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Isoprenoid Depletion Alters Expression of Inflammatory Markers in Monocytes and Macrophages

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Submitted in partial fulfillment of Honors Requirements for the Department of Biology
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The Department of Biology at Dickinson College hereby accepts this senior honors thesis by Katelyn Swade, and awards departmental honors in Biology.

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ABSTRACT

Mevalonate kinase deficiency (MKD) occurs in patients with mutations in the mevalonate kinase (MVK) gene, which is responsible for the phosphorylation of mevalonate in the mevalonate pathway. Patients with MKD exhibit a systemic inflammatory phenotype marked by recurrent episodes of disturbed cytokine production and fever attacks. The mevalonate pathway is responsible for the production of non-sterol isoprenoid compounds such as farnesyl- and geranylgeranylpyrophosphate (FPP and GGPP). FPP and GGPP are lipid moieties that are critical for the proper functioning of cellular molecules including the Ras, Rho, and Rab families of small GTP-binding proteins. Phenotypes of MKD have been linked to GGPP depletion in monocytes and macrophages and are exacerbated in the presence of inflammatory stimuli such as lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. The goal of this study was to compare the response of different cell types to LPS following isoprenoid depletion. MKD can be modeled in cell culture by treating cells with statins, a class of drugs that inhibit 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, the enzyme that acts directly upstream of mevalonate. When RAW 264.7 murine macrophages were treated with lovastatin and stimulated with LPS, expression of the pro-inflammatory markers IL-1β, TNF-α, and CD14 increased. An inhibitor of geranylgeranyl transferase (GGTase) I had a similar effect as lovastatin on the expression of these markers. Since GGTase I is the enzyme responsible for the transfer of GGPP to Rho GTPases, we hypothesized that one or more members of this protein family are responsible for these effects. Treatment with lovastatin followed by stimulation with LPS led to increased activation of Rho, but no detection of Rac1 activation was present. In addition, an inhibitor of Rho protein function partially blocked the increased levels of IL-1β and TNF-α following lovastatin treatment, indicating that Rho protein activation is playing a role in these effects. In order to further examine the role of isoprenoid depletion in human monocytes and macrophages, monocytes were isolated from human blood by centrifugal elutriation and differentiated to monocyte-derived macrophages (MDMs) by treatment with macrophage colony stimulating factor (M-CSF). In contrast to the RAW 264.7 cells, IL-1β mRNA levels show a suppressed increase in human monocytes following LPS stimulation in the presence of lovastatin and no changes in CD14 mRNA levels were observed in either human monocytes or MDMs. These findings indicate substantial cell specific differences in the response to LPS following isoprenoid depletion and require further investigation to determine the contribution of different cell types to the phenotypes of MKD. Ultimately, a better mechanistic understanding of these processes has the potential to impact treatment of inflammatory diseases such as MKD.
INTRODUCTION

Mevalonate Kinase Deficiency

Mevalonate Kinase Deficiency (MKD) is an autosomal recessive disorder marked by a strong innate immune system response, including recurrent episodes of disturbed cytokine production and fever attacks. MKD is extremely rare, with less than 200 reported cases worldwide from its initial discovery in 1985. The gene mutation that causes MKD (12q24.11) is known to have over 100 pathological mutations thus far in its discovery. MKD is a newly discovered disorder that is poorly understood overall. For this reason, MKD is likely under-diagnosed. A better understanding of the disorder and its pathology could lead to more targeted therapies and pharmaceutical treatments for MKD and similar inflammatory disorders.

Phenotypes

MKD has two distinct phenotypes, Mevalonic Aciduria (MA) and Hyperimmunoglobulinemia D with Periodic Fever Syndrome (HIDS). Overlap in the underlying genetic basis of these phenotypes shows that both MA and HIDS represent the same deficiency, with MA being a more severe disorder with cognitive and neurological defects, and HIDS being a less severe defect of the same mutation. Both MA and HIDS are caused by a mutation in the mevalonate kinase gene (MVK) that leads to a decrease in the amount of enzyme, mevalonate kinase, in the body of MKD patients. The mevalonate kinase activity in patients with MA is excessively low, almost undetectable. HIDS patients have decreased levels of mevalonate kinase within their bodies as compared to healthy individuals, but levels are detectable and higher than patients with MA. MKD is marked by a strong innate immune response for patients. Symptoms of MA include febrile crisis, delays in development, dysmorphic features, cataracts, and anemia. Typically, these episodes occur up to 25 times per year and last for 3-6 days. Often patients with MA do not survive past adolescence. Symptoms of HIDS include febrile attack, abdominal pain, skin rashes, diarrhea, vomiting, and headaches. These episodes generally occur every 2-6 weeks and last 3-7 days. These immune responses are significant and show that the symptoms of MA and HIDS are not desirable.

Mevalonate Pathway

MKD is an inborn error of cholesterol and nonsterol isoprenoid biosynthesis and is caused by a mutation in the MVK gene. This mutation inhibits the function of the enzyme, mevalonate kinase, which is responsible for the ATP-dependent phosphorylation of mevalonate to 5-phosphomevalonate in the mevalonate pathway (Figure 1) and is a key component of many cellular processes in the body. A threshold of around 40% mevalonate kinase activity causes the body to respond to the depletion. If this phosphorylation does not occur, the body will excrete mevalonate in the form of mevalonic acid. If the mevalonate is not being processed or used in cellular
functioning, the body wants to get rid of the mevalonate as it is not useful. A high level of mevalonic acid in an individual’s urine is a strong indication of MKD. The MVK mutation thus inhibits the remainder of the pathway, including important isoprenoid intermediates and subsequent protein families. Specifically, the depletion of two isoprenoids, farnesyl- and geranylgeranylpyrophosphate (FPP and GGPP), is thought to be the reason for the inflammatory response generated in patients with MKD. FPP and GGPP are lipid moieties that are responsible for the prenylation of small GTP binding protein families, such as Ras, Rho, and Rab. Prenylation of small Rho GTPases can lead to altered activation of these proteins and phenotypes of MKD have been linked to altered activation states of small GTP binding proteins. An exploration of the mechanisms of the inflammatory response due to this effect can lead to a better understanding of MKD.

