Glycolysis Is an Intrinsic Factor for Optimal Replication of a Norovirus

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ABSTRACT The metabolic pathways of central carbon metabolism, glycolysis and oxidative phosphorylation (OXPHOS), are important host factors that determine the outcome of viral infections and can be manipulated by some viruses to favor infection. However, mechanisms of metabolic modulation and their effects on viral replication vary widely. Herein, we present the first metabolomics and energetic profiling of norovirus-infected cells, which revealed increases in glycolysis, OXPHOS, and the pentose phosphate pathway (PPP) during murine norovirus (MNV) infection. Inhibiting glycolysis with 2-deoxyglucose (2DG) in macrophages revealed that glycolysis is an important factor for optimal MNV infection, while inhibiting the PPP and OXPHOS showed a relatively minor impact of these pathways on MNV infection. 2DG affected an early stage in the viral life cycle after viral uptake and capsid uncoating, leading to decreased viral protein production and viral RNA. The requirement of glycolysis was specific for MNV (but not astrovirus) infection, independent of the type I interferon antiviral response, and unlikely to be due to a lack of host cell nucleotide synthesis. MNV infection increased activation of the protein kinase Akt, but not AMP-activated protein kinase (AMPK), two master regulators of cellular metabolism, implicating Akt signaling in upregulating host metabolism during norovirus infection. In conclusion, our findings suggest that the metabolic state of target cells is an intrinsic host factor that determines the extent of norovirus replication and implicates glycolysis as a virulence determinant. They further point to cellular metabolism as a novel therapeutic target for norovirus infections and improvements in current human norovirus culture systems.

IMPORTANCE Viruses depend on the host cells they infect to provide the machinery and substrates for replication. Host cells are highly dynamic systems that can alter their intracellular environment and metabolic behavior, which may be helpful or inhibitory for an infecting virus. In this study, we show that macrophages, a target cell of murine norovirus (MNV), increase glycolysis upon viral infection, which is important for early steps in MNV infection. Human noroviruses (hNoV) are a major cause of gastroenteritis globally, causing enormous morbidity and economic burden. Currently, no effective antivirals or vaccines exist for hNoV, mainly due to the lack of high-efficiency *in vitro* culture models for their study. Thus, insights gained from the MNV model may reveal aspects of host cell metabolism that can be targeted for improving hNoV cell culture systems and for developing effective antiviral therapies.

KEYWORDS calicivirus, carbon metabolism, glycolysis, noroviruses, oxidative phosphorylation, pentose phosphate pathway

Viruses are obligate intracellular parasites. Thus, their biology is entirely dependent on the physiology of the host cells they infect. One increasingly appreciated aspect of virus-host interactions is cellular metabolism (1–4). Historically, cellular metabolism

has been considered mainly in terms of its role in cellular energy homeostasis. However, metabolism and metabolic cross talk are increasingly being appreciated as crucial aspects in a range of cellular processes such as proliferation and cell death (5), the activation and functioning of the immune system (6, 7), autophagy (8, 9), and in the establishment of infectious disease (10). Indeed, a wide range of pathogens, including parasites (11), bacteria (12–14), and viruses (3), have been shown to affect and to be affected by their hosts' metabolic activity. Of note, the controlled modulation of metabolism in immune cells has been shown to be a key feature in adaptive and innate immune responses (6, 14–16), and these findings have given rise to an entire field referred to as "immunometabolism" (17–20). For example, macrophages adopt a variety of metabolic profiles depending upon the specific signals they sense (21, 22). Specifically, sensing through different Toll-like receptors (TLR) in myeloid cells can initiate any combination of up- and/or downregulation of glycolysis and oxidative phosphorylation (OXPHOS) (23). Thus, metabolic processes are a vital feature of the immune system for effectively combating viral infections or are an Achilles' heel of the host cell that can be manipulated by invading pathogens for their own advantage.

Eukaryotic cellular metabolism encompasses a wide range of catabolic and anabolic processes, and various aspects of host metabolism have been linked to viral infections. In particular, the major pathways of central carbon metabolism, glycolysis and oxidative phosphorylation (OXPHOS), have been investigated for their role in viral infection. For example, Kaposi's sarcoma herpesvirus (KSHV) suppresses aerobic glycolysis and OXPHOS to foster cellular, and thus, viral, survival (24). In contrast, an array of diverse viruses such as herpes simplex virus 1, HIV-1, rubella virus, white-spot syndrome virus, dengue virus, rhinovirus, hepatitis C virus, influenza virus, and adenovirus (25–33) have been shown to initiate a host cell response characterized by an increase in glycolysis, resulting in a more hospitable intracellular environment for viral replication. However, the specific ways in which viral infections initiate metabolic responses, and how these responses affect viral infection, vary substantially. Disentangling the unique metabolic responses of host cells upon viral infection, especially in regard to glycolysis and OXPHOS, may help in the development of broadly acting antiviral therapies.

Human noroviruses (hNoV) are nonenveloped, positive-sense, single-stranded RNA viruses of the *Caliciviridae* family that cause the majority of acute nonbacterial gastroenteritis globally (34–37). In addition to the public health burden, the economic burden of hNoV infections is enormous, with global costs estimated at $60 billion annually (35, 36). Currently, there are no licensed vaccines or antivirals that are effective against hNoV infections. Although advances have been made in developing *in vitro* model systems for studying hNoV (38–42, 95), the field still lacks a highly efficient, easy-to-use cell culture model. Therefore, murine norovirus (MNV) remains a powerful tool for investigating general norovirus biology (43–45). The goal in the current study was to identify aspects of host cell metabolism that are important for modulating MNV replication. Such findings may enable the development of more efficient hNoV culture systems and/or antiviral therapies and vaccines for hNoV in the future (46).

With these goals in mind, we performed the first metabolomic and energy profiling analysis of norovirus infection. Our analysis demonstrated that MNV infection of macrophages causes changes in the host cell metabolic profile characterized by an increase in central carbon metabolism. Inhibition of glycolysis with 2-deoxyglucose (2DG) severely attenuated MNV, but not human astrovirus VA1, infection *in vitro*. Inhibition occurred at the level of replication, as we observed a lag in the appearance of viral proteins in infected cells with a concomitant lag in viral genome replication but no effect on viral uptake or uncoating. Inhibition of MNV infection by 2DG was not rescued by the addition of nucleotides and was independent of type I interferon responses. Energetic profiling combined with experiments inhibiting the PPP and OXPHOS with 6-aminonicotinamide and oligomycin A, respectively, revealed that these pathways have a minor role with MNV compared to glycolysis. Investigations of two master regulators of cellular metabolism, Akt and AMPK, revealed that MNV infection caused an increase in Akt activation, while inhibition of Akt signaling reduced both

cellular glycolysis and MNV infection. Overall, our findings identify glycolysis as an intrinsic host factor important for optimal MNV infection of macrophages. Since noroviruses have a tropism for immune cells (47) and specific immune cell subsets are characterized by different metabolic profiles (48, 49), these findings may have implications for viral pathogenesis and the development of improved hNoV culture systems.

RESULTS

Targeted metabolomics survey identifies multiple metabolites that increase during MNV-1 infection in RAW 264.7 cells. Viral infections can cause changes in host cell metabolism that are important for viral replication (1, 3). In our efforts to identify host cell factors that are important for successful norovirus (NoV) infection, we hypothesized that infection of macrophages with murine norovirus (MNV) causes changes in central carbon metabolism of host cells that are beneficial or required for optimal viral infection. MNV-1 (CW3 isolate) is an acute strain of murine norovirus that has a natural tropism for macrophages *in vivo* and is particularly efficient at infecting transformed murine macrophage RAW 264.7 (RAW) cells (44). Thus, we performed a targeted metabolomics profiling of MNV-infected RAW cells to identify changes in the amount of host cell metabolites from glycolysis, the tricarboxylic acid (TCA) cycle, and others.

