inhibited IL-1β Production in Mice and Protected Mice against LPS-induced Endotoxic Shock—Finally, we examined the in vivo effects of PL and PLP. We induced IL-1β production in ICR mice by i.p. injection of a low dose of LPS (2 μg/kg body weight (bw)) followed by ATP (50 μmol/kg bw) (41, 42), or in C57BL/6 mice by a high dose of LPS (20 mg/kg bw) alone (43). In both experimental systems, serum and/or peritoneal IL-1β levels were suppressed by injecting PL or PLP at 20 mg/kg bw (Fig. 7, A–D). In contrast, PL or PLP did not significantly suppress serum or peritoneal TNF-α levels (Fig. 7, E and F).

Injecting a high dose of LPS induces lethal endotoxic shock in mice. Components of the NLRP3 inflammasome (i.e. NLRP3 and ASC) play essential roles in this disease model (44–46), although IL-1β and IL-18 are dispensable (47, 48). To test whether PL and PLP can rescue mice from lethal endotoxic shock, C57BL/6 mice pretreated with PBS (control), PL, or PLP were given an injection of LPS at 50 mg/kg bw. Mice pretreated with PBS (n = 15) died within 2 days after LPS injection; notably, the survival was improved in mice pretreated with PL or PLP (n = 15 each group) (Fig. 7G). Taken together, these results suggest that PL and PLP may inhibit the activity of the NLRP3 inflammasome in vivo.

Discussion

In the present study, we showed that the B6 vitamers PL and PLP inhibit both TLR-induced NF-κB activation (signal 1) and NLRP3-mediated caspase-1 activation (signal 2), thereby abolishing IL-1β production in macrophages. PLP is a required cofactor for many metabolic enzymes. However, the PLP con-
The inhibitory effect of PL and PLP on signal 1 was not TLR4-specific, because PL and PLP inhibited IL-6 and TNF-α production induced by ligands for other TLRs (Fig. 2). Although TLR4 activates both the MyD88-dependent and the TRIF-dependent NF-κB activation pathways, TLR2, TLR7, and TLR9 specifically activate the MyD88-dependent pathway, and TLR3 only activates the TRIF-dependent pathway (49). Thus, it is likely that the target of PL and PLP in the NF-κB pathway is a common downstream component of the MyD88- and TRIF-dependent pathways. In this context, it is worth noting that these pathways commonly induce the phosphorylation cascade of TAK1-IKK-IκB, thereby degrading IκB and activating NF-κB (49). Our results (Fig. 1K) indicate that PL and PLP inhibited the LPS-induced TAK1 phosphorylation, IKK-IκBα pathway, and JNK phosphorylation. Taken together, it is likely that PL and PLP inhibit signal 1 by targeting TAK1 or a molecule upstream of TAK1.

Our experiments using bacterial species that selectively activate NLRP3, NLRC4, or AIM2 suggested that PL and PLP specifically inhibited the NLRP3 inflammasome. Thus, it is likely that PL and PLP target NLRP3 or a more upstream event in signal 2. Consistent with this possibility, PL and PLP sharply inhibited ASC speck formation, which occurs immediately

FIGURE 4. PL and PLP selectively suppress the NLRP3 inflammasome. A and B, peritoneal macrophages were incubated with PL or PLP for 24 h, and then infected with S. aureus (S. a.) with a multiplicity of infection (MOI) of 100 or with S. typhimurium (S. t.) with a MOI of 20. C and D, peritoneal macrophages were primed with LPS for 16 h, and then incubated with PL or PLP for 2 h and finally infected with S. aureus (MOI 50) or S. typhimurium (MOI 20). E, peritoneal macrophages were infected with L. monocytogenes (L. m., MOI 2) for 4 h, and then incubated with PL or PLP for 2 h, after which penicillin G (100 units/ml) was added to facilitate intracellular bacterial DNA release. F and G, WT and ASC−/− peritoneal macrophages were primed with Pam3CSK4 (1 μg/ml) for 2 h, incubated with PL or PLP for 1 h, and finally transfected with LPS (2 μg/ml) using FuGENE HD for 18 h. IL-1β in culture supernatants was quantified by ELISA (A–F). Cell death was evaluated by assaying LDH release (G). Data show mean ± S.D.; n = 3. Asterisks indicate significant differences (**, p < 0.01) from the control group (c). All experiments were repeated at least three times, and representative data are shown.

