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Understanding the Effect of Dietary Palmitic Acid on Glycolysis During Innate Immune Memory in Macrophages

Khaleda A. Aqaei
Portland State University

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TITLE: Understanding the effect of dietary palmitic acid on glycolysis during innate immune memory in macrophages

AUTHORS: Khaleda Aqaei and Brooke A. Napier

A. ABSTRACT:

Trained immunity is long-term innate immune memory induced by a primary stimulus, which leads to hyper-inflammation upon secondary stimulation with a homologous or heterologous ligand. Trained immunity is mediated by epigenetic and metabolic reprogramming of the target cell and leads to modification of gene expression and cellular function. Classically, trained immunity is initiated by β-glucans, an inflammatory molecule found on the exterior of fungal species. Interestingly, our lab has recently described that dietary fatty acids can initiate trained immunity, working through similar pathways as β-glucans. Specifically, our data show that a pre-treatment with a specific dietary saturated fatty acid (SFA), palmitic acid (PA), initiates trained immunity in macrophages, leading to a hyper-inflammatory response to a secondary challenge with numerous microbial ligands. Macrophages are tissue-specific innate immune cells that play a key role in orchestrating inflammatory diseases and infection. Though we have reported PA-dependent trained immunity leads to a hyper-inflammatory response, it is unknown if PA induces metabolic changes associated with induction of trained immunity. Specifically, enhanced glycolysis occurs during the trained immune response. Thus, the goal of this study is to determine if PA enhances glycolysis, like classical stimulants of trained immunity, during enhanced inflammation upon *secondary* stimulation with LPS. We aim to quantify the expression of three key glycolytic genes within mouse macrophages with PA-induced trained immunity: 1) *glut1*, which allows glucose to enter the cell, and 2) *hk2* and 3) *pfkp*, which encode for two rate-limiting enzymes in glycolysis. We hypothesized that PA induces glycolysis during trained immunity in macrophages. We found that after 24h of treatment with PA followed by a secondary heterologous stimulus, PA induced expression of *glut1,* but not *hk2* or *pfkp*. Additionally, if we treated macrophages with PA for 24h, rested it for 24h, and then added a secondary stimulus, there was no longer a

significant increase in expression of *hk2*. Our results indicate that PA may enhance glycolysis during the trained immune response, however if the macrophage is rested for 24h after exposure to PA, it no longer induces a glycolytic response. These data highlight the dynamic glycolytic response of the macrophage during PA-dependent trained immunity. The capacity for PA to directly impact innate immune metabolism associated with inflammatory pathways may inform dietary interventions and treatments for patients with acute or chronic inflammatory diseases.

B. BACKGROUND:

The vertebrate immune system has traditionally been divided into innate and adaptive arms. Innate immunity is the branch of the immune system that responds rapidly to infection and is activated within minutes of detecting non-self or infections. The initial innate immune response is required to initiate the adaptive immune response, which requires several days to mount an effective humoral (antibody driven) and cellular response (T-cell driven) (Netea. *et al*., 2011). Each of these branches of the immune system have specific cell types to carry out immunological functions required for responding to and clearing and infectious agent.

Monocytes are innate immune cells that originate in the bone marrow and migrate throughout the body to sites of infection, where they differentiate into tissue-resident macrophages. Macrophages orchestrate inflammation in response to infection, and help clear infection by immediately responding to, engulfing, and destroying pathogens Arango (Duque*, et al.,* 2014, Martin, *et al.,* 2014). Macrophages express pattern recognition receptors (PRRs) on their cell membrane and within the cell that allow them to recognize specific pathogen-associated molecular patterns (PAMPs) found on many pathogens. One example of a PAMP is the molecule lipopolysaccharide (LPS), which is found on the outer membrane of many Gram-negative bacterial species (Matsuura, 2013). The recognition of LPS by PRRs on macrophages leads to inflammation and is considered a non-specific feature of innate immunity, because many different species of Gram-negative bacteria can be recognized this way (Netea, *el al.,* 2020). When a macrophage detects LPS, it rapidly induces an inflammatory response, which is mediated by the

production and secretion of signaling molecules called cytokines. Cytokines are synthesized by the macrophage and work in an autocrine, paracrine, and endocrine manner to alert other innate and adaptive immune cells of an invasion by a pathogen (Arango Duque and Descoteaux, 2014; Murphy, Weaver, Janeway, 2017, Janeway's Immunobiology).