A targeted mass spectrometry analysis of metabolites isolated from MNV-1-infected RAW cells (multiplicity of infection [MOI], 5) after 8 h of infection (approximately one replication cycle) revealed multiple metabolites that were significantly increased in infected cells compared to mock-infected cells, or unchanged, but no metabolites that were significantly decreased during infection (Fig. 1; see also Tables S1 and S2 in the supplemental material). In particular, an increase in select metabolites from glycolysis (fructose-bisphosphate, 2- and 3-phosphoglycerate, and dihydroxyacetone-phosphate), the pentose phosphate pathway (PPP) (6-phosphogluconate), and the TCA cycle (citrate/isocitrate and malate) suggest that glycolysis, the PPP, and potentially OXPHOS are increased during MNV infection (Fig. 1A). Notably, overall levels of ATP were higher in infected cells than in mock-infected cells (Fig. 1A), indicating an overall increase in RAW cell metabolism as a result of viral infection. The detection of a significant increase in metabolites in cell culture is particularly noteworthy, since MNV-infected cultures represent a heterogeneous population of infected and uninfected cells (50).

Another group of metabolites that increased in RAW cells during MNV infection includes inosine-monophosphate (IMP), hypoxanthine, and xanthine (Fig. 1B). These metabolites are part of a pathway involved in adenosine catabolism that can result in the production of uric acid, a potent immune signal (16), and potentially reactive oxygen intermediates, which can have signaling and antimicrobial activity. Upregulation of enzymatic activity in this pathway and an increase in the resulting metabolites have been observed in the liver of mice infected with several RNA and DNA viruses (51), in the lungs and tissues of influenza virus-infected mice (52), and in mice infected with rhinovirus (53), and thus may represent a generalized cellular response to viral infection.

Last, the metabolites uridine triphosphate (UTP), UDP-glucose, and UDP-D-glucuronate were also increased in MNV-infected RAW cells (Fig. 1C). These metabolites are part of the glucuronic acid pathway that can lead to the generation of proteoglycans and other glycosylated forms of proteins (54) that have various roles, including as potential extracellular signals (55, 56). Indeed, many hNoV strains, including the clinically relevant genogroup II, genotype 4 viruses, are able to bind to host extracellular glycans, e.g., histo-blood group antigens (57, 58). Collectively, our metabolomics survey suggests that macrophages respond to MNV infection by increasing (i) the energy- and metabolite-generating pathways glycolysis, OXPHOS, and the PPP; (ii) adenosine catabolism, which may be a part of the general innate immune response; and (iii) the glucuronic acid pathway, which may have effects on cellular protein glycosylation.

2DG reduces MNV-1 infection in RAW cells and bone marrow-derived macrophages. Metabolomics profiling of MNV-infected RAW cells suggested that glycolysis and OXPHOS are increased during viral infection. However, whether this increase
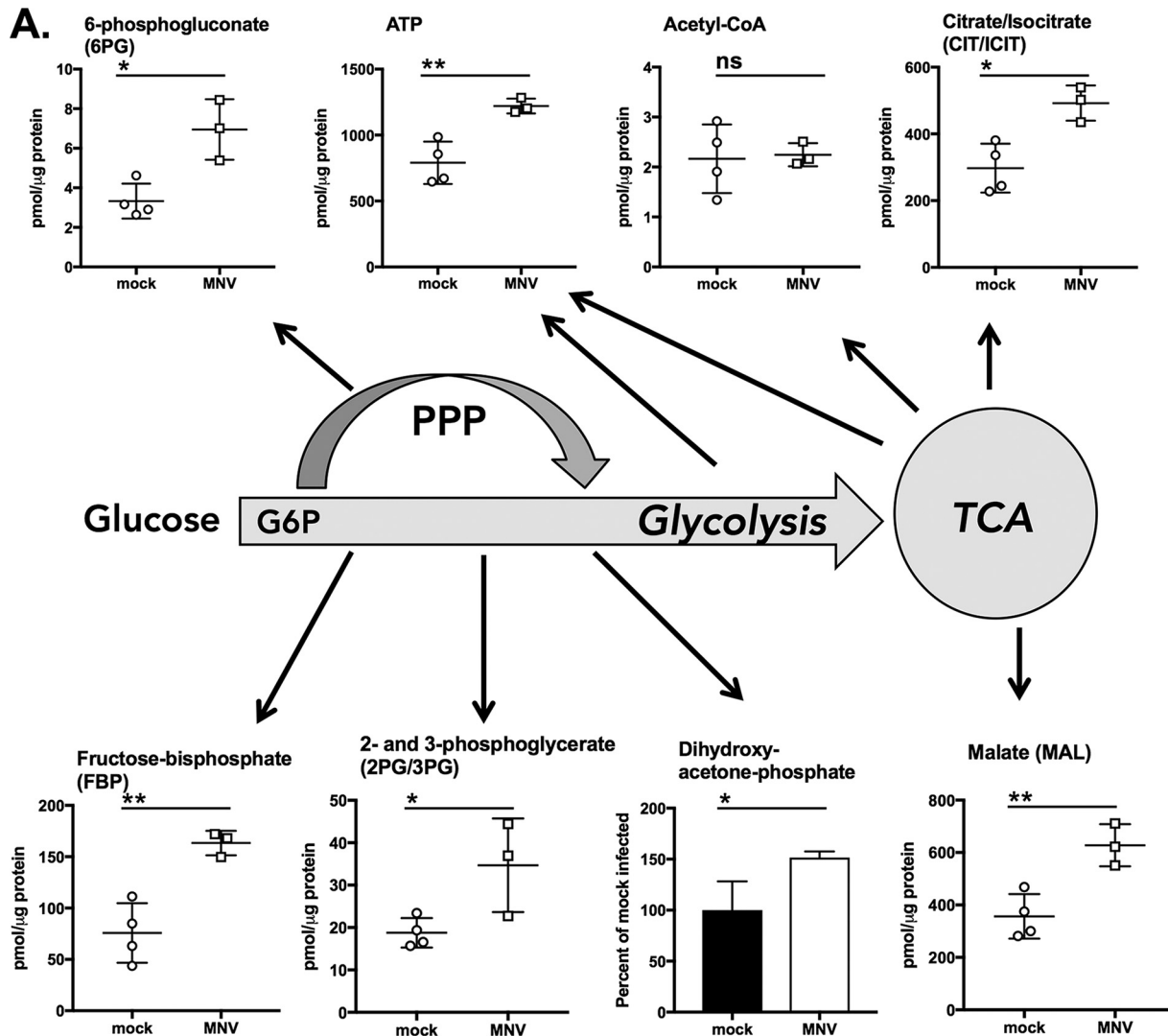
FIG 1 Metabolomics survey of RAW 264.7 cells infected with MNV-1 reveals several metabolic pathways that are increased during infection. (A) Measurements of select metabolites from central carbon metabolism, including glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (TCA). (B and C) Metabolites from xanthine biosynthesis (purine metabolism) (B) and the UDP-glucuronate pathway (glucuronic acid pathway) (C). Schematics of the metabolic pathways shown are simplified for clarity. All metabolites assayed are listed in Tables S1 and S2 with mean and standard deviation for the results from three MNV-1-infected samples (MOI, 5) and four mock-infected samples (mock cell lysate). Infection was for 8 h. ◆ indicates where in the pathway UTP is consumed. Horizontal lines indicate statistical comparison of MNV-infected versus mock-infected cells. Analyses were performed in MetaboAnalyst using Student's t test. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

creates an intracellular environment more supportive for viral replication, or rather represents an antiviral immune strategy of the host cell, is unclear from such a survey. To test whether host cell glycolysis is supportive for effective MNV infection of macrophages in generating building blocks, viral infection was measured *in vitro* in the presence of the potent and commonly used glycolysis inhibitor 2-deoxyglucose (2DG), a glucose analog that blocks early glycolysis (59, 60).