**MKD Patients Exhibit Increase In Cytokine Production**

Inhibition of the mevalonate pathway shows an increase in expression of cytokines, including those of the interleukin-1 (IL-1) family through enhancement of caspase-1 activity in patients. Caspase-1 is the enzyme responsible for the maturation of the cytokines IL-1β and IL-18. Increases in serum levels of procalcitonin (PTX3), an acute phase protein induced by the IL-1 family can be seen in the majority of MKD patients. MKD is thought to be a multicytokine disease and cytokine response may play a pivotal role in characterizing MA and HIDS.

**Statins**

Statins are a class of drugs that lower plasma cholesterol levels and are therefore commonly prescribed to individuals with hypercholesterolemia. Statins are a popular pharmaceutical within the American population as an estimated 34 million Americans have elevated levels of cholesterol and are diagnosed with hypercholesterolemia. Statins are marketed under the trade names Lipitor (atorvastatin), Lescol (fluvastatin), Mevacor (lovastatin), Pravachol (pravastatin), and Zocor (simvastatin). Patients with hypercholesterolemia have high levels of cholesterol in the blood, often leading to cardiovascular diseases due to a buildup of cholesterol plaques within cardiovascular blood vessels. Cholesterol is transported throughout the body via lipoproteins of varying densities. High levels of the low-density lipoproteins (LDL) in circulation lead to an increased risk of cardiovascular disease for an individual. Statins work by entering cells and inhibiting the rate-limiting step of cholesterol biosynthesis, the conversion of HMG CoA to mevalonate (Figure 1). Once depleted, the cell can no longer synthesize adequate amounts of cholesterol to maintain cellular functioning, so the cell sends LDL receptors to the surface and pulls cholesterol out of the plasma. Thus, statins reduce overall serum cholesterol levels and reduce the risk of cardiovascular dysfunction. However, the cholesterol biosynthetic pathway is responsible for other processes in addition to the synthesis of cholesterol, like the production of isoprenoids that could be associated with immune functions.
Since the mevalonate pathway inhibition by statins occurs just one step upstream of the block by the mutation in MVK, immune cells treated with statins can mimic the inflammatory response of MKD. Lovastatin treated healthy PBMCs exhibit similar secretion of IL-1β as PBMCs isolated from patients with HIDS. Additionally, cytokine secretion and expression in PBMCs of patients with HIDS or MA increase when cells are also treated with lovastatin19. Therefore, statins can mimic or enhance the inhibition of the mevalonate pathway in immune cells.

**Bacterial Stimulus**

Both MA and HIDS have two states, an active and an inactive state. One hypothesis could be that some trigger or environmental stimulus needs to occur to initiate the active state. This trigger is most commonly replicated by introducing lipopolysaccharide (LPS) to cell lines. LPS is a component of the outer membrane of Gram-negative bacteria and works as a pathogen associated molecular pattern (PAMP) responsible for activating a specific signal transduction cascade. In this cascade, LPS binds to an LPS-binding protein, which then binds to a receptor complex comprised of toll-like receptor 4 (TLR4), MD2, and CD14. Activation of this receptor complex initiates signal transduction pathways leading to the release of inflammatory mediators. Immune cells of patients with MKD or immune cells treated with statins that are stimulated by LPS exhibit an exacerbated inflammatory response as seen in patients with MKD20.

As mentioned above, CD14 is a receptor for LPS and acute phase protein that can be found as two isoforms. One form is membrane bound (mCD14) and contains a glycosylphosphatidylinositol (GPI)- anchor, while the other form is soluble, lacks the GPI anchor, and is released from myeloid cells21. Lovastatin treatment increases mCD14 and decrease sCD14 after LPS stimulation. These alterations in CD14 expression may contribute to inflammation following isoprenoid depletion22.

**Mouse Models**

Treating mice with alendronate, an inhibitor of the conversion of FPP to GGPP, and bacterial muramyl-dipeptide, a PAMP like LPS, can mimic MKD by triggering a systemic inflammatory response in mice. This replication was successfully modeled in both BALB/c and C57BL/6 mice, indicating that the results are not strain-specific. No significant difference was found in the comparison of mice strains23. In these models, an altered activation state of small GTPase proteins leads to changes in cytokine production, including IL-1β, TNF-α, and IL-6. Additionally, adding back exogenous isoprenoids, like FPP and GGPP, can reverse the inflammatory response in mouse models of MKD24-26. These results provide further in vivo support for the role of FPP and GGPP in MKD.
Cellular Models of MKD

RAW 264.7 murine macrophages have been used to model MKD. Isoprenoid depletion by lovastatin in RAW 264.7 macrophages leads to increased TNF-α release, CD14 mRNA and cell-associated protein levels, but decreased release of sCD14 following LPS stimulation\(^2\). Co-incubation of lovastatin treated RAW 264.7 cells with either mevalonate or GGPP blocked alterations in TNF-α and CD14 expression and release, indicating that these effects are likely dependent on inhibition of isoprenoid biosynthesis\(^2\). GGTI-298 is a drug that inhibits geranylgeranyl transferase I, the enzyme responsible for transferring GGPP to Rho GTPases. Treatment of macrophages with GGTI-298 was shown to increase TNF-α release and CD14 mRNA and protein expression, similar to using lovastatin, leading to the hypothesis that one or more Rho GTPases are responsible for regulating TNF-α release and CD14 expression following LPS stimulation. Interestingly, GGTI-298 treatment does not reduce the release of sCD14 following LPS stimulation, leading to the hypothesis that this effect is independent of Rho GTPases.