In contrast, adaptive immune cells identify specific proteins called antigens that are unique to only one species of pathogen and use antigens to develop antibodies and cellular immunity that is built to recognize and neutralize one specific pathogen. Additionally, adaptive immunity can mount a memory response to this one specific pathogen (Netea, *et al*., 2011); and, it has been thought that only adaptive immune cells have the ability to remember pathogens.

Historically, it was believed that innate immune cells did not retain memory of pathogens following infection; however, it has been recently appreciated that innate immunity has nonspecific memory that is regulated by different mechanisms than adaptive immune memory (Netea, et al., 2011). In 2012, Netea's group found macrophages are capable of organizing a memory response called trained immunity (Netea, *et al*., 2013), and it is mediated by distinct metabolic and epigenetic alterations that have not been fully characterized (Netea, *et al*., 2020).

Trained Immunity: Trained immunity is an adaptation of the innate host defense system that allows macrophages to develop non-specific, and long-lived memory to a group of pathogens (Divangahi & Netea, *et al*., 2020). This memory phenomenon has been shown to occur in both *in vitro* (cell culture) and *in vivo* model systems of mice, as well as humans in clinical settings (Netea, *et al*., 2011). Specifically, β-glucans have been demonstrated to induce enhanced inflammatory signaling capacity within human monocytes and macrophages via metabolic and epigenetic alterations (Dominguez-Andres, et al., 2019). Similarly, the Bacillus Calmette–Guérin (BCG) vaccine, the vaccine against *Mycobacterium tuberculosis* infection, is known to enhance monocyte and macrophage responses to secondary infection with unrelated pathogens in human patients (Kleinnijenhuis, 2011). The impacts of BCG- and β-glucan-induced trained immunity within human monocytes and macrophages involve a well-known metabolic feature called

the Warburg Effect, whereby enhanced aerobic glycolysis allows for the activated cell to meet the energy demands necessary for enhanced inflammatory cytokine production and secretion in response to infection (Warburg, 1923; EM Palsson-Mcdermott, O'Neill *et al*., 2015, Cell Metabolism; Cheng, *et al*., 2014, Science).

Interestingly, trained immunity has also been shown to be a feature of macrophages that have been exposed to non-microbial stimuli or "sterile" triggers. For example, atherosclerotic mice that have been fed a Western Diet (WD), a diet enriched in saturated fats and sugar, exhibit a long-lasting reprogramming of innate immune cells with enhanced inflammatory capacity (Christ *et al*., 2019). Human macrophages have also been shown to exhibit a long-lasting enhanced inflammatory response to LPS following brief exposure to oxidized low-density lipoprotein, (oxLDL is a molecule leads to epigenetic reprogramming of monocytes, eventually causing long-term elevated pro-inflammatory cytokine production), to induce trained immunity (Bekkering *et al*., 2014). Recently, the mechanisms of trained immunity induced by sterile triggers have been shown to involve metabolic alterations that remodel the epigenetic landscape of human and mouse monocytes and macrophages (Bekkering, et al., 2018, Cell; Dominguez-Andres *et al*., 2019; Saeed*, et al*., 2014). While these studies have identified key metabolic pathways, including glycolysis, that play important roles in trained immunity, no one has shown the impact of dietary constituents on these pathways in macrophages. Specifically, saturated fatty acids (SFAs) are known to have immunomodulatory impacts on innate immune cells and alter inflammatory responses (Napier, *et al.,* 2019). However, the mechanisms underlying the capacity of SFA to modulate metabolism within macrophages undergoing the trained immunity response have not been explored.

<u>Palmitic Acid and Trained Immunity</u>: Palmitic acid (PA) is a long-chain saturated fatty acid that is found in diets enriched in animal fats including meat, milk, and butter, (Carta, *et al*., 2017). Many groups have shown that PA can induce inflammation in macrophages and monocytes, (Napier *et al*., 2019, Korbecki, *et al*. 2019, Laine, *et al*., 2007, Tzeng*, et al.,* 2019, Schwartz*, et al*., 2010).