RAW cells were infected with MNV-1 at an MOI of 5 for 1 h. Medium containing 10 mM 2DG was then added postinfection to exclude direct effects of the compound on virions. After an 8-h incubation (one viral replication cycle), a >2-$\log_{10}$ decrease in the number of infectious viral particles in 2DG-treated cells was observed by plaque assay (Fig. 2A). RAW cells are a transformed cell line and generally engage in active "Warburg-effect" glycolysis (61). We therefore repeated the experiment in primary bone marrow-derived macrophages (BMDM) isolated from BALB/c mice to determine
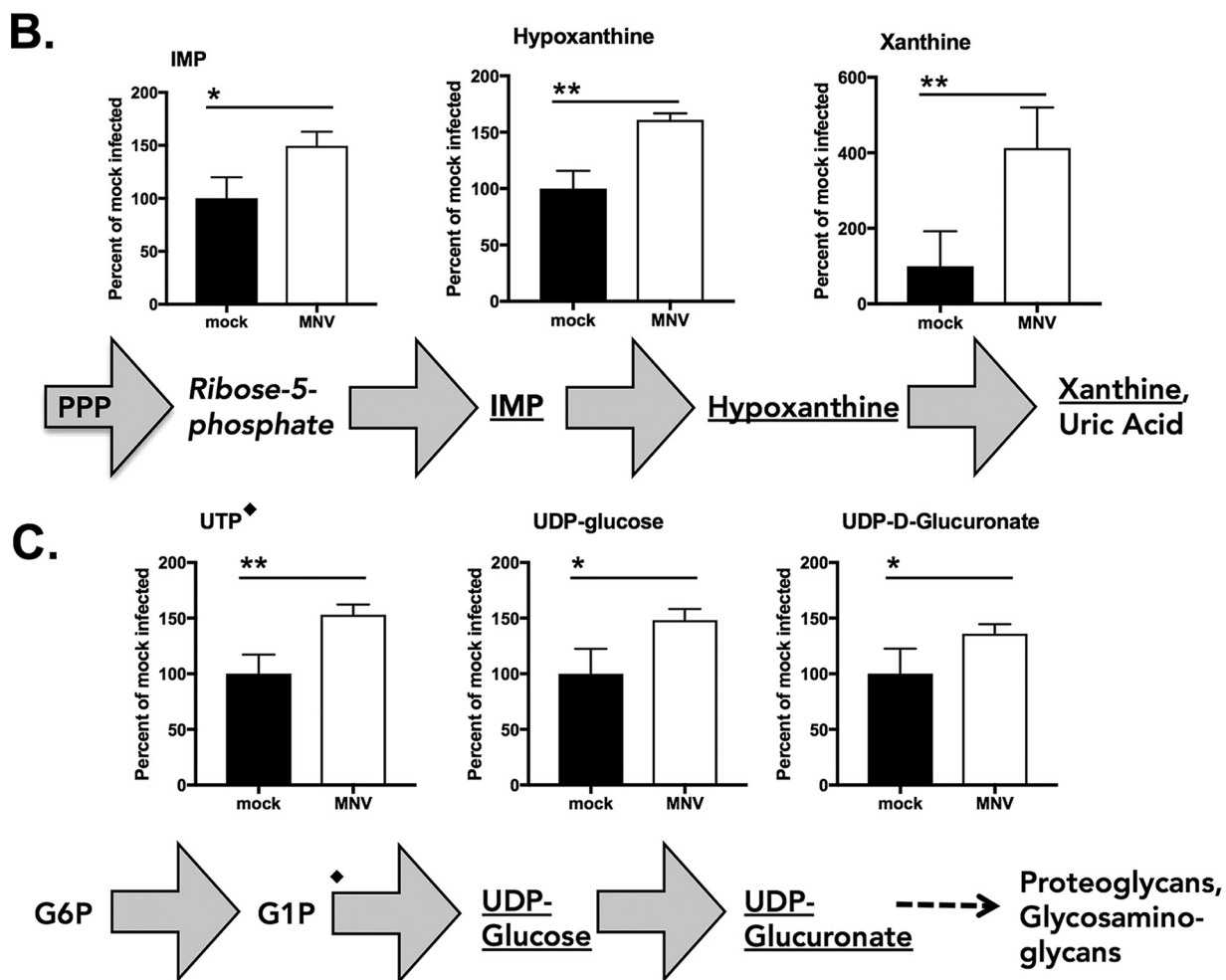
B.

C.

FIG 1 (Continued)

whether glycolysis is also relevant in nontransformed cells. 2DG treatment of BMDM caused an average 1-$\log_{10}$ decrease in viral loads after 8 h (Fig. 2B). 2DG treatment did not inhibit RAW viability during an 8-h treatment (Fig. 2C) but did reduce RAW cell viability by about 30% after 24 h (Fig. S1A).

Since RAW cells were grown in medium replete with glucose (~25 mM), putatively creating a competitive metabolic situation between glucose and 2DG, we next determined the minimal concentration of 2DG that significantly inhibited viral infection in RAW cells. Findings from a dose-response study performed in the presence of glucose demonstrated that 2DG inhibited MNV-1 infection in a dose-dependent manner, with the lowest significant inhibition at 4.0 mM (Fig. 2D).

To determine the point during the infectious cycle that 2DG exerts its inhibitory effect on MNV, a time-of-addition study was performed. RAW cells were infected with MNV-1 and 2DG added to the medium at various times postinfection. The results showed that 2DG had a significant effect on MNV infection when added to the culture up to 2 h postinfection (Fig. 2E), suggesting that glycolysis is important for early steps in the viral life cycle.

These data are consistent with the notion that glycolysis provides necessary building blocks for viral replication. Thus, to determine whether 2DG exerts a generalized antiviral response in any transformed cell line against any virus, we tested viral infection of a different single-stranded RNA (ssRNA) virus, human astrovirus VA1, which is readily propagated in Caco-2 cells (62). Surprisingly, 2DG did not significantly inhibit human
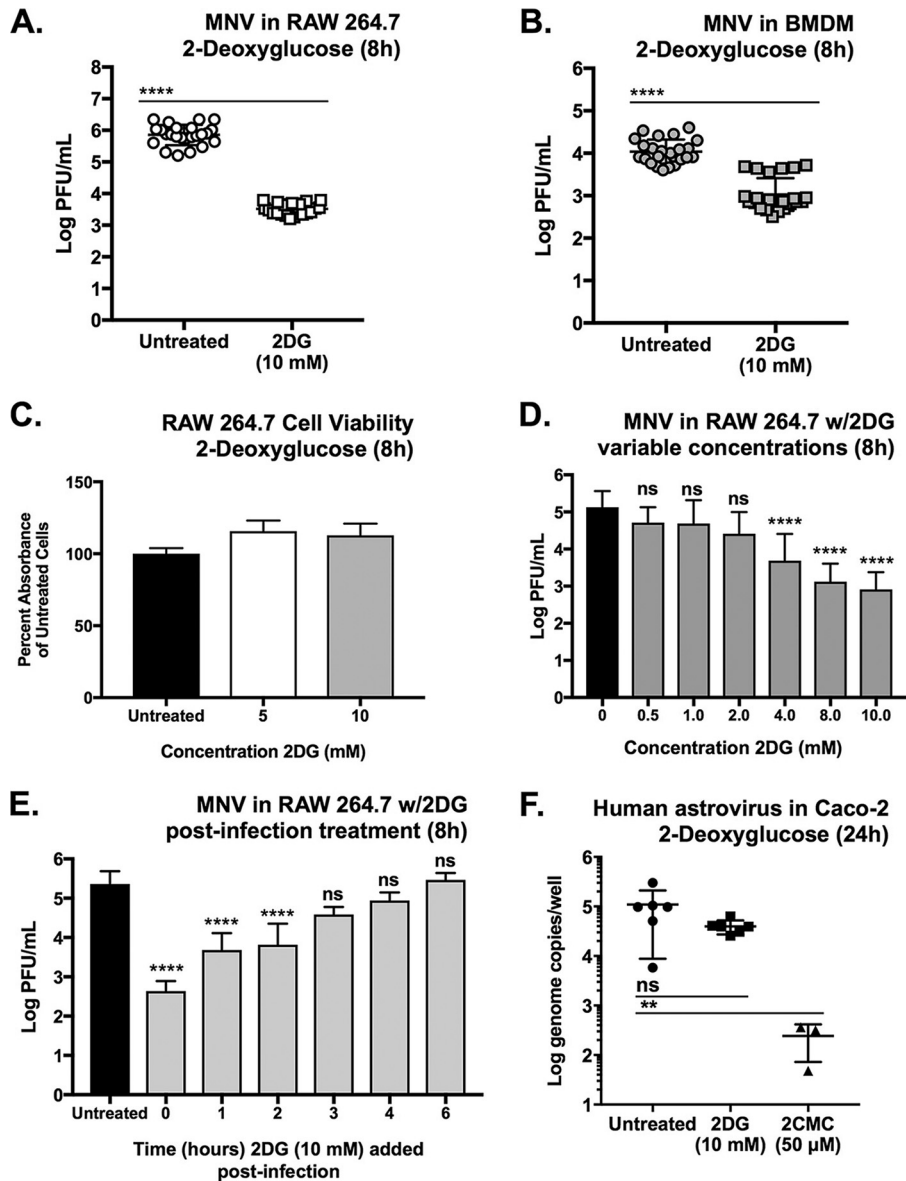
FIG 2 Effects of 2-deoxyglucose (2DG) on MNV-1 and human astrovirus VA1 infection *in vitro*. (A and B) 2DG (10 mM) reduces MNV-1 infection in RAW cells (~2 $\log_{10}$) (A) and primary bone-marrow derived macrophages (~1 $\log_{10}$) (BMDM-BALB/c mice) (B). Infections were for 8 h at an MOI of 5. (C) Cell viability assay (resazurin reagent) showing that 2DG does not reduce RAW cell viability during 8 h of exposure. Cell viability at 24 h is shown in Fig. S1A. (D) Effects of different concentrations of 2DG on MNV-1 infection in RAW cells. (E) MNV-1 infection in RAW cells with 2DG added at different times postinfection. (F) 2DG does not affect infection of human astrovirus VA1 in Caco-2 cells (2DG 10 mM; 2'-*C*-methylcytidine [2CMC] positive control, 50 μM). Toxicity of 2DG on Caco-2 cells is shown in Fig. S1E. (A, B, D, and E) Measurements were by plaque assay. (F) Astrovirus in was measured by RT-qPCR of viral RNA. (A, B, and F) Mann-Whitney test was used. (E) Kruskal-Wallis test with Dunn's multiple-comparison test was used. ****, $P < 0.0001$; **, $P < 0.01$; ns, not significant. Experiments represent combined data from at least three independent experiments except for panel F, which represents two experiments. Horizontal lines indicate statistical comparisons made.