In order to extend the work done in murine macrophages to human cells, the Mono-Mac 6 (MM6) human monocyte cell line was used. Similar to the RAW 264.7 cells, MM6 cells treated with lovastatin followed by LPS exposure increased total CD14 mRNA levels (Marrero, 2013 honors thesis).

Overall, it can by hypothesized that the lack of prenylation of one or more Rho family members leads to alterations to the inflammatory response in MKD. The 3 most studied Rho GTPases are RhoA, Rac1, and cdc42. In particular, Rho and Rac1 have been investigated in cellular models of MKD. In THP-1 cells, the increased expression of IL-1β is an effect of the interplay between the inactivation of Rho and the activation of Rac1 as the inactivation of Rho causes the activation of Rac1\(^2\). In fibroblasts of patients with MKD, Ras and Rho protein expression increases with simvastatin inhibition\(^2\). RhoA and Rac1 are activated in the soluble non-prenylated form, but inactive in the membrane-bound form in cells of MKD patients\(^3\). In bovine pulmonary arterial endothelial cells, Rac1 is activated with simvastatin inhibition\(^3\). Therefore, it appears isoprenoid depletion leads to activation of Rac1 across different cell lines, which could be a potential cause of alterations to the inflammatory response in MKD.

Many different cell lines have been used to model MKD, but it is not always clear how the results relate to primary human monocytes. Therefore, this study has two distinct aims. First, this study aims to further characterize the response to isoprenoid depletion in RAW 264.7 macrophages. Additionally, this study aims to characterize the response of primary human monocytes to LPS following isoprenoid depletion in order to further explore potential cell line and cell type differences in this system. These aims will be accomplished by inhibiting the mevalonate pathway at various points (as highlighted in Figure 1) and observing the resulting inflammatory response.
MATERIALS AND METHODS

Cell Culture

RAW 264.7 mouse macrophages were cultured in RPMI 1640 with glutamine and 10% FBS.

Human Mono Mac 6 (MM6) monocytes were cultured in RPMI 1640 with glutamine, 10% fetal bovine serum (FBS), 1 X non-essential amino acids (NEAA), and OPI media supplement (containing oxalacetic acid, sodium pyruvate, and insulin from Sigma #O-5003).

Human primary monocytes were isolated from healthy donors using counter-current centrifugal elutriation by collaborator Mark Wallet at the University of Florida Department of Pathology, Immunology and Laboratory Medicine. Monocytes were cultured for 7 days in DMEM, supplemented with 10% human serum and 1 ng/mL M-CSF to differentiate to monocyte-derived macrophages (MDMs)32.

Cell Treatment and Data Analysis

Western Blot Analysis of Small Rho GTPases

RAW 264.7 macrophages were treated with lovastatin (10µM) or left untreated for 16-18 hours and subsequently stimulated with LPS (100 ng/ml). Supernatants were harvested at time points of 0-2 hours using lysis/binding/wash buffer (25mM Tris•HCl, pH 7.2, 150mM NaCl, 5mM MgCl2, 1% NP-40 and 5% glycerol) with Thermo Scientific Pierce EDTA-Free Protease Inhibitor Tablets (Life Technologies #88266).

After determining protein concentrations of the supernatants with QuickStart™ Bradford Protein Assay Kit (Bio-Rad #500-0201), samples were selectively enriched for Rho using the Thermo Scientific Active Rho Pull-Down and Detection Kit (Life Technologies #16116) or for Rac using the Thermo Scientific Active Rac1 Pull-Down and Detection Kit (Life Technologies #16118). Total cell lysates were analyzed to ensure GTP signal detection.

Western blots were read and analyzed using the Pierce™ ECL Western Blotting Substrate (Life Technologies #32106).

Cytokine Expression in Mouse Macrophages, Human Monocytes, and Human Primary Monocytes and Macrophages

RAW 264.7 macrophages, MM6 human monocytes, and both differentiated (MDMs) and undifferentiated (monocytes) cells were treated withLovastatin (10 µM) for 16-18 hours followed by stimulation with LPS (1, 10, or 100 ng/ml) for varying amounts of time. Additionally, RAW 264.7 macrophages were treated withLovastatin (10µM) and small GTPase inhibitors for 16-18 hours followed by stimulation with LPS (100 ng/ml) for 6 hours. These GTPase inhibitors include a cdc42/Rac1 duel inhibitor (10 µM) from
EMD Millipore (ML141-Calbiochem, #217708), a Rac1 inhibitor (50µM) from EMD Millipore (CAS 117856-17-6- Calbiochem, #553502), and a highly purified C3 transferase Rho inhibitor (5µg/ml) from Cytoskeleton, Inc. (Cat. # CT03-A).

RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma Aldrich #RTN70). 65-1000 ng of total RNA was converted to cDNA using the High-Capacity RNA-to-cDNA kit (Life Technologies #4368814). cDNA was used in a real-time pcr reaction using Taqman® primers and probes (Life Technologies) directed against IL-1β, TNF-α, or CD14. β-actin was used as an endogenous control for the mouse samples and human EIF2B1 was used as the endogenous control for the human samples. The comparative C_T method was used for quantification.

**sCD14 and IL-1β Release in Murine Macrophages and Human Monocytes**

RAW 264.7 macrophages, MM6 human monocytes and both differentiated (MDMs) and undifferentiated primary human monocytes were treated with either lovastatin (10 µM) or GGTI-298 (10 µM) for 16-18 hours and subsequently stimulated with LPS (1, 10, or 100 ng/ml). Supernatants were harvested at time points up to 24 hours and assayed for sCD14 or IL-1β using Quantikine® ELISA kits (R&D Systems). ELISA results for the cell lines were divided by A280 readings on cell lysates to correct for plating error.

