UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigating the role of GIV/Girdin in the circadian rhythm macrophages

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by

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The thesis of Yashaswat Singh Malhotra is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Elucidating the role of GIV/Girdin in the rhythmic functions of macrophages

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Macrophages, a.k.a ‘big eaters’ are key cells in our immune system. These effector cells of the innate immune system phagocytose bacteria and secrete both pro-inflammatory and antimicrobial mediators. In addition, macrophages also play a pivotal role in eliminating diseased and damaged cells. Macrophages have a strong intrinsic circadian rhythm that regulates the diurnal rhythmicity of their immune responses, including immunosurveillance, infiltration to sites of injury, pathogen recognition, pathogen clearance, and the timely resolution of inflammation. A healthy

macrophage circadian rhythm is crucial for appropriate defensive responses and is completely lost during the immunosenescence chapter of life one experiences on aging. Here we have stumbled upon a novel regulator of the macrophage circadian rhythm, GIV/Girdin, a multi-modular G protein activator and a potent inhibitor of cAMP. Using publicly available datasets, we show that GIV expression undergoes rhythmic cycling that is lost in aged mice. GIV depleted Raw 264.7 macrophages show significant variation in the expression of their peripheral clock genes. We also demonstrated that peritoneal macrophages display significant diurnal variation in their expression of Cry1 and Klf-4, two genes crucial for a healthy macrophage circadian rhythm and time-of-day dependent immune responses. In-vivo studies revealed that the loss of GIV significantly alters the circadian variation in phagocytic activity against *E.coli* as well as the infiltration of inflammatory macrophages to sites of peritoneal injury. In this thesis, we seek to dissect the extensive role that GIV might play in modulating the circadian rhythm of macrophages and how we might manipulate this new player pharmacologically to improve macrophage immune responses against pathogens.

I. INTRODUCTION

The circadian clock is a timekeeping system that allows organisms to adapt their physiological and behavioral rhythms to anticipate changes in their environment, (Allada & Chung, 2010). Precisely maintained, the circadian rhythm regulates a multitude of overt and covert responses, including activity, roaming behavior, homeostasis, metabolic changes, tissue recovery and many more (Bae et al., 2019; Serin & Acar Tek, 2019). Over the past two decades, a mountain of evidence for circadian oscillations of components of the immune system has emerged, highlighting the role of the circadian rhythm in disease onset and pathogenesis (Arjona et al., 2012; Lange et al., 2010; Scheiermann et al., 2013). The diurnal rhythmicity of immune responses includes leukocyte trafficking, immunosurveillance, pathogen recognition, phagocytic capacity, and defensive responses (Blacher et al., 2022; Dimitrov et al., 2007; Fonken et al., 2015; W. He et al., 2018; Nguyen et al., 2013). Specifically, macrophages display strong circadian behaviors, with approximately 8% of their transcriptome being under circadian regulation (Keller et al., 2009). This temporal regulation of immune responses leads to strictly gated time of day appropriate immune-active and immune-tolerant phases.

In mammals, including mice and humans, the circadian rhythm and its downstream response elements are centrally entrained in the master pacemaker of the suprachiasmatic nucleus (SCN) by daily variations in light. The SCN in turn provides principal cues for synchronizing endogenous clocks in peripheral tissues (Cermakian & Sassone-Corsi, 2000; Lowrey & Takahashi, 2000; Moore MD, 1997;

Panda et al., 2002). The circadian rhythm of peripheral immune cells, including macrophages is regulated at a molecular level by transcriptional - translational feedback loops that comprise positive and negative regulatory networks (Shirato & Sato, 2022). The positive arm comprises the brain and muscle ARNT-like 1 (Bmal1) and circadian transcription factor circadian locomotor output cycles kaput (Clock). Bmal1 and Clock heterodimerize and bind to E-box sites on DNA, resulting in the transcription of clock-controlled genes (CCG). These CCGs include the negative regulators of the circadian molecular clock, period (Per1-3) and cryptochrome (Cry1-2). Upon accumulation of Per and Cry proteins, they translocate into the nucleus to disrupt Bmal1 : Clock heterodimerization, thereby inhibiting their own production. A second feedback loop is also involved in the regulation of the molecular clock, with BMAL1 - CLOCK heterodimerization transactivating the nuclear receptor Reverb that represses transcription of Clock and Bmal1 (Langmesser et al., 2008; Timmons et al., 2020).