astrovirus infection *in vitro* (Fig. 2F), suggesting that the MNV phenotype in RAW cells and in BMDM is specific to MNV.

Taken together, these data demonstrate that host cell glycolysis contributes to optimal MNV infection in macrophages. They further suggest that glycolysis is an intrinsic host factor that modulates infection in a virus-specific manner.

MNV infection of RAW cells causes an increase in overall metabolism with a higher proportion of ATP derived from glycolysis. The metabolomics data showed
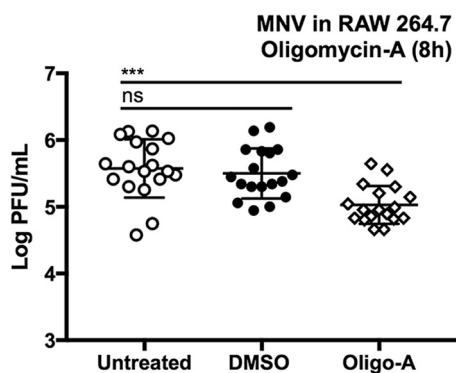
FIG 3 Effect of oligomycin A (Oligo-A) on MNV-1 replication in RAW 264.7 cells. Treating cells with oligomycin A (1 μM) caused decreased MNV replication in RAW cells ($\sim$0.5 log) after 8 h. Horizontal lines indicate statistical comparisons made. Kruskal-Wallis test with Dunn's multiple-comparison test, ***, $P < 0.001$; ns, not significant.

that two metabolites of the TCA cycle were increased after MNV infection, so we tested the effect of inhibiting OXPHOS in RAW cells with oligomycin A (Fig. 3) and observed a small but significant reduction in MNV replication ($\sim$0.5 log) after 8 h. This smaller role for OXPHOS in MNV infection led us to ask what contribution glycolysis and OXPHOS make to the increased levels of ATP in MNV-infected RAW cells observed in Fig. 1A. We performed the real-time ATP synthesis rate assay on a Seahorse extracellular flux analyzer, which revealed that a higher proportion of ATP was generated by glycolysis in MNV-infected cells than in mock-infected cells (34% versus 28%, respectively) (Fig. 4A) and that a smaller proportion was generated by OXPHOS (66% versus 72%, respectively). However, the overall energetic rate was higher in MNV-infected cells, showing that MNV infection increases overall carbon metabolism, particularly glycolysis (Fig. 4B and C). As expected, 2DG reduced the proportion of glycolysis-derived ATP in both mock-infected and infected cells (19%), causing these cells to rely energetically primarily on OXPHOS and to have an overall lower rate of metabolic flux. These data demonstrate that although both glycolysis and OXPHOS are increased during MNV infection, glycolysis seems to play a more prominent role for MNV during infection than does OXPHOS.

2DG treatment inhibits MNV-1 negative-strand vRNA and viral nonstructural protein production. Postinfection treatment of RAW cells with 2DG suggested that host cell glycolysis is important for early stages of MNV infection (Fig. 2E). To more accurately pinpoint the stage in the viral infectious cycle at which glycolysis is important, RAW cells were transfected directly with viral RNA (vRNA) in order to bypass the steps of binding, uptake, and virion uncoating. 2DG treatment of transfected RAW cells resulted in about a 2-$\log_{10}$ reduction in infectious virus particle production after 12 h (Fig. 5A) and a 1-$\log_{10}$ reduction at 24 h (Fig. S2A), suggesting that 2DG does not affect virion binding or genome uncoating of MNV.

MNV is a single-stranded, positive-strand, nonenveloped virus, and so the viral life cycle involves uptake of viral particles, uncoating of the positive-strand vRNA, and direct translation of the positive-sense genome to produce the nonstructural proteins (including the viral RNA polymerase), followed by viral negative-RNA-strand synthesis for eventual production of new positive-strand vRNA, structural coat proteins, and progeny virion assembly (Fig. 5B). To measure vRNA production during 2DG treatment, we isolated RNA over the course of a 12-h infection in RAW cells and assessed the relative amounts of total positive- and negative-strand vRNA (Fig. 5C to E) (63). At 4 h postinfection (hpi), no difference in the quantity of positive-strand and total vRNA was observed (Fig. 5C and E), indicating that the same amount of virus infected the cells and confirming that 2DG has no significant effect on viral binding and entry. However, there is a significant reduction in the amount of negative-strand vRNA at 4 hpi in 2DG-treated cells (Fig. 5D). At 8 and 12 hpi, there is also
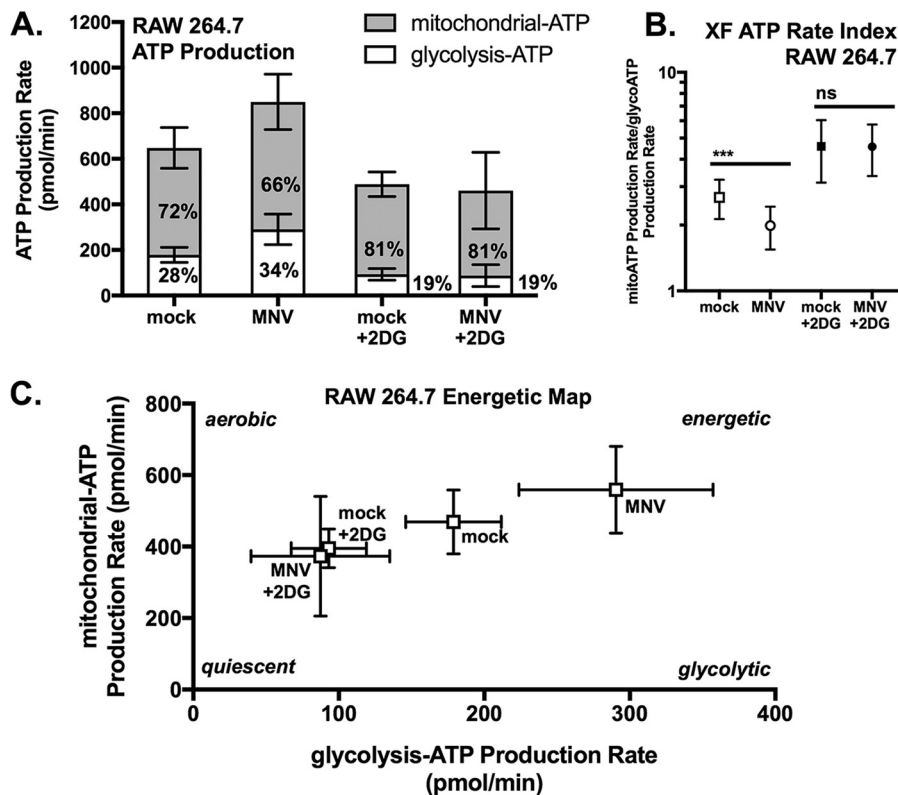
FIG 4 Seahorse XF real-time ATP rate analysis of RAW 264.7 cells. (A) Metabolic flux analysis on the Seahorse XF shows an increase in glycolysis and OXPHOS in RAW cells after 8 h of MNV infection compared to mock infection, with a higher proportion of ATP derived from glycolysis during infection (see Table S3 for descriptive statistics and tests). Cells treated with 10 mM 2DG had overall lower ATP derived from glycolysis than did nontreated cells for both mock- and MNV-infected cells. (B) XF ATP rate index calculated from data in panel A. (C) Energetic map of the four conditions tested charting mitochondrial ATP (mitoATP) versus glycolysis-generated ATP (glycoATP). (B) Mann-Whitney test where ***, $P < 0.001$; ns, not significant. All Seahorse data shown are compiled from three independent experiments using at least 5 technical replicates per experiment per condition and using 3×10^4 RAW cells/well. Horizontal lines indicate statistical comparison made.