**RESULTS**

**Lovastatin and GGTI-298 Increase Expression of IL-1β and TNF-α in Mouse Macrophages**

In order to further evaluate RAW 264.7 macrophages as a model for MKD, the mevalonate pathway was inhibited with lovastatin (10µM, 16-18 hours) or GGTI-298 (10µM, 16-18 hours) treatment, followed by LPS (100 ng/ml, 4 hours) stimulation. In order to demonstrate the inflammatory response following inhibition, levels of cytokine expression were analyzed in these cells, specifically levels of TNF-α and IL-1β. Total RNA was converted into cDNA then analyzed by quantitative pcr (Figure 3). Lovastatin inhibiton and LPS stimulation leads to a moderate increase (2.14 fold) in TNF-α and a large increase in IL-1β (10.74 fold) as compared to control and carrier (DMSO) cells. GGTI-298 blocks geranylerganyl transferase I (GGTase I) thereby blocking GGPP from prenylating Rho family GTPases, including Rho, Rac1, and cdc42. GGTI-298 inhibition and LPS stimulation leads to a slight increase in TNF-α (1.58 fold) and a large increase in IL-1β (7.1 fold) as compared to control and carrier (DMSO) cells. Perillyl alcohol (POH) inhibits Rab GGTase[32]. Treatment with POH does not alter expression of TNF-α and IL-1β in this model.

**Small Rho GTPases Role in Inflammatory Response**

In order to further characterize the inflammatory response, the role of small Rho GTPases was studied by pull-down and Western blot analysis. Samples were selectively enriched for cdc42, Rho, or Rac1 with an active pull-down and detection kit. Samples were compared to a G-protein activating positive control, GTPγS, and a GDP negative control. Rac1 activation was not detectable after treatment with LPS (100ng/µl, 0-2
hours) or lovastatin (10μM) (Figure 4). It appeared that Rho activation decreased following lovastatin treatment in the absence of LPS compared to carrier treated cells. Treatment with lovastatin (10μM) and LPS stimulation (100ng/ml, 0-90 minutes) resulted in increased activation of Rho compared to carrier cells stimulated with LPS (Figure 5). This increase was most prominent after 30 minutes of LPS stimulation.

If the activation of Rho after LPS stimulation is leading to increased IL-1β and TNF-α expression in lovastatin treated cells, then the inhibition of Rho should block these effects. In order to test this, the role of Rho activation was further characterized by real-time analysis of cells treated with lovastatin (10 μM) and one of three small GTPase inhibitors (5μg/ml C3 transferase for Rho, 50 μM Rac1 Inhibitor, or 10 μM cdc42/Rac1 ML141 inhibitor) followed by LPS stimulation (100ng/ml, 4 hours). Samples were analyzed for IL-1β and TNF-α expression. Rho inhibition, but not Rac1 or cdc42, resulted in a decrease in IL-1β and TNF-α in lovastatin treated cells (Figure 6) indicating that the increased Rho activation following LPS stimulation in lovastatin treated cells (Figure 5) contributes to increased expression of these cytokines.

**CD14 mRNA Levels in Murine Macrophages and Human Monocytes**

One possible explanation for increased signal transduction and cytokine production is that RAW 264.7 cells are sensitized to LPS. CD14 is a cell surface receptor that greatly sensitizes cells to LPS21 and has been reported to increase following lovastatin treatment22. In order to further characterize this effect, RAW 264.7 macrophages or MM6 monocytes were treated with GGTI-298 (10μM) for 16-18 hours followed by stimulation with LPS (100 ng/ml) for 4 hours. Total RNA was isolated and converted to cDNA which was used in a real-time pcr reaction with Taqman primers/probes (Applied Biosystems) directed against CD14 or 18S ribosomal RNA. CD14 mRNA levels were normalized against 18S mRNA and set relative to non-treated cells. As previously reported, CD14 mRNA levels increased with lovastatin or GGTI-298 treatment in RAW 264.7 macrophages22. In addition, this effect also occurred in MM6 human monocytes. This increase was exacerbated in the presence of LPS, indicating that LPS treatment can enhance the intense inflammatory response caused by mevalonate pathway inhibition (Figure 7).

**sCD14 Release in Murine Macrophages and Human Monocytes**

Both RAW 264.7 macrophages and MM6 human monocytes were treated with either lovastatin (10 μM) or GGTI-298 (10 μM) for 16-18 hours and subsequently stimulated with LPS (100 ng/ml). Supernatants were harvested at time points up to 24 hours and assayed for sCD14 using Quantikine® ELISA kits (R&D Systems). ELISA results were divided by A280 readings on cell lysates to correct for plating error. Samples were evaluated by ELISA for levels of supernatant associated CD14 (Figure 8). sCD14 is found to act differently than mCD14 in RAW 264.7 macrophages and expression is likely not dependent on the inhibition of small Rho GTPases. Lovastatin treatment leads to decreased release of sCD14 following LPS stimulation in RAW 264.7 macrophages compared to control and carrier cells, while GGTI-298 treatment does not
seem to differ compared to control and carrier cells. Both lovastatin and GGTL-298 treatment led to increased release of sCD14 following LPS stimulation as compared to control and carrier cells in MM6 human monocytes, indicating a difference between RAW 264.7 and MM6 cell lines in this effect.