FIGURE 5. PL and PLP suppress signal 1 and signal 2 in human cells. A and B, THP-1 macrophages were treated with PL or PLP for 2 h, incubated with LPS for 4 h, and finally stimulated with nigericin (Nig, 5 μM) for 0.5 h. C and D, THP-1 macrophages were primed with LPS for 4 h, treated with PL or PLP for 2 h, and finally stimulated with nigericin (5 μM) for 0.5 h. A–D, IL-1β and TNF-α in culture supernatants were measured by ELISA. Data show mean ± S.D.; n = 3. Asterisks indicate significant differences (**, p < 0.01) from the control group (c). All experiments were repeated at least three times, and representative data are shown.
downstream of NLRP3 activation. K⁺ efflux and mitochondrial ROS generation have been suggested as common upstream events of the activation of NLRP3 by various activators. Our results indicated that PLP inhibited ATP-induced mitochondrial ROS generation, which may contribute to PLP's inhibition of signal 2. However, PL did not affect mitochondrial ROS generation. In addition, neither PL nor PLP inhibited ATP- and nigericin-induced K⁺ efflux.

Because it was previously reported that NLRP3 is recruited to mitochondria upon activation (50), we investigated whether PL and PLP affect the mitochondrial localization of NLRP3 in peritoneal macrophages treated with LPS and/or ATP. In our study, a portion of NLRP3 localized at the mitochondria after the induction of NLRP3 expression by LPS, and ATP stimulation did not change the amount of mitochondrial NLRP3. Furthermore, neither PL nor PLP affected the amount of mitochondrial NLRP3, when PL or PLP was added after LPS stimulation but before ATP stimulation (data not shown).

We also sought to determine whether PL and PLP affected the interaction of NLRP3 and ASC or post-translational modification of NLRP3 such as ubiquitination or tyrosine phosphorylation (51). However, in our study, these responses of endoge-
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Experimental Procedures

Mice—ICR mice and C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). All protocols for animal studies were approved by the Kanazawa University Committee on Animal Welfare.

Reagents—PL hydrochloride, PN hydrochloride, PLP (Nacalai Tesque, Kyoto, Japan), PM dihydrochloride (Calbiochem), LPS from *Escherichia coli* K235 and from *E. coli* 0111:B4, ATP, nigericin, poly(I:C) (Sigma-Aldrich), Pam3CSK4, R837 (Invivogen, San Diego, CA), CpG oligodeoxynucleotides (Genset Oligos, La Jolla, CA), and MSU (Wako, Osaka Japan) were purchased.

To test the inhibitory activity of vitamin B6 on other TLR ligands, mouse macrophages were cultured with a B6 vitamer for 2 h and then with various TLR agonists for 6 h. THP-1 macrophages were treated sequentially with a B6 vitamer for 2 h, LPS for 4 h, and nigericin (5 μM) for 30 min.

To test the inhibitory activity of vitamin B6 on signal 2, mouse macrophages were treated with LPS for 16 h, with a B6 vitamer for 2 h, and with various inflammasome activators for 6 h. THP-1 macrophages were treated with LPS for 4 h, with a B6 vitamer for 2 h, and with nigericin for 30 min.
To induce noncanonical NLRP3 inflammasome formation, peritoneal macrophages were primed with 1 μg/ml Pam3CSK4 for 5 h, washed once with fresh medium, and then treated with or without 500 μM PL or PLP for 1 h. Finally, cells were transfected with LPS (2 μg/ml) using FuGENE HD (Promega, Madison WI) and cultured for 18 h.

**Bacterial Infection—S. aureus** (Smith strain, kindly provided by Dr. Nakamichi, Kanazawa University, Kanazawa, Ishikawa, Japan) and *S. typhimurium* (ATCC 14028) in the log phase were used for infection. *L. monocytogenes* (EGD, serovar 1/2a) was cultured in brain-heart infusion broth (Eiken Chemical, Tokyo, Japan), collected in the log phase, washed with PBS, suspended in PBS supplemented with 10% glycerol, and stored in aliquots at −80 °C. Bacterial stocks were thawed and diluted in RPMI 1640 medium just prior to infecting macrophages (35).

The peritoneal macrophages were placed in antibiotic-free medium in 96-well plates and infected with the bacteria. The plates were briefly centrifuged to improve interactions between the cells and bacteria. Penicillin G (100 units/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml, Thermo Fisher Scientific) were added 1 h after infection with *S. aureus* or *S. typhimurium*, and the cells were further cultured for 5 h. To activate AIM2, peritoneal macrophages were infected with *L. monocytogenes*. Finally, penicillin G (100 units/ml) was added to the culture to facilitate intracellular bacterial DNA release, and the cells were further cultured for 3 h.