significantly less vRNA overall for all species of RNA assessed with 2DG treatment (Fig. 5C to E). The phenotype was the same in primary BMDM, with the only difference being the overall lower magnitude of viral infection than in RAW cells (Fig. S2B and C), which is consistent with previous observations (44). These data demonstrated that although vRNA replication occurs in 2DG-treated cells, a lag occurred in the transcription of negative-strand vRNA.

Since expression of nonstructural proteins is required for negative-strand vRNA synthesis, we assessed the quantity of MNV nonstructural protein using anti-ProPol/NS6&7 and anti-capsid antibodies by Western blotting. Cells treated with 2DG contained no detectable polymerase (Pol) or VP1 proteins at 7 hpi, while reduced amounts of these proteins were present at 12 hpi (Fig. 5F). These data indicate that host cell glycolysis is important for an early step in viral replication after delivery of the viral RNA into the cytosol.

Taken together, our data are consistent with a role for glycolysis in an early, postentry stage in the viral life cycle. Whether 2DG affects the primary round of translation of the viral RNA released from the incoming capsid, or if it affects the formation of viral replication complexes and production of new negative-sense RNAs, remains to be determined.

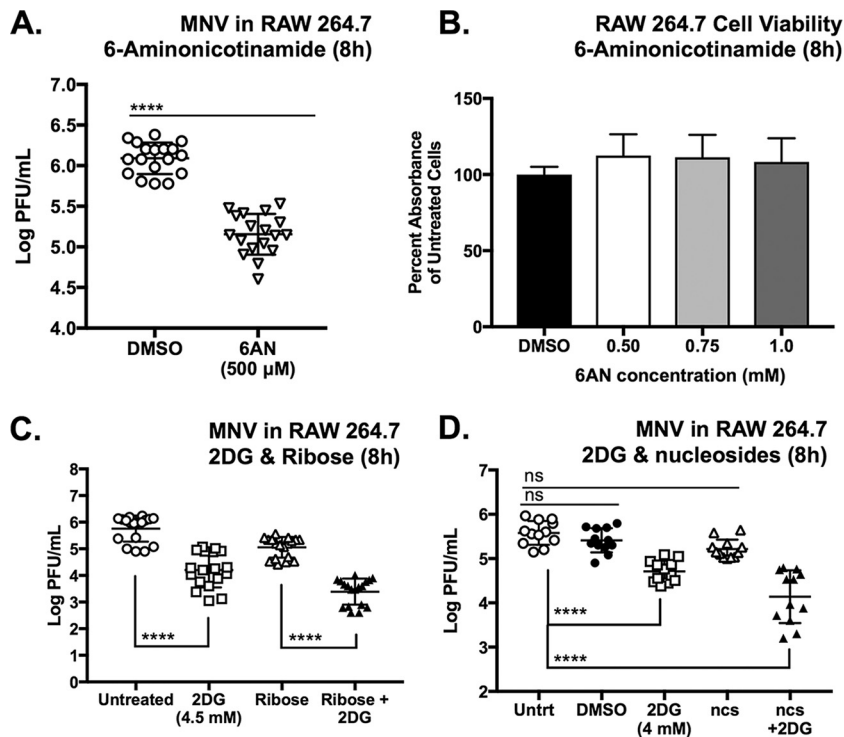**Inhibiting the pentose phosphate pathway reduces MNV infection of RAW cells.** The metabolomics survey outlined in Fig. 1 demonstrated that 6-phosphogluconate, the first metabolite produced from glucose-6-phosphate in the oxidative half of the pentose phosphate pathway (PPP), was more abundant in MNV-infected cells. This sug-
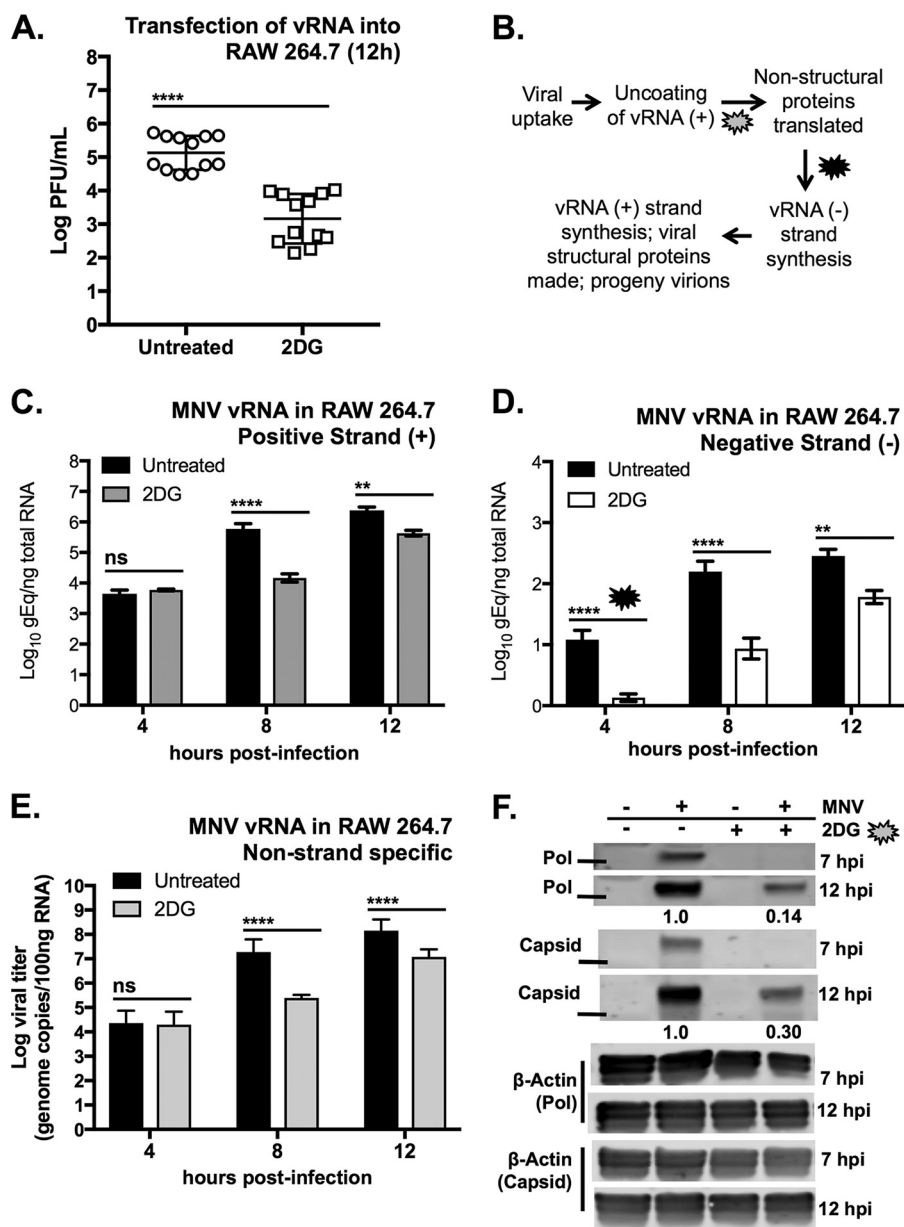
FIG 5 2DG treatment inhibits MNV infection early after viral uptake and uncoating. (A) MNV-1 viral RNA (vRNA) was transfected into RAW cells and then treated with 10 mM 2DG. Data are from two independent experiments. (B) A simplified overview of the events in the MNV-1 life cycle. Callouts indicate points of the viral life cycle that may be affected during 2DG treatment. (C and D) Strand-specific RT-qPCR of positive (+) (C) and negative (−) (D) MNV vRNA strands from RAW cells infected with MNV-1 for 4, 8, and 12 h with and without 2DG treatment (10 mM). (E) Taq-Man RT-qPCR of total MNV-1 viral RNA (non-strand specific) from the same RNA samples used for panels C and D. Data are combined from three independent experiments with three replicates per experiment. (F) Western blot analysis of nonstructural (Pol) and structural (capsid) viral proteins after 7- and 12-h infection of RAW cells in untreated and 2DG-treated cells. β-Actin was used as a loading control for overall protein content. Solid line indicates 50-kDa ladder. Data shown are representative Western blots from two independent experiments. Numbers below blots indicate densitometry measurement of protein in 2DG relative to untreated cells at 12 h (average of two experiments). Mock-infected cells served as a negative control. (A) Mann-Whitney test was used. (C to E) Two-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test used. Horizontal lines indicate statistical comparison made. ****, $P < 0.0001$; **, $P < 0.01$; ns, not significant.