_Cytokine Expression in Human Primary Monocytes and Macrophages_

In order to further investigate macrophage and monocyte differences, human primary monocytes were isolated from healthy donors (n = 3) using counter-current centrifugal elutriation by collaborator Mark Wallet at the University of Florida Department of Pathology, Immunology and Laboratory Medicine. Monocytes were cultured for 7 days in DMEM, supplemented with 10% human serum and 1 ng/mL M-CSF to differentiate to monocyte-derived macrophages (MDMs). Monocytes and macrophages were treated with lovastatin (10μM) for 18 or 48 hours. Monocytes and macrophages were then stimulated with 0ng/ml, 1ng/ml, 10ng/ml, or 100ng/ml LPS for 6 hours. RNA and supernatants were harvested and assayed for _IL-1β, TNF-α, or CD14_ mRNA levels by real-time pcr or secreted levels of _IL-1β_ using a Quantikine® ELISA kit (R&D Systems).

Primary monocytes and monocyte-derived macrophages from Donor 1 were treated with lovastatin (10μM) for 18 hours and stimulated with LPS (100 ng/ml) for 6 hours. Unlike RAW 264.7 cells, human primary monocytes and MDMs do not exhibit an increase in _IL-1β or TNF-α_ expression following LPS stimulation. Carrier (DMSO) cells stimulated with LPS show a large increase in cytokine expression. Lovastatin treated monocytes stimulated with LPS show an increase in cytokine expression that is less of an increase than carrier (DMSO) cells stimulated with LPS, while lovastatin treated MDMs stimulated with LPS do not seem to differ in cytokine expression compared to carrier (DMSO) MDMs stimulated with LPS (Figure 9).

After results from Donor 1 showed this response to LPS, a further investigation of the dose-dependent response of LPS stimulation was evaluated in Donors 2 and 3. Cells were treated with carrier (DMSO) or lovastatin (10μM) for 18 hours, then stimulated with 0, 1, 10, or 100 ng/ml of LPS. Donors 2 and 3 both show that LPS stimulation causes a dose-dependent increase in cytokine expression in primary monocytes. Additionally, 0 and 1 ng/ml of LPS stimulation does not cause a change in cytokine expression in lovastatin treated cells compared to the carrier (DMSO) treated cells. 10 ng/ml and 100 ng/ml of LPS stimulation seems to cause a suppressed _IL-1β_ expression in lovastatin treated cells compared to carrier (DMSO) treated cells (Figure 10).

_IL-1β_ release has been reported to increase following isoprenoid depletion independent of changes in mRNA levels in THP-1 human monocytes due to an increase in caspase-1 processing of pro-IL-1β. Therefore, we examined secreted levels by ELISA. IL-1β levels increased depending on LPS dosage in human monocytes. Both carrier (DMSO) and lovastatin treated monocytes with LPS stimulation show an increase in IL-1β release that is dose-dependent. 100ng/ml LPS stimulation leads to the most IL-1β release. IL-1β release seems to be greater 24 hours post lovastatin treatment compared
Throughout literature and these findings, cell line difference is apparent. In this data, GTP support a lovastatin treated cells with a Rho inhibitor can decrease activation of Rho following LPS stimulation. LPS stimulation are novel and indicate that isoprenoid depletion results in increased cytokine expression at baseline compared to control cells, but the results following LPS stimulation are novel and indicate that isoprenoid depletion results in increased cytokine expression at baseline compared to control cells. Our data that the co-incubation of lovastatin treated cells with a Rho inhibitor can decrease IL-1β and TNF-α mRNA levels support a possible role for Rho activation in these effects.

In this data, GTP-bound Rho and Rac1 levels were measured in RAW 264.7 cell lines. Throughout literature and these findings, cell line differences show varying responses to monocytes treated with lovastatin for 48 hours. Macrophages do not seem to display a major change in IL-1β release overall (Figure 11).

**DISCUSSION**

**Inhibition of the Mevalonate Pathway Leads to Increased Cytokine Expression in Mouse Macrophages**

As literature suggest, inhibiting the mevalonate pathway at the rate-limiting step of HMG-CoA to mevalonate thus blocking the prenylation of small Rho GTPases causes a cytokine response indicative of inflammation. Undoubtedly, this work across cell lines shows a prominent increase in inflammatory mediators in cells treated with lovastatin or GGTI-298. This response can be seen in RAW 264.7 mouse macrophages, MM6 human monocytes, and primary monocytes. Human primary macrophage cells do not show an increase in IL-1β expression or release after lovastatin treatment, and therefore are not responding similarly.

Many studies have examined the effects of lovastatin treatment on the expression of inflammatory markers; however, the extension of this work to include GGTI-298 treatment provides crucial information to the understanding of the inflammatory response that can be seen. Concluding that GGTI-298 inhibition can result in the same cytokine response as lovastatin treatment has many implications for the source of the inflammatory response. Since a block further down in the pathway causes the same response, it can be implied that some downstream effects from GGPP’s prenylation of small Rho GTPases are likely the source of this increase in cytokine expression. The goal of the results of this study is to provide some insight for a further understanding of this pathway’s relation to inflammation. Therefore, the role of small Rho GTPases is important to evaluate.

**Small Rho GTPases Play a Critical Role in the Inflammatory Response**

The three most common small Rho GTPases, RhoA, Rac1, and cdc42, could be potential sources for changes in the expression of inflammatory markers upon the inhibition of the mevalonate pathway. Notably, the interplay of these three proteins could be the key to understanding the exact mechanism of this response. This work found that Rac1 activation was not detectable, but lovastatin did alter Rho activation. Additionally, LPS stimulation caused an increase in levels of GTP bound Rho after lovastatin treatment and cells that were not treated with lovastatin but stimulated with LPS decreased in detectable activation of Rho (Figure 5). Our results support the finding that lovastatin reduces Rho activation at baseline compared to control cells, but the results following LPS stimulation are novel and indicate that isoprenoid depletion results in increased activation of Rho following LPS stimulation. Our data that the co-incubation of lovastatin treated cells with a Rho inhibitor can decrease IL-1β and TNF-α mRNA levels support a possible role for Rho activation in these effects.