In mice the immune tolerant phase is from ZT0- ZT12 (Zeitgeber time, where ZT0 represents the time where the lights are turned on and ZT12 represents the time when lights are turned off) and is associated with tissue recovery and a dampened immune response. On the other hand, the immune active phase is from ZT12 to ZT24 and is associated with heightened inflammatory and defensive responses to pathogens (Downton et al., 2020; Lind & Thusgaard, 1985).

(a) (b)

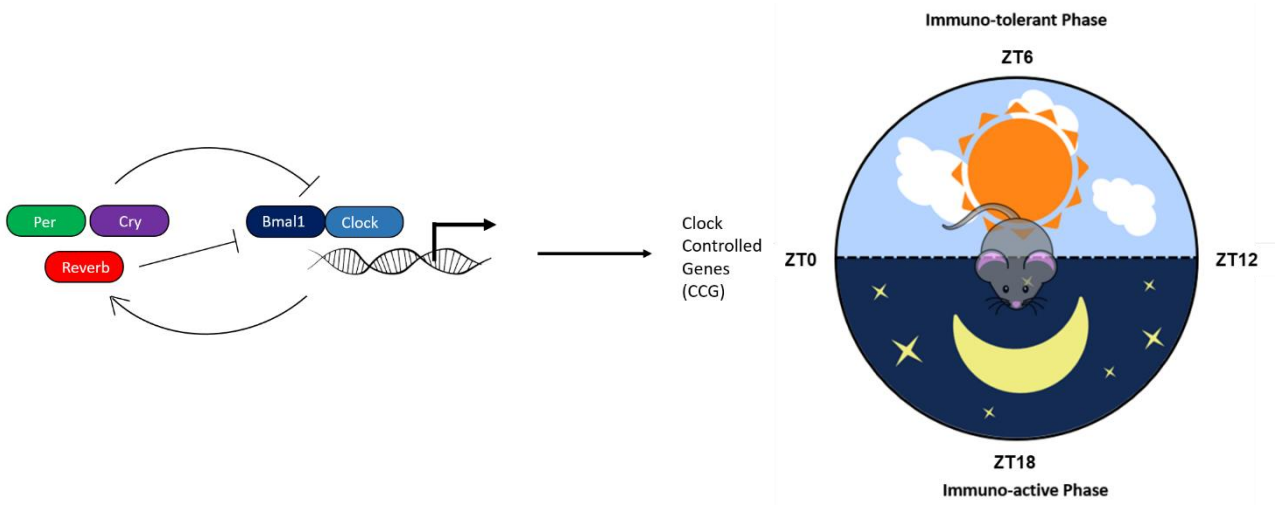


Figure 1: Peripheral clock genes and immune phases in mice. Graphical representation of the transcription-translation feedback loop of the peripheral clock (1a) and time-of-day dependent immune phases seen in mice.

While knock out studies have demonstrated that various peripheral clock genes can directly control aspects of innate immunity (Nguyen et al., 2013; (Huo et al., 2017; Wang et al., 2020; Yang et al., 2015), recent evidence has highlighted that the loss of circadian gene regulation in immune cells and effector functions of macrophages can occur even when the rhythmic expression of peripheral clock genes remain intact, as is seen during aging (Blacher et al., 2022; Mattis & Sehgal, 2016). This suggests the presence of alternative or additional mechanisms leading to a loss of circadian regulation of immune cell functions and poorly differentiated immune environments. Here we show the unexpected role of GIV, a member of the non-receptor Guanine nucleotide Exchange Modulator (GEM) family of proteins, in maintaining the circadian rhythm and time dependent immune response of

macrophages. GIV is a member of the G proteins superfamily, who along with their activators, constitute a major signaling hub in eukaryotes and remain the core targets of modern medicines (~40% of the market) (Ghosh & Mullick, 2021); although many of these drugs are used to tackle the health consequences of a disrupted circadian rhythm, including cardiovascular diseases, inflammatory bowel disease, mood disorders and reproductive problems, none of them aim to reinstate the circadian rhythmicity of immune cells. Our studies are expected to demonstrate a novel and fundamental role of a new cell signaling pathway in our immune cells that regulates macrophage circadian function and have the potential to usher a new therapeutic target to maintain the same.