gested that the PPP, which branches off glycolysis at the early stage of glucose phosphorylation (64), may also be important for MNV infection in RAW cells. In addition, since 2DG interferes at the level of glucose phosphorylation, the viral inhibition caused by 2DG could also be due to interference with the PPP.

FIG 6 The pentose phosphate pathway makes a minor contribution to MNV infection of RAW cells. (A) 6-Aminonicotinamide (6AN) (500 μM), the inhibitor of 6-phosphogluconate dehydrogenase, reduces MNV infection in RAW cells (MOI, 5) ($\sim$1.0 log). (B) Resazurin cell viability assay of RAW cells treated with indicated concentration of 6AN for 8 h (see Fig. S1B for data for 24 h). (C and D) Supplementing MNV-infected RAW cells with 50 mM ribose (C) or 50 μM nucleosides (ncs) (D) does not alleviate the viral growth inhibition caused by 4.5 or 4.0 mM 2DG treatment after 8 h of infection. (D) Nucleosides used were 50 μM each adenosine, guanosine, thymidine, cytidine, and uridine. RAW cells were treated overnight before infection with nucleosides and supplemented with nucleosides after infection with MNV. (A) Mann-Whitney test used. (C and D) Kruskal-Wallis test with Dunn's multiple-comparison test used. ****, $P < 0.0001$; ns, not significant. Dimethyl sulfoxide (DMSO) is vehicle control used in vol/vol match to 6AN or ncs treatment. Data represent a combination of the results from three independent experiments. Lines indicate statistical comparisons made.

Therefore, to test the importance of the PPP for MNV infection, we used 6-amino-nicotinamide (6AN), an inhibitor of the PPP enzyme glucose-6-dehydrogenase. Treatment with 500 μM 6AN after MNV-1 infection caused a 1-$\log_{10}$ reduction in the production of infectious MNV-1 after 8 h (Fig. 6A). 6AN was nontoxic to RAW cells up to 1.0 mM during 8 h (Fig. 6B), whereas all concentrations of 6AN tested caused an approximately 30% reduction in cell viability after 24 h (Fig. S1B).

Inhibition of MNV infection by 2DG may be partially due to its effect on the PPP by depleting ribose nucleotides, one of the major end products of the PPP. Alphaviruses, which rely on host cell glycolysis via PI3 kinase signaling, are partially rescued for viral replication with ribose supplementation when PI3 kinase signaling is inhibited (65). Therefore, we infected RAW cells with the minimal amount of 2DG that still causes a significant reduction in MNV infection (4 mM) and supplemented the cultures with ribose alone (Fig. 6C) or presupplemented cells with a mix of five ribonucleosides (Fig. 6D). Neither treatment was sufficient to increase viral titers during 2DG inhibition. These data suggest that, at least under the conditions tested, viral inhibition from 2DG is caused by cellular changes other than nucleotide availability.

2DG viral inhibition is independent of the type I interferon response. The mechanism of viral inhibition by 2DG could be due to a variety of cellular perturbations that are caused by a decrease in glycolysis. MNV infection in RAW cells induces a strong innate immune response, including interferon induction (66). Type I interferons in turn
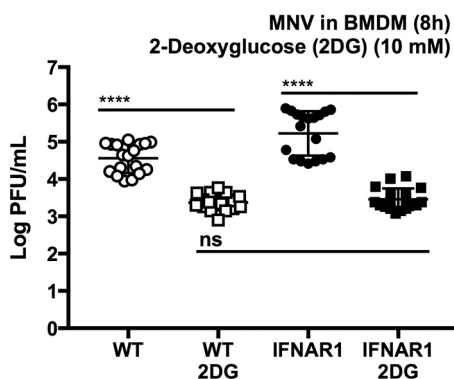
FIG 7 2DG inhibition of MNV infection is independent of the type I interferon response. 2DG treatment (10 mM) reduces MNV infection in both WT BMDM and in BMDM lacking the type I interferon receptor (IFNAR1-knockout cells) (WT-2DG versus IFNAR-2DG). Kruskal-Wallis test with Dunn's multiple-comparison posttest. ****, $P < 0.0001$; ns, not significant. Data represent a combination of the results from three independent experiments. Horizontal lines indicate statistical comparisons.

are able to affect host cell metabolism (67, 68) and exhibit a strong anti-MNV response (69–71). Therefore, we determined whether 2DG inhibition of viral replication was dependent on type I interferon responses. Wild-type C57BL/6 BMDM and BMDM lacking the type I interferon receptor (IFNAR1$^{-/-}$) were infected with MNV-1 for 1 h and then treated with 10 mM 2DG. After 8 h, both WT and IFNAR1$^{-/-}$ cells had reduced viral titers following 2DG treatment compared to untreated cells (Fig. 7). These data demonstrate that the inhibition of MNV infection by 2DG is independent of the antiviral type I interferon response.

MNV-1 infection increases activation of Akt but not AMPKα. To identify cellular signaling pathways that underlie the observed metabolic changes during MNV infection, we focused on two master regulators of metabolic control in cells, PI3 kinase/Akt and AMPK (72–80). In mammals, AMPK is able to sense the energetic status of cells, specifically the ratios of AMP and ADP relative to ATP, and can promote fatty acid oxidation and the expression of mitochondrial proteins (81–83). Western blot analysis of RAW cells revealed very low levels of total AMPKα protein and no increases in phosphorylation at Thr172 between mock- and virus-infected cells or between untreated and 2DG-treated cells were observed (Fig. 8A and S3). These data demonstrate that AMPK is not involved in the energetic changes in RAW cells that we have observed during MNV infection.

Another protein that has been implicated in energy sensing in multiple cell types is Akt. This kinase has been shown to play a key role in stimulating glycolysis and glucose metabolism via multiple mechanisms (72, 76, 84). In addition, Akt signaling is often altered during the infectious cycle of numerous viruses (85). Western blot analysis of Akt activation during MNV-1 infection demonstrated that Akt phosphorylation at Ser473 was slightly elevated at 2 hpi (~2-fold) (Fig. 8B and S3) above the baseline level of Akt activation in mock-treated RAW cells. Akt was further activated as indicated by the higher levels of Ser473 phosphorylation at 7 hpi (~10-fold higher) (Fig. 8B) and 12 hpi (Fig. S4). 2DG treatment prevented the increase in Akt phosphorylation (Fig. 8B).