In this data, GTP-bound Rho and Rac1 levels were measured in RAW 264.7 cell lines. Throughout literature and these findings, cell line differences show varying responses.
in Rho and Rac1 activation. The 2010 study suggests both Rac1 and RhoA are activated by statin treatment, while the 2014 study suggests the inactivation of RhoA leads to an activation of Rac1 that is responsible for the increase in IL-1β expression. This work indicates that the small Rho GTPase proteins in different cells could respond differently to pathway inhibition.

*mCD14 and sCD14 Function in Distinct Ways*

As previously discussed, CD14 is a pattern recognition receptor of LPS and exists in two protein isoforms. One form is membrane bound (mCD14) and contains a glycosylphosphatidylinositol (GPI)-anchor, while the other form is soluble, lacks the GPI anchor, and is released from myeloid cells. Since LPS represents the bacterial stimulus patients with MKD encounter before exhibiting systemic inflammatory symptoms, CD14 is important to this study. Increases in CD14 are indicative of an inflammatory response and can be measured, in addition to cytokine response, to evaluate the mechanisms of inflammation in these patients and models of the deficiency.

mCD14 and sCD14 exhibit distinct patterns of expression upon mevalonate pathway inhibition. Lovastatin and GGTI-298 treatment leads to increased CD14 mRNA expression in RAW 264.7 and MM6 cells (Marrero, unpublished data, Figure 7). Additionally, LPS exacerbates the increase in CD14 expression in these cells. These results show us that the increase in inflammatory response is likely due to the inhibition of small Rho GTPases in both RAW 264.7 and MM6 cells.

sCD14 responds differently than mCD14 to lovastatin and GGTI-298 treatment. There is not an abundant amount of literature that examines the role of sCD14 in MKD or models of MKD. These findings could prove interesting in looking at the trafficking and function of CD14 within cells. In RAW 264.7 mouse macrophages, sCD14 levels decreased following lovastatin treatment and LPS stimulation compared to control and carrier cells. However, in these cells GGTI-298 treatment did not show a change in sCD14 release as compared to control and carrier cells. Further, in MM6 human monocytes, lovastatin and GGTI-298 treatment lead to an increase in sCD14 release following LPS stimulation as compared to control and carrier cells. These results indicate two main findings: sCD14 release is not completely consistent across cell lines and sCD14 release is likely not dependent on inhibition of small Rho GTPases in RAW 264.7 macrophages and therefore functions differently than CD14 mRNA levels.

*Cell Differences are Likely Critical to Mimicking MKD*

Throughout the discussion, a common theme of differences in cell line and cell type becomes evident. Many publications examine the effects of mevalonate pathway inhibition on inflammatory response in one cell type and typically do not extend their work across cell types. As more is discovered about this pathway inhibition and inflammatory response relationship, cell lines and cell types should be further explored. Literature on small Rho GTPase function is not consistent across cell lines or cell type.
In this study, RAW 264.7 mouse macrophages, MM6 human monocytes, and human primary monocytes and macrophages were considered. This study, in particular, found that lovastatin treatment of human primary monocytes increased IL-1β release following LPS stimulation, indicating that the inhibition of the mevalonate pathway leads to an inflammatory response in these cells. Moreover, human primary MDMs did not respond similarly. No major change in IL-1β release is noted after lovastatin treatment and LPS stimulation in these primary macrophage cells compared to the carrier (DMSO) treated cells with LPS stimulation. In contrast to the increased secretion of IL-1β, IL-1β mRNA levels appear to decrease following lovastatin treatment in primary human monocytes with LPS stimulation as compared to the carrier cells. This is in direct conflict with the increased IL-1β mRNA levels observed in RAW 264.7 cells following lovastatin treatment. In addition, while CD14 mRNA levels increase following lovastatin treatment in RAW 264.7 and MM6 cells, no changes in CD14 mRNA levels were observed in primary human monocytes or MDMs. Additionally, sCD14 release decreases upon lovastatin treatment and LPS stimulation in RAW 264.7 mouse macrophages, but increases upon lovastatin treatment and LPS stimulation in human MM6 monocytes.

Potential differences in cell source could be responsible for these observed distinctions. The human and mouse CD14 promoters share 59.5% identity in the 1000 base pair promoter region, while the conserved sequence directly after the "ATG" start site showed 69.2% homology (Marrero, unpublished data). Consistencies across humans and mice in the CD14 gene are present; however, this comparison is a prime example of how mouse and human genes are not completely homogenous.

Additionally, monocyte and macrophage differences are likely playing a role in these observed distinctions. The human primary cells used in this study were differentiated according to protocol by collaborator, Mark Wallet, at the University of Florida. Three donors were used and slight differences in cell response were observed among donors. The cells were left in DMEM media for 7 days and supplemented with 10% human serum and 1 ng/ml M-CSF to differentiate. In the 1999 study, THP-1 cells were left to differentiate for only 4 days using phorbol myristate acetate (PMA) as the differentiation agent. The process of differentiation may have lead to phenotypic macrophage distinctions. While differentiated cells appeared to “stick” to the plate, the defining characteristic of macrophages, the various ways to differentiate cells could lead to different macrophage cell populations.

While it is known that monocytes and macrophages both have inflammatory functions, studies suggest differences in the two cell types that could be contributing to the observed differences in inflammatory response after pathway inhibition. Monocytes respond to damage or infection and differentiate to macrophages or dendritic cells that travel to the site of damage. The distinction between monocytes and macrophages creates subtle differences that could be connected to the response differences in these findings. Specifically, monocytes may contribute to inflammation through T cell activation, causing changes in cell surface receptors and function.
In conclusion, it is clear that primary human monocytes and macrophages respond differently to isoprenoid depletion compared to cell lines such as RAW 264.7 and MM6. While all of these cell types are likely useful models for understanding the inflammatory response, future work should consider differences in monocyte and macrophage populations in order to better understand the source of inflammation in MKD.