Our group has collected preliminary data that demonstrates macrophages treated with a physiologically relevant concentration of PA (1mM) for 24h, and then stimulated

with a secondary heterologous inflammatory stimulus (LPS 10 ng/mL) show a significantly higher production of key inflammatory cytokines (TNF, IL-6, and IL-1β) (Fig 1A-C), compared to macrophages treated with LPS alone. These are the first data that suggest that PA can induce trained immunity in macrophages, and currently we are actively understanding the mechanisms behind this immune regulation.

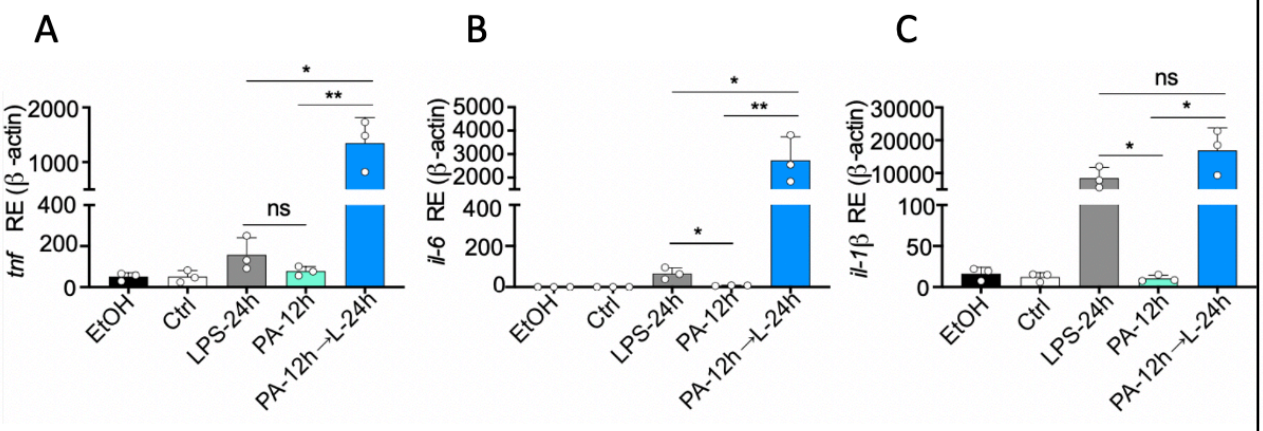

Figure 1. Palmitic acid induces a hyper-inflammatory response to secondary challenge with LPS in macrophages. Primary bone marrow-derived macrophages (BMDMs) were isolated from aged-matched females and males. **A-C** BMDMs were plated at $1x10^6$ cells/mL and treated with either ethanol (EtOH; media with 1.69% ethanol), media (LPS Ctrl; media only), or LPS (100 ng/mL) for 12 or 24h. Next, BMDMs were treated with either EtOH, media, LPS (10 ng/mL) for 24 h, PA (500 μM; diluted in 0.8% EtOH) for 12h with or without secondary challenges with LPS. After indicated time points, RNA was isolated and expression of **a** *TNF*, **b** *il-6*, **c** il-*1β* was measured via qRT-PCR for all plates, all treatments were performed in triplicate. For all panels, a student's *t*-test was used for statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p< 0.001$. For all panels, error bars show the mean ± SD. (Seufert*, et al.* in preparation).

C. <u>AIM</u>:

The goal of this study is to determine if PA enhances glycolysis during the PA-induced trained immunity response and if so, does the glycolysis persist long-term. Specifically, we aim to **1)** quantify the expression of three key glycolytic genes within mouse macrophages treated with PA, followed by LPS, and **2)** quantify the expression of three key glycolytic genes within mouse macrophages treated with PA, then rested for 24h, followed by LPS. **We hypothesize that PA induces glycolysis in macrophages during PA-induced trained immunity.**

D. APPROACH:

Summary: We used an *in vitro* model of mouse macrophages to simulate PA-induced trained immunity using PA as the first inflammatory stimulus and lipopolysaccharide (LPS) as the secondary, heterologous inflammatory stimulus. Mouse bone marrow-derived macrophages (BMDMs) were extracted from the femurs of mice, cultured, and frozen back, as described previously (Seufert *et al*., 2021, in preparation). Next, we 1) treated BMDMs with PA for 24 hours (h) and treated with LPS for 24 h or we 2) treated BMDMs with PA for 24 hours (h), rested for 1 day in fresh media and treated with LPS for 24h. After treating the cells, we isolated the mRNA from the cell lysates and determined the expression of genes that code for glucose transport and glycolytic enzymes (Schematic of workflow: Fig 2).