Because Akt phosphorylation was elevated during MNV infection and 2DG blocked Akt activation and viral replication, we asked whether inhibition of Akt signaling would inhibit MNV infection in RAW cells, linking Akt signaling with a change in host cell glycolysis. Treating cells with 15 μM MK2206, a potent inhibitor of Akt phosphorylation, completely prevented Akt phosphorylation at Ser473 (Fig. 8B) but did not affect AMPKα phosphorylation (Fig. 8A). Treating RAW cells with 15 μM MK2206 after the 1-h MNV adsorption phase reduced viral production after 8 h by about 1 log$_{10}$ (Fig. 8C). Furthermore, 2DG and MK2206 reduced RAW cell glycolysis irrespective of infection, as measured by assaying endpoint lactate production (Fig. 8D). Both compounds are nontoxic at these
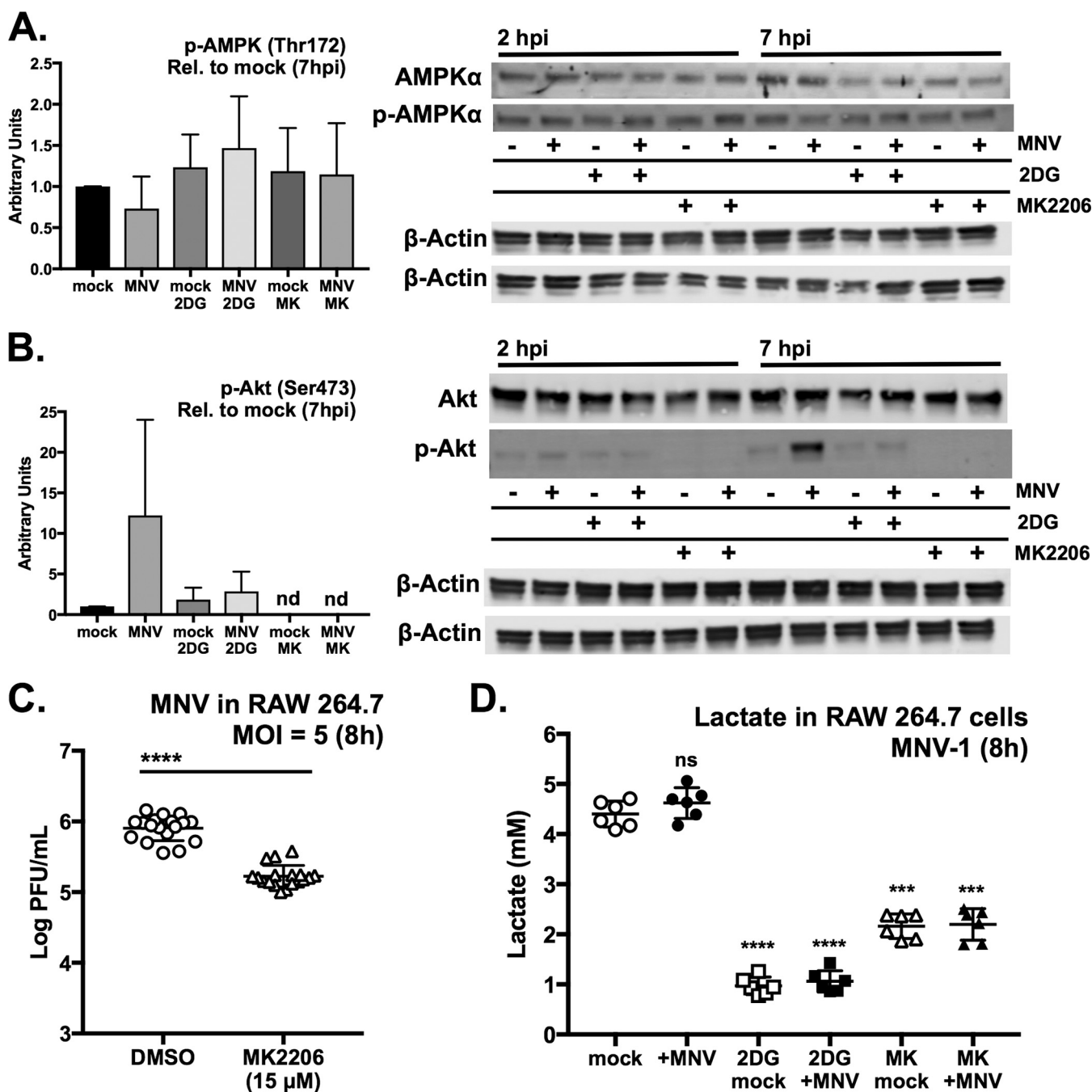
FIG 8 MNV upregulates glycolysis via Akt signaling. (A and B) Western blot analysis of RAW cells infected with MNV (MOI, 5) for 2 and 7 h for AMPKα and phospho-AMPKα (Thr172) (A) and Akt and phospho-Akt (Ser473) (B). Treatments were 10 mM 2DG and 15 μM MK2206. Western blot from 12 hpi is shown in Fig. S3. β-Actin was used as loading control and for densitometry normalization. Graphs on the left represent densitometry analysis comparing phosphoprotein relative (Rel.) to mock-infected cells at 7 hpi. Graphs of densitometry analysis for 2 hpi are in Fig. S4. (C) Inhibition of Akt phosphorylation with MK2206 reduces MNV-1 infection of RAW cells by about 0.75 log$_{10}$. Horizontal line indicates statistical comparison made. (D) Measurement of glycolysis via lactate production in mock- and MNV-infected RAW cells after an 8-h infection (MOI, 5). Cells were treated with 10 mM 2DG and 15 μM MK2206. (C) A Mann-Whitney test was used. (D) One-way ANOVA with Dunnett's multiple-comparison test (graph shows data for one of two independent experiments with three replicates each). ****, $P < 0.0001$; ***, $P < 0.001$; ns, not significant; nd, not detected.

concentrations (Fig. S1C and D). These experiments demonstrate that Akt activation is a feature of MNV infection of RAW cells and that Akt plays a role in maintaining glycolysis in these cells. Akt activation during MNV infection is consistent with a previous transcriptomic study of monocytes transfected with the nonstructural protein NS1-2, which implicated NS1-2 in affecting PI3K-Akt signaling pathways (86). Taken

together, these data are consistent with a model whereby MNV infection upregulates glycolysis via Akt signaling.

DISCUSSION

When viruses infect cells, they are entirely dependent on the intracellular landscape of their hosts in order to replicate efficiently. Indeed, the intracellular metabolic state of target cells acts as an intrinsic host factor, and a variety of metabolic pathways are important for successful viral infection (3). However, different viruses cause diverse metabolic effects in various cell types, and the mechanisms of viral engagement with host metabolic processes vary greatly (24–32). Thus, defining the specific host cell metabolic features that are required for individual viruses may reveal key host cell vulnerabilities that could be helpful for the future development of effective and safe antiviral therapies (46). Noroviruses lack effective therapies. In this study, we uncover central carbon metabolism, most prominently glycolysis, as an intrinsic factor that is important for optimal infection of macrophages by MNV at early points during replication, suggesting a potential new antinorovirus target.

Maintaining homeostasis of glucose metabolism in mammalian physiology is of importance in virtually every tissue, and glycolysis and OXPHOS are considered to be "central" carbon metabolism since they are hubs for multiple metabolic pathways and play a vital role in energy homeostasis. Therefore, it is not surprising that some viruses have evolved to take advantage of different aspects of these conserved pathways to their benefit. Interestingly, glycolysis may be increased or decreased in response to viral infection, with similar beneficial outcomes for the virus. For instance, dengue virus increases both glucose uptake and transcription of the important enzyme hexokinase 2 (28), while herpes simplex virus 1 activates glycolysis by increasing transcription and activation of the enzyme phosphofructokinase-1 (PFK-1) (33), with both viruses relying on active glycolysis for optimal infection. On the other hand, Kaposi's sarcoma-associated herpesvirus (KSHV) causes a suppression of both aerobic glycolysis and OXPHOS in transformed cells under nutrient stress, which thereby inhibits cell death and enhances viral survival in this model of the tumor microenvironment (24). Our observation that astrovirus infection was not affected by the treatment of Caco-2 cells with 2DG highlights that not all viruses require glycolysis in transformed cells, which generally conduct a significant level of Warburg effect glycolysis at baseline (61). Thus, it was notable that 2DG inhibited MNV infection in nontransformed primary cells, highlighting the fact that glycolysis has proviral functions during norovirus infection. These results illustrate that the relationship of target cell metabolism to viral infection is cell type specific and virus specific. Therefore, future studies are needed to determine whether these findings extend to other MNV (e.g., dendritic cells, B cells, and T cells) or hNoV (e.g., enterocytes and B cells) target cell types (40, 44, 87, 88, 95).