Possible Known Treatments

No known single treatment has been found for all MKD patients; however, many studies have explored drug therapies that show levels of remission in some patients. Among these studies, promising results have been obtained in trials that treat patients with farnesyl transferase inhibitors (FTIs) or IL-1-targetting drugs. FTIs, like Tipifarnib (Tip), Lonafarnib (Lon), and Manumycin A, are marketed as experimental anticancer drugs that prevent the proper functioning of Ras proteins. FTIs could inhibit cytokine secretion by redirecting the available mevalonate intermediates to GGPP synthesis in a model of MKD and diminishing the inflammatory response\textsuperscript{36,37}. IL-1-targetting drugs, such as anakinra and canakinumab, have shown promising effects for remission in clinical trials of patients with HIDS or MA. IL-1-targetting drugs work by inhibiting the receptor binding of IL-1 receptors and thus decrease the secretion and expression of cytokines in the IL-1 family\textsuperscript{38-40}. Clinical trials show promising outlook for possible treatments for MKD, but finding a completely effective method to cure MKD will entail many more studies.

Conclusions/Further Directions

The data in this study explore a wide range of possibilities for understanding the effects of isoprenoid depletion on inflammation and create potential for future explorations.

Primarily, further exploration of the interplay of small Rho GTPases is crucial to the understanding of MKD and the role of pathway inhibition on inflammatory response. Rho, Rac1, and cdc42 effects should be investigated further. The mechanistic understanding of the inflammatory response could lead to targeted drug therapies for patients with MKD and disorders like MKD.

Further, cell line and type consistency with this work is necessary to understand how patients with MKD are affected. Clear differences in cell type response are observed, causing complications in literature consistency. Distinctions of responses of mouse macrophages, mouse monocytes, human macrophages, and human monocytes will benefit the extension of this work in implications for a method of therapy for MKD.

Additionally, the relationship between statin inhibition and MKD is evident in these findings. Lovastatin and GGTI-298 treatment mimic the disorder and cause a distinct inflammatory response. Understanding the mechanisms of MKD could lead to a better understanding for how the increasingly popular cholesterol drugs, statins, perform secondary functions in the body in addition to decreasing LDL cholesterol levels.
REFERENCES


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FIGURES

Figure 1. The mevalonate pathway is responsible for the conversion of acetyl CoEnzyme A to cholesterol through a series of isoprenoid intermediates. These intermediates could be critical in determining the mechanisms of the inflammatory response in MKD.
Figure 2. LPS is a PAMP that triggers the inflammatory response through a MyD88-dependent and independent pathways\textsuperscript{43}. 
Figure 3. Lovastatin and GGTI-298 Increase Expression of IL-1β and TNF-α in Mouse Macrophages. RAW 264.7 macrophages were treated with either lovastatin (10 µM), GGTI-298 (10 µM), or POH (100 µM) for 16-18 hours and subsequently stimulated with LPS (100ng/ml) for 4 hours. RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma Aldrich #RTN70). 1 µg of total RNA was converted to cDNA using the High-Capacity RNA-to-cDNA kit (Life Technologies #4368814). cDNA was used in a Real-time PCR reaction using Taqman® primers and probes (Life Technologies) directed against IL-1β and TNF-α. IL-1β and TNF-α levels were normalized against β-actin mRNA and set relative to non-treated cells. Lovastatin treatment increased TNF-α and IL-1β levels as compared to control and carrier (DMSO) cells, showing that pathway inhibition at the conversion of HMG-CoA to mevalonate causes an increase in cytokine expression. GGTI-298 treatment increased TNF-α and IL-1β levels as compared to the control and carrier (DMSO) cells, showing that pathway inhibition at the geranylgeranylation of Rho GTPases causes an increase in cytokine expression. POH treatment did not increase TNF-α and IL-1β levels, showing that pathway inhibition at the geranylgeranylation of Rab proteins does not show an increase in cytokine expression in this model.
Figure 4. **Rac1 Activation is Not Detectable in Mouse Macrophages.** RAW 264.7 macrophages were treated with lovastatin (10μM) for 16-18 hours or stimulated with LPS (100ng/ul) for time points of 15 minutes to 2 hours. After determining protein concentrations of the lysates with QuickStart™ Bradford Protein Assay Kit (Bio-Rad #500-0201), samples were selectively enriched for Rac-GTP using the Thermo Scientific Active Rac1 Pull-Down and Detection Kit (Life Technologies #16118). Total cell lysates were analyzed to ensure Rac1 signal detection. Western blots were read and analyzed using the Pierce™ ECL Western Blotting Substrate (Life Technologies #32106). GTP bound Rac1 can be detected in the GTPγS control. Rac1 was not detectable in lovastatin treated or LPS stimulated RAW 264.7 macrophages.
Figure 5. **Lovastatin Alters Rho Activation in Mouse Macrophages.** RAW 264.7 macrophages were treated with lovastatin (10μM) for 16-18 hours and subsequently stimulated with LPS (100ng/ml). Lysates were harvested at time points of 0, .5, or 1.5 hours. After determining protein concentrations of the lysates with the QuickStart™ Bradford Protein Assay Kit (Bio-Rad #500-0201), samples were selectively enriched for Rho-GTP using the Thermo Scientific Active Rho Pull-Down and Detection Kit (Life Technologies #16118). Total cell lysates were analyzed to ensure Rho signal detection. Western blots were read and analyzed using the Pierce™ ECL Western Blotting Substrate (Life Technologies #32106). In macrophages treated with a carrier (DMSO) in place of lovastatin, LPS stimulation decreases detectable Rho-GTP levels as time of stimulation increases. In lovastatin treated macrophages, LPS stimulation increases detectable levels of Rho-GTP as time of stimulation increases.
Figure 6. Rho Inhibition Decreases Expression of IL-1β and TNF-α in Mouse Macrophages. RAW 264.7 macrophages were treated with lovastatin (10μM) and Rho, Rac1, or Rac1/cdc42 inhibitors for 16-18 hours, and then stimulated with LPS (100ng/ul) for 4 hours. RNA was isolated (Sigma Aldrich #RTN70) and converted to cDNA (Life Technologies #4368814). cDNA was used in a real-time pcr reaction using Taqman® primers and probes (Life Technologies) directed against IL-1β or TNF-α with β-actin as an endogenous control. Lovastatin and Rho inhibitor treatment was found to suppress the increase in IL-1β and TNF-α mRNA expression as compared to control, carrier (DMSO), lovastatin and Rac inhibitor, and lovastatin and cdc42 inhibitor treated cells.
Figure 7. **Lovastatin and GGTI-298 Treatment Increase CD14 mRNA Levels in Murine Macrophages and Human Monocytes.** RAW 264.7 macrophages or MM6 monocytes were treated with GGTI-298 (10µM, 16-18 h) then stimulated with LPS (100 ng/ml) for 4 hours. Total RNA was isolated and converted to cDNA to be used in a real-time PCR reaction with Taqman primers and probes (Applied Biosystems) directed against CD14 or 18S ribosomal RNA. CD14 mRNA levels were normalized against 18S mRNA and set relative to non-treated cells. RAW 264.7 macrophages and MM6 monocytes show increases in mCD14 with GGTI-298 treatment. In both RAW 264.7 (A) and MM6 (B) cells, LPS stimulation results in an even greater increase in mCD14 with GGTI-298 treatment.
Figure 8. Lovastatin and GGTI-298 Alter the Release of sCD14 in Murine Macrophages and Human Monocytes. RAW 264.7 macrophages and MM6 monocytes were treated with either lovastatin (10 μM) or GGTI-298 (10 μM) for 16-18 hours and subsequently stimulated with LPS (100 ng/ml) up to 24 hours. Supernatants were harvested at time points up to 24 hours and assayed for sCD14 using Quantikine ELISA kits (R&D Systems). ELISA results were divided by A280 readings on cell lysates to correct for plating error. In RAW 264.7 macrophages, lovastatin treatment led to a decreased release of sCD14 following LPS stimulation as compared to control and carrier (DMSO) cells (A). GGTI-298 treatment in RAW 264.7 macrophages did not seem to alter sCD14 expression compared to control or carrier (DMSO) cells (B). In MM6 monocytes, lovastatin (A) and GGTI-198 (B) treatment led to an increased release of sCD14 following LPS stimulation compared to control and carrier (DMSO) cells.
Figure 9. **Effect of Lovastatin in Primary Monocytes and MDMs From Donor 1.**