Extraction and treating of BMDM: Mouse bone marrow-derived macrophages (BMDMs) were isolated from aged-matched female and male wild-type C57BL/6 mice. Mice were euthanized and femurs were dissected and centrifuged to collect bone marrow. Bone marrow was resuspended in DMEM supplemented with M-CSF, and BMDMs were cultured for 6 days prior to storage at -80℃ with 90% FBS and 10% DMSO. Next, BMDMs were thawed and cultured for 5 days, and then collected, pelleted, washed, and resuspended in media (DMEM) containing 10% FBS, 2% bovine serum albumin (BSA; ProLiant Biologicals), and 10% M-CSF. In order to initiate PA-induced trained immunity, we first plated cells at 2.5 x 10^5 cells/well in 24-well tissue culture plates and treated them with either ethanol (EtOH; media with 1.69% ethanol), media (Naive Ctrl; media only), or palmitic acid (1mM; diluted in 1.69% EtOH, Sigma-Aldrich) for 24 h. This concentration of PA was used because it reflects the concentration of PA found in the blood of obese humans (Korbecki, *et al*., 2019). After the 24h PA treatment, all media was removed from the wells, and the cells rested for 1 day in fresh media to explore for memory, after one day if the cells epigenetically modify and respond to inflammation by enhancing the trained immunity.

Next, we either immediately added LPS as the secondary, heterologous stimulus to induce hyper-inflammation in macrophages or, rested the cells for 24 h and then treated

with LPS for 24 h. Specifically, cells were treated with 10 ng/mL LPS (Ultrapure LPS, *E. coli* 0111: B4, Invivogen) and incubated at 37° for an additional 24 h. After indicated time points, mRNA was isolated and expression of glycolysis genes (*glut1*, *hk2,* and p*fkp*) were measured via qRT-PCR, as discussed below.

RNA extraction and real-time qRT-PCR: To assess the expression of glycolytic genes, we first isolated mRNA from treated BMDMs. First, we lysed the BMDMs with trizol in the fume hood then we flash froze in the liquid nitrogen for 20 seconds and stored them within -80 ℃. Next, we continued mRNA extractions using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Then, we created cDNA from mRNA samples using the SuperScript III First-Strand synthesis system (Invitrogen).

Next, we used previously published gene-specific primers that target glycolytic genes of interest (Liu *et al*., 2020, Cell Host & Microbes). [glut1 Forward: TCAACACGGCCTTCACTG; glut1 Reverse: CACGATGCTCAGATAGGACATC; hk2 Forward: TGATCGCCTGCTTATTCACGG; hk2 Reverse: AACCGCCTAGAAATCTCCAGA; pfkp Forward: CGCCTATCCGAAGTACCTGGA; pfkp Reverse: CCCCGTGTAGATTCCCATGC] Using these gene-specific primers we amplified transcripts using FastStart Universal SYBR Green Master (Bio-Rad). Importantly, we had 4 types of treatments in this experiment: 1) naive (+/- rest), 2) naive + LPS, 3) PA (+/-) rest and 4) LPS and PA ctrl, and we calculated relative expression of glycolytic genes to the housekeeping gene, [β-actin Forward: CTGTCCCTGTATGCCTCTG; β-actin Reverse: ATGTCACGCACGATTTCC]. Additionally, each experiment described within this section was repeated in biological and experimental triplicate.