Another notable aspect of the relationship between carbon metabolism and infection is the finding that glycolysis may facilitate infection outside of a canonical metabolic role. HIV-1 causes an increase in expression of hexokinase-1 (HK1) accompanied by a decrease in enzymatic activity (89). Our findings with 2DG, which targets the enzymatic activity of hexokinase, points to a metabolic, rather than nonmetabolic, role for glycolysis during norovirus infection. Specifically, glucose-6-phosphate (G6P), located at the intersection of glycolysis and PPP, is a major hub for macrophage metabolic regulation of MNV infection given that inhibition of the PPP also reduced viral infection.

One particular caveat of host cell metabolic profiling studies is the complexity of metabolic responses that immune cells can adopt in response to various stimuli. This is of particular relevance for macrophages. Although the M0/M1/M2 system of categorizing macrophage metabolic states is a useful construct for generalizing inflammatory versus noninflammatory activity, these cells actually establish a complex range of metabolic phenotypes (22, 90, 91). For example, although the bacterial product lipopolysaccharide (LPS) causes an increase in glycolysis and a decrease in OXPHOS in human monocytes, a different bacterial product, Pam3CysSK4 (P3C), causes both

pathways to increase (23). Thus, two different bacterial products signaling through different Toll-like receptors (TLRs) establish unique metabolic profiles. This finding emphasizes that unique pathogens elicit complex host metabolic responses and that the range of molecular signals that immune cells respond to *in vivo* may determine the susceptibility of cell types to certain infections. The metabolomics survey and energetic profiling in this study demonstrated that MNV infection elicits an increase in both glycolysis and OXPHOS, and that the increase in overall metabolism disproportionately favors glycolysis. In addition, since macrophages are target cells of MNV *in vivo* (44, 87), it is conceivable that their metabolic status during infection influences the establishment of norovirus infection at the cellular levels, with potential influences on viral pathogenesis. However, future studies are needed to test this.

Another important aspect of macrophage metabolism is how metabolic rewiring controls functional outputs, such as microbial killing mechanisms and cytokine/chemokine production (7), which in turn could indirectly affect viral infection. Akt has been implicated in regulating reactive oxygen species (ROS) generation (75). Although MNV infection increased Akt activation, we did not observe an increase in general ROS in RAW cells with 2DG treatment (Fig. S5). Furthermore, blocking glycolysis with 2DG did not cause a significant difference in the production of the inflammatory cytokine tumor necrosis factor alpha (TNFα) in 2DG-treated RAW cells during MNV infection (Fig. S6). Combined with the finding that the effect of 2DG is independent of type I interferon (IFN) signaling, these data suggest that the antiviral effect of 2DG is not mediated via immune signaling. However, whether MNV affects general macrophage functions via Akt activation and metabolic rewiring of these cells will need to be tested in future studies.

A general caveat to the use of pharmacologic inhibitors in biological systems is their potential to induce side effects. Although 2DG has been commonly used as a prototypical glycolysis inhibitor (59, 60), it may also affect other aspects of cell behavior that can influence infectivity. For example, 2DG has been shown to induce ROS-triggered autophagy via AMPK (92). This pathway is unlikely to be involved in the antiviral activity of 2DG in our studies considering the lack of ROS induction upon 2DG treatment in RAW cells (Fig. S5) and the lack of AMPK induction during infection (Fig. 8A). Another study showed that 2DG can be damaging for certain viral infections via initiation of an endoplasmic reticulum (ER) stress response in mice (93). Similarly, 2DG decreases porcine epidemic diarrhea virus infection *in vitro* via triggering the unfolded protein response and reducing protein translation (94). Our work showed that 2DG inhibited MNV infection early during the viral life cycle, affecting the translation of nonstructural proteins and the transcription of new viral genomes. However, the virus does eventually begin to replicate genomes and produce viral proteins even in the presence of 2DG. Thus, the mechanism by which 2DG causes this lag in the MNV life cycle could be via a rapid cellular stress response, a decrease in specific metabolites, or a combination of the two, and additional studies are needed to clarify the relative contributions of both.

Last, it should be noted that a variety of metabolites, including ornithine, 3-phosphoserine, and creatinine, among others, were also increased during MNV infection. While these molecules could be important host factors for viral infection, they were not explored further here. Such investigations and an extension of metabolic findings to human noroviruses are planned for the future. Human norovirus has remained stubbornly intractable to robust cultivation *in vitro*. Although there has been some success in infecting transformed B cells (40) and human intestinal enteroids (95) with human norovirus, viral loads are typically low, and an infectious, passageable cell culture-derived virus stock is not readily available (96). Identifying host cell factors such as metabolites and specific metabolic activities may therefore aid in optimizing *in vitro* cultivation systems for human noroviruses.

In conclusion, we have shown that glycolysis in macrophages is an intrinsic factor promoting optimal infection of a norovirus. Our data are consistent with a model whereby MNV activates the protein kinase Akt to increase central carbon metabolism in macrophages. The glycolysis inhibitor 2DG inhibits norovirus (but not astrovirus)

infection, independent of the type I IFN response, by limiting an early step in the viral life cycle that results in reduced nonstructural protein production and viral RNA synthesis. These findings reveal cellular metabolism as a potential therapeutic target for norovirus and suggest a new strategy for improving human norovirus culture systems.

MATERIALS AND METHODS

Detailed methods can be found in Text S1 in the supplemental material.

Compounds and reagents. Please refer to Text S1 for details on the compounds and reagents used.

Cell culture and virus strains. RAW 264.7 and Caco-2 cells were obtained from the ATCC. The plaque-purified MNV-1 clone (GV/MNV1/2002/USA) MNV-1.CW3 (43) (referred to here as MNV-1) was used at passage 6 in all experiments.

Virus infections, virus transfection, and plaque assay. All MNV infections were done in the RAW 264.7 cell line, BALB/c primary bone marrow-derived macrophages (BMDM from male mice), or BMDM from wild-type (WT) and IFNAR1-knockout cells on a C57BL/6 background. Transfections and viral enumerations were performed in a manner similar to in previous studies (62, 97, 98). Please refer to Text S1 for details.

Cell viability assay. Cell viability was tested using resazurin reagent, according to the manufacturer's recommendations (catalog no. 30025-1; Biotium).

RNA extraction and RT-qPCR. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) experiments were performed as per manufacturer's directions using chloroform extraction (TRIzol) or the Zymo Research Direct-zol RNA MiniPrep Plus kit (catalog no. R2072).

Strand-specific RT-qPCR. Strand-specific RT-qPCR for MNV was performed as previously described (63).

Protein extraction, SDS-PAGE, and immunoblotting. The protein extraction, SDS-PAGE, and immunoblotting experimental conditions and antibodies used are detailed in Text S1.

Metabolomics assay. Samples were analyzed at the Michigan Regional Comprehensive Metabolomics Resource Core (MRC^2) at the University of Michigan by mass spectrometry as detailed in Text S1.

Lactate assay. Cell supernatants were assessed for lactate using the Cayman Chemical glycolysis cell-based assay kit (catalog no. 600450), as per the manufacturer's protocol.

ELISA. Cytokine levels were determined at the University of Michigan Rogel Cancer Center Immunological Monitoring Core by enzyme-linked immunosorbent assay (ELISA) (Duosets; R&D Systems, Minneapolis, MN), as detailed in Text S1.

Statistical analysis. Metabolomics data were analyzed in MetaboAnalyst 4.0. For all other experiments, data were analyzed in Prism7 using tests as indicated in the figure legends.

Agilent Seahorse XF real-time ATP rate assay. RAW 264.7 cells were assessed using the Agilent Seahorse XF real-time ATP rate assay kit 103592-100 on a Seahorse XFe96 extracellular flux analyzer, as per the manufacturer's directions, as detailed in Text S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio .02175-18.

TEXT S1, DOCX file, 0.1 MB.
FIG S1, PDF file, 0.3 MB.
FIG S2, PDF file, 0.3 MB.
FIG S3, PDF file, 0.1 MB.
FIG S4, PDF file, 0.1 MB.
FIG S5, PDF file, 0.1 MB.
FIG S6, PDF file, 0.2 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.
TABLE S3, DOCX file, 0.1 MB.

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