Primary monocytes and monocyte-derived macrophages (MDMs) from Donor 1 were treated with lovastatin (10μM) for 18 hours and stimulated with LPS (100 ng/ml) for 6 hours. Total RNA was isolated and converted to cDNA to be used in a real-time PCR reaction with Taqman primers and probes (Applied Biosystems) directed against *IL-1β* or *TNF-α*. *IL-1β* and *TNF-α* mRNA levels were normalized against *EIF2B1* mRNA and set relative to non-treated cells. No change in cytokine (*IL-1β* or *TNF-α*) expression was observed for monocytes or MDMs treated with lovastatin but not stimulated with LPS as compared to the carrier (DMSO) treated cells. Carrier (DMSO) cells stimulated with LPS show a large increase in cytokine expression. Lovastatin treated monocytes stimulated with LPS show an increase in cytokine expression that is less of an increase than carrier (DMSO) cells stimulated with LPS, while lovastatin treated macrophages stimulated with LPS do not seem to differ in cytokine expression compared to carrier (DMSO) MDMs stimulated with LPS.
Figure 10. **LPS Stimulation Causes Dose-Dependent Increase in IL-1β and TNF-α Expression in Primary Monocytes.** Cells were treated with carrier (DMSO) or lovastatin (10μM) for 18 hours, then stimulated with 0, 1, 10, or 100 ng/ml of LPS. Total RNA was isolated and converted to cDNA to be used in a real-time qPCR reaction with Taqman primers and probes (Applied Biosystems) directed against IL-1β. IL-1β mRNA levels were normalized against EIF2B1 mRNA and set relative to non-treated cells. Donors 2 and 3 both show LPS stimulation causes a dose-dependent increase in cytokine expression in primary monocytes. Additionally, 0 and 1 ng/ml of LPS stimulation does not cause a change in cytokine expression in lovastatin treated cells compared to the carrier (DMSO) treated cells. 10 ng/ml and 100 ng/ml of LPS stimulation seems to cause a suppressed cytokine expression in lovastatin treated cells compared to carrier (DMSO) treated cells.
Figure 11. **Human Primary Monocytes and Macrophages Increase IL-1β Release with Lovastatin Treatment.** Human primary monocytes were isolated from healthy donors using counter-current centrifugation. Monocytes were cultured for 7 days in DMEM, supplemented with 10% human serum and 1 ng/mL M-CSF to differentiate to monocyte-derived macrophages (MDMs). Monocytes and macrophages were treated with lovastatin (10μM, 16-18 h) and subsequently stimulated with 0ng/ml, 1ng/ml, 10ng/ml, or 100ng/ml LPS. Supernatants were harvested 6 h post LPS treatment and assayed for IL-1β using a Quantikine® ELISA kit (R&D Systems) for IL-1β. IL-1β secretion levels increased depending on lovastatin dosage in human monocytes. Lovastatin treatment seems to be associated with an increase in IL-1β secretion compared to carrier cells in these monocytes. (A) No major differences were observed in human macrophages (B).