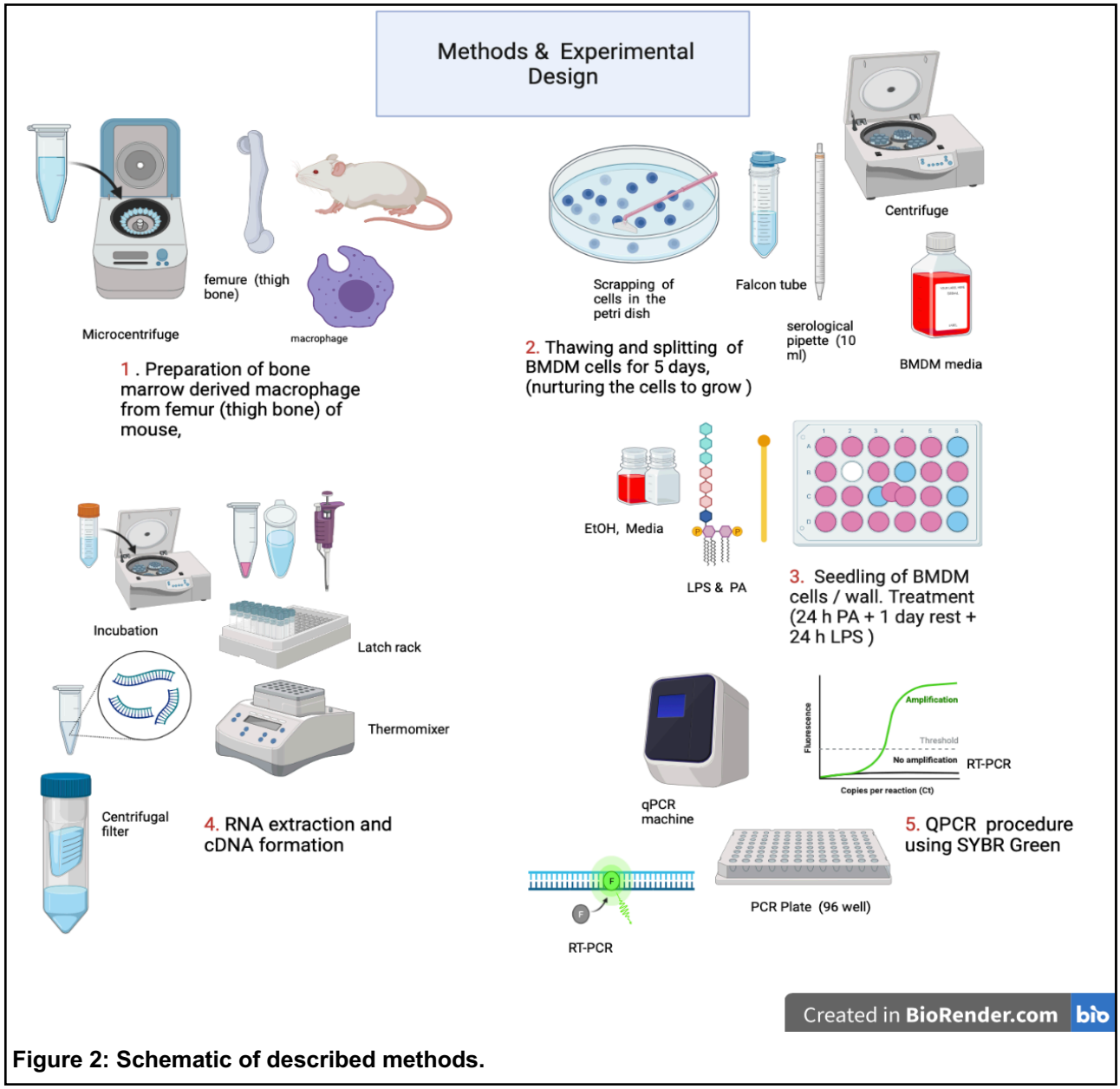

Figure 2: Schematic of described methods.

E. RESULTS:

Palmitic acid (PA) upregulates glycolysis in macrophages: To understand if PA is inducing glycolysis during trained immunity, we first induced trained immunity and then measured the expression of key glycolytic enzymes. The upregulation of key glycolytic enzymes can inform us if PA can induce glycolysis during trained immunity.

Thus, we first treated primary bone marrow-derived macrophages (BMDMs) with without 1mM of PA for 24h, then subsequently treated with LPS (10 ng/mL) for an additional 24 h. After these two treatments, we isolated mRNA from the BMDMs and measured expression of key glycolytic enzymes *hk2, glut1,* and *pfkp* using qualitative Real-Time PCR. We found that all 3 glycolytic enzymes were expressed to significantly higher levels in LPS treated macrophages, as previously published (Fig 3 A-C), (Tannahill, *et al*., 2013). Interestingly, we found that *glut1* was significantly up-regulated in PA-LPS treated macrophages (Fig 3B), suggesting that the expression of *glut1* is up-regulated during the PA-dependent trained immunity response. However, both *hk2* and *pfkp* were not found to be significantly up-regulated in the PA-LPS treated BMDMs (Fig 3 A, C). These data suggest there is a bifurcation in regulation of these 3 glycolytic enzymes during PA-induced trained immunity, and that *glut1* is positively regulated by the PA-dependent trained immune response.