**Real-time PCR**—Total RNA was purified from mouse macrophages using TRIZOL reagent (Thermo Fisher Scientific), and cDNA was synthesized using the First Strand cDNA Synthesis kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the StepOne Real-Time PCR system (Thermo Fisher Scientific) with the following primers: *Il1b*, 5′-TGGGCCCTCAA-AGGAAAAG-3′ and 5′-GGTGCTGATGTACCAGTT-3′; *Il6*, 5′-AGACAAAGCCAGAGCTTCTTACG-3′ and 5′-TGCCGAGTAGTCTCATAAAGTG-3′; *Cc2*, 5′-GGTCCCTGCTGATGCTGTTGGG-3′ and 5′-CCCTTCTGGGTCAGCACAG-3′; *Ptgs2*, 5′-GCCAGGGCTGAATCTCCAAACA-3′ and 5′-GCTCAGGAGGCCACTGATACCTA-3′; *Nlrp3*, 5′-GTGGTGACCTCGTGATGTTG-3′ and 5′-TCTTCTGGAGGCGCTTCAA-3′; and *Gapdh*, 5′-CATTGACCCCCCTTATTGACC-3′ and 5′-TGGAGGATGGTGATGGATT-3′.

**ELISA and LDH Analysis**—The concentrations of mouse and human IL-1β, IL-6, and TNF-α in culture supernatants were determined using OptEIA ELISA kits (BD Pharmingen) according to the manufacturer’s protocols. The mouse IL-1β ELISA kit was purchased from MBL (Nagoya, Japan). Cell death was determined by measuring LDH activity in the culture medium using the CytoTox96 NonRadioactive Cytotoxicity Assay (Promega).

**Western Blotting Analysis**—Cells were lysed in Tris-buffered saline containing 1% Nonidet P-40 and Complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. Lysates were centrifuged at 12,000 × g for 10 min to remove debris and then boiled in Laemmli sample buffer for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated for 1 h in a blocking buffer (5% skim milk, 0.05% Tween 20 in Vitamin B6 Suppresses NLRP3 Inflammasome

**Determination of Mitochondrial ROS Levels**—Cells (6 × 10^5 cells/well in a 24-well plate) were lysed in 3% ultrapure HNO₃ (600 μl) for 30 min on ice. Lysates were diluted 10 times with ultrapure H₂O, and potassium concentrations were determined by inductively coupled plasma mass spectrometry (SPQ-9000, Seiko Instruments, Chiba, Japan) using KCl solution as a standard.

**Determination of Intracellular Potassium Levels**—Mitochondrial ROS levels were measured using MitoSOX Red (Thermo Fisher Scientific) according to the manufacturer’s protocol. Data were acquired with a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (FlowJo, Ashland, OR).

**In Vivo Experiments**—The Food and Nutrition Board of the Institute of Medicine proposed a tolerable upper limit of 100 mg/day of vitamin B6 for adult humans (53). Based on this and the conversion between human doses and animal-equivalent doses according to body surface area (54), we estimated a tolerable vitamin B6 dose of 20 mg/kg bw/day for mice; this was the dose used for the in vivo experiments in the present study. ICR mice were i.p. injected first with PL or PLP, injected 2 h later with LPS (from E. coli 0111:B4, 2 μg/kg bw), and injected 90 min later with 10 μl/kg bw of 5 mM ATP (50 μmol/kg bw). Serum and peritoneal lavage samples were collected 1 h after the ATP injection. C57BL/6 mice were i.p. injected with LPS (20 mg/kg bw) with or without PL or PLP, and serum and peritoneal lavage samples were collected 3 h later. Lethal endotoxic shock was induced in C57BL/6 mice by i.p. LPS injection (50 mg/kg bw, from E. coli 0111:B4, Sigma-Aldrich).
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Statistical Analysis—Data were analyzed using GraphPad Prism 6.05 (GraphPad Software, La Jolla, CA). Difference between a control and an experimental group was examined by one-way analysis of variance and Dunnett's test. Difference between mouse survival curves was evaluated by the log-rank (Mantel-Cox) test, p < 0.05 was considered significant.