Next, we were interested to understand the persistence of this PA-dependent increase in glycolysis during trained immunity. Thus, we treated BMDMs and without 1mM of PA for 24h, rested for 24h with media only then subsequently treated with LPS (10 ng/mL) for an additional 24 h. We found that BMDM treated with 1 mM of PA followed by 24h rest did not show significantly higher expression of any of the 3 glycolytic enzymes (Fig 3 D-F). These data suggest that PA induces the expression of some glycolytic enzymes during trained immunity, but if the macrophages are rested for 24h after the PA treatment, this allows the macrophage to recover to homeostatic expression of glycolytic enzymes. These data highlight the plasticity of the PA-dependent glycolytic influx during the trained immune response in macrophages.

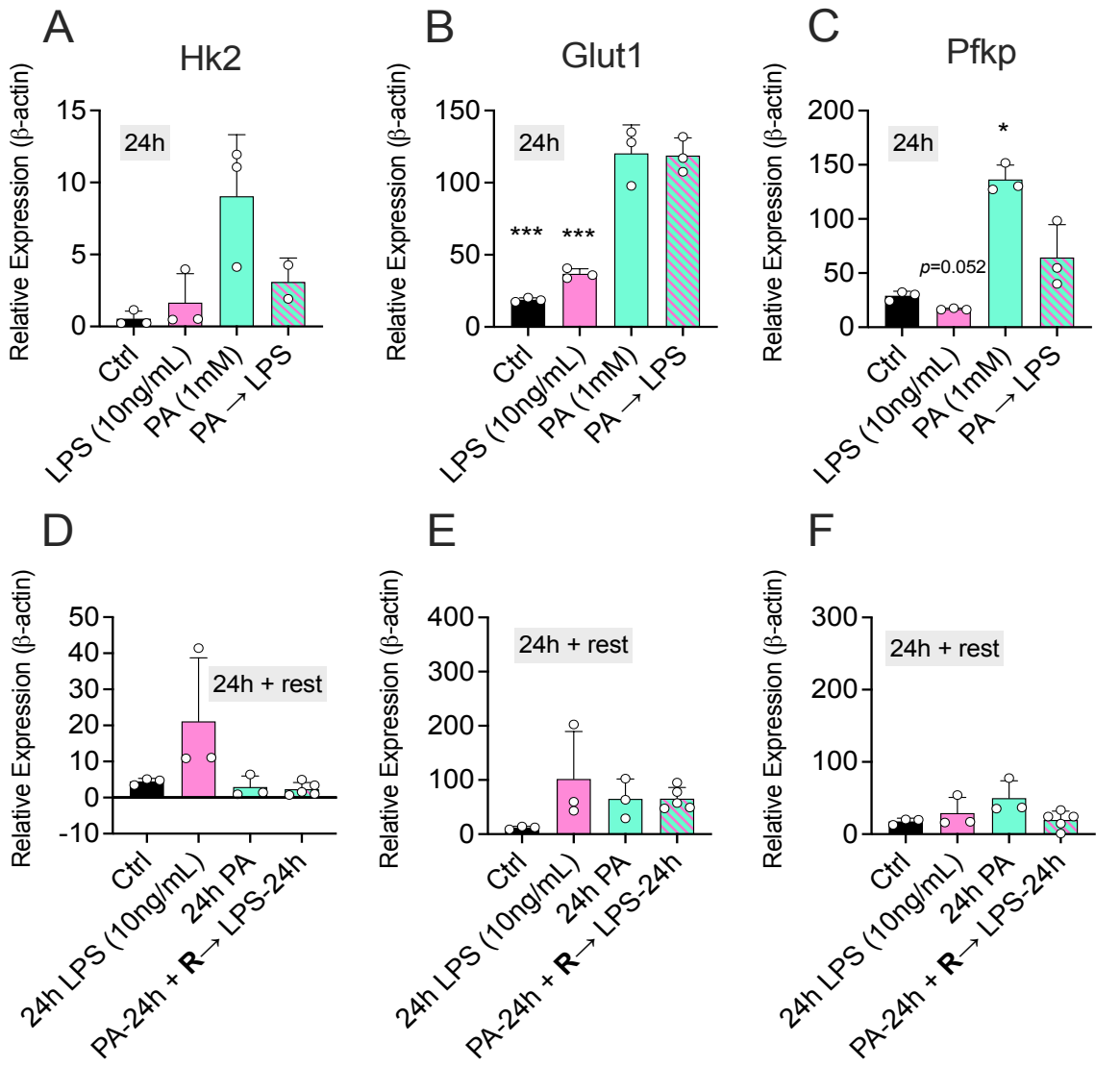

Figure 3: Palmitic acid induces expression of *hk2* and *glut1*, but not *pfkp*, during trained immunity. Primary bone marrow-derived macrophages (BMDMs) were isolated from aged-matched females and males C57BL/6 mice. **A-f** BMDMs were plated at 1x10^6 cells/mL and treated with either ethanol (EtOH; media with 1.69% ethanol), media (LPS Ctrl; media only), or LPS (10 ng/mL) for 24h or palmitic acid (1mM; diluted in 1.69% EtOH) for 24 h, with and without a secondary challenge with LPS. After indicated time points, RNA was isolated and expression of **a** *hk2*, **b** *glut1*, **c** pfkp was measured via qRT-PCR. Next, BMDMs were treated with either EtOH, media, or LPS (10 ng/mL) for 24 h, rested 24 h or PA (1mM; diluted in 1.69% EtOH) for 24 h, with and without a secondary challenge with LPS. After indicated time points, RNA was isolated and expression of **d** *hk2,* **e** *glut1*, **f** *pfkp* was measured. For all plates, all treatments were performed in triplicate. For all panels, a student's *t*-test was used for statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For all panels, error bars show the mean ± SD.).

F. DISCUSSION:

We have found that PA may induce glycolysis by enhancing the expression of glycolytic gene: *glut1* (Fig 3). However, the treatment of bone marrow-derived macrophages with 24h PA, 24h rested (Fig 3. D-F) and exposed to a secondary inflammatory stimulus (LPS), no longer upregulated *glut1* expression (Fig 3). This suggests that resting of macrophages with only media for additional 24h can potentially impact on expression of glycolytic genes in glycolysis metabolisms. Overall, these data indicate that PA treatment of macrophages not only increases cytokine expression (Fig 1) after exposure to LPS, but that it also confers an increase expression of some glycolytic enzymes. Our data support previously reported findings that high-fat diets containing PA may play an important role in the metabolic regulation of inflammatory diseases such as sepsis (Palsson-McDermott, *at al.,* 2011, Napier *et al*., 2019). Specifically, the enhancement of LPS-induced glycolysis by PA occurs in conjunction with expression of pro-inflammatory cytokines (Fig 2-3).

Although trained immunity provides protection against a secondary non-specific infection, innate immune cell memory can also impair immune function, as observed in septic immune paralysis (Netea*, el al.,* 2020) or persistent inflammatory activation evident in atherosclerosis (Netea, *et al.,* 2020). These data suggest that trained immunity is a double-edged sword, where it can be beneficial for resistance to infection, but detrimental in the context of inflammatory diseases (A.R. DiNardo, *et al*., 2021). Thus, PA-induced trained immunity may aggravate tissue damage during inflammatory disease and may contribute to obesity-induced metabolic disorder (Charles-Messance, *et al.,* 2020). Our future studies will focus on identifying additional metabolic pathways and cellular mechanisms that are targeted by PA-treatment that lead to enhanced glycolysis and inflammation in the trained immunity response. These data will shed light on the potentially beneficial and dangerous effects of PA-induced trained immunity by PA-regulated metabolic alterations.

In conclusion, further data is essential in understanding the metabolic regulation of macrophages during PA-induced trained immunity which will be important for elucidating the mechanism of cellular regulation and hyper-inflammation in innate immune

cells during inflammation. Palmitic acid is not only up-regulating trained immunity inflammation in macrophages and enhancing severity of inflammatory disease such as sepsis, but it can also impact on metabolism and leads to metabolic disorders in patients.

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