Author Contributions—P. Z. contributed to the experimental design, performed most of the experiments, and wrote the manuscript; K. T. performed or supervised experiments involving bacterial infection; T. K. and H. K. provided technical assistance; S. S. helped collect samples; M. H. and H. I. performed and supervised inductively coupled plasma mass spectrometry analyses; N. K. contributed to the experimental design and critical review of the manuscript; and T. S. designed and supervised the research project and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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References
1. Saibeni, S., Cattaneo, M., Vecchi, M., Zighetti, M. L., Locchi, A., Lombardi, R., Meucci, G., Spina, L., and de Franchis, R. (2003) Low vitamin B6 plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants. Am. J. Gastroenterol. 98, 112–117
2. Sakakenny, L., Roubenoff, R., Obin, M., Fontes, J. D., Benjamin, E. J., Bujanover, Y., Jacques, P. F., and Selhub, J. (2012) Plasma pyridoxal-5-phosphate is inversely associated with systemic markers of inflammation in a population of U.S. adults. J. Nutr. 142, 1280–1285
3. Huang, S. C., Wei, J. C., Wu, D. J., and Huang, Y. C. (2010) Vitamin B6 supplementation improves pro-inflammatory responses in patients with rheumatoid arthritis. Eur. J. Clin. Nutr. 64, 1007–1013
4. Komatsu, S. I., Watanabe, H., Oka, T., Tsuge, H., Nii, H., and Kato, N. (2001) Vitamin B-6-supplemented diets compared with a low vitamin B-6 diet suppress azoxymethane-induced colon tumorigenesis in mice by reducing cell proliferation. J. Nutr. 131, 2204–2207
5. Galluzzi, L., Vacchelli, E., Michels, J., Garcia, P., Kepp, O., Vitale, I., and Kroemer, G. (2013) Effects of vitamin B6 metabolism on oncogenesis, tumor progression and therapeutic responses. Oncogene 32, 4995–5004
6. Douaud, G., Refsum, H., de Jager, C. A., Watanabe, H., Hirota, Y., Nagai, M., and Fukuoka Kinki Parkinson’s Disease Study Group (2010) Dietary intake of folate, vitamin B-6, vitamin B-12, and vitamin D is associated with risk of Parkinson’s disease in Japan. Br. J. Nutr. 104, 757–764
7. Yanaka, N., Koyama, T. A., Komatsu, S., Nakamura, E., Kanda, M., and Kato, N. (2005) Vitamin B6 suppresses NF-κB activation in LPS-stimulated macrophages. Int. J. Mol. Med. 16, 1071–1075
8. Garlanda, C., Dinarello, C. A., and Mantovani, A. (2013) The interleukin-1 family: back to the future. Immunology 139, 1003–1018
9. Franchi, L., Muñoz-Planillo, R., and Núñez, G. (2012) Sensing and reacting to microbes through the inflammasomes. Nat. Immunol. 13, 325–332
10. Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Safdelf, J., et al. (1995) Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell 80, 401–411
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Griffiths, R. J., Stam, E. J., Downs, J. T., and Otterness, I. G. (1995) ATP induces the release of IL-1 from LPS-primed cells in vivo. J. Immunol. 154, 2821–2828

Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., and Zhou, R. (2015) Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. Cell 160, 62–73

Mariathasan, S., Newton, K., Monack, D. M., Vucic, D., French, D. M., Lee, W. P., Roose-Girma, M., Erickson, S., and Dixit, V. M. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature 430, 213–218

Sutterwala, F. S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G. S., Grant, E. P., Bertin, J., Coyle, A. J., Galán, J. E., Askenase, P. W., and Flavell, R. A. (2006) Critical role for NALP3/CIA51/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 24, 317–327

Imamura, R., Wang, Y., Kinoshita, T., Suzuki, M., Noda, T., Sagara, J., Taniguchi, S., Okamoto, H., and Suda, T. (2010) Anti-inflammatory activity of PYNOD and its mechanism in humans and mice. J. Immunol. 184, 5874–5884

Shornick, L. P., De Togni, P., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R. W., Ferguson, T. A., and Chaplin, D. D. (1996) Mice deficient in IL-1β manifest impaired contact hypersensitivity to trinitrochlorobenzene. J. Exp. Med. 183, 1427–1436

Sakao, Y., Takeda, K., Tsutsui, H., Kaisho, T., Nomura, F., Okamura, H., Nakanishi, K., and Akira, S. (1999) IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock. Int. Immunol. 11, 471–480

Kawai, T., and Akira, S. (2007) Signaling to NF-κB by Toll-like receptors. Trends Mol. Med. 13, 460–469

Subramanian, N., Natarajan, K., Clatworthy, M. R., Wang, Z., and Germain, R. N. (2013) The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation. Cell 153, 348–361

Juliana, C., Fernandes-Alnemri, T., Kang, S., Farias, A., Qin, F., and Alnemri, E. S. (2012) Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. J. Biol. Chem. 287, 36617–36622

Alves, J. N., Pires, K. M., Lanzetti, M., Barroso, M. V., Benjamín, C. F., Costa, C. A., Resende, A. C., Santos, J. C., Ribeiro, M. L., Porto, L. C., and Valença, S. S. (2013) Critical role for CCR2 and HMGB1 in induction of experimental endotoxic shock. Arch. Biochem. Biophys. 537, 72–81

Institute of and Medicine, U. S. (1998) Vitamin B6. In Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline, pp. 150–195, National Academies Press, Washington, D. C.

U. S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research (CDER) (2005) Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, pp. 6–7, U. S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research, Rockville, MD