G-protein signaling via GIV/ GIV: A non-canonical G-protein signaling hub

The guanine nucleotide-binding (G) protein α subunit (Gα)- interacting vesicle-associated protein (GIV, also known as “Girdin”) is a cytoplasmic guanine nucleotide exchange modulator (GEM) (Ghosh & Mullick, 2021). GEMs possess the intrinsic ability to interact with and bi-functionally regulate G-proteins, serving as an activating guanine nucleotide exchange factor (GEF) for Gαi using the same motif that allows them to function as a guanine nucleotide dissociation inhibitor (GDI) for Gαs. In doing so, GEMs, such as GIV possess the unique capability of coupling G-protein signaling downstream of a diverse class of receptors, such as receptor tyrosine kinases (RTKs), Integrins, Frizzled receptors (FZDRS), etc (Gupta et al., 2016). Previously, our group has demonstrated that GIV is a potent inhibitor of cAMP (Getz et al., 2019)

and has found GIV to be implicated in the progression of a diverse set of diseases, including atherosclerosis, NASH, liver fibrosis, colitis, cancer, and infertility (Ghosh et al., n.d.; Lopez-Sanchez et al., 2014; Malhotra et al., n.d.; Reynoso et al., 2021; Sahoo et al., 2021).

More recently, our group has also uncovered that GIV is a crucial regulator of immune cell signaling and inflammatory processes. GIV is highly expressed in immune cells, especially macrophages, where it can differentially interact with distinct pathogen recognition receptors (PRRs). Specifically, GIV binds to TLR-4 to suppress LPS induced proinflammatory signaling pathways (Swanson et al., 2020). GIV also couples with cytoplasmic NOD2 where it is required for bacterial sensing and protective host responses (Katkar et al., 2022). Given these findings, we wanted to investigate if GIV may modulate the diverse spectrum of circadian rhythm based immune responses in macrophages.

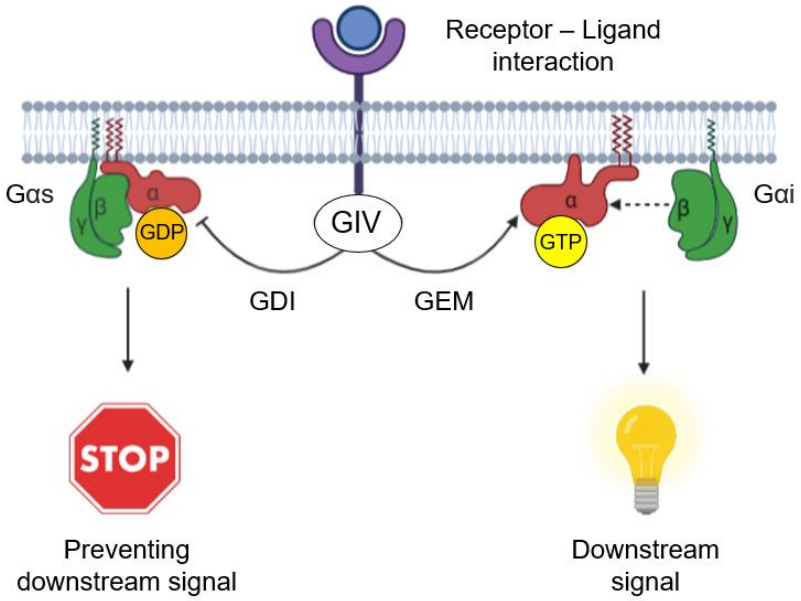


Figure 2: Signaling via GIV. Graphical representation of signalling via GIV. GEMs, including GIV, serve as bifunctional modulators of G-proteins; GIVs can function as GEFs, activating signaling via Gαi and function as GDIs, preventing activation of signalling via Gαs.

II. MATERIALS AND METHODS

Generation of conditional GIV KO mouse lines

Girdin floxed mice were a generous gift from Dr. Masahide Takahashi (Nagoya University, Japan). LysMcre mice (B6.129P2-Lyz$^{2tm1(cre)Ifo}$/j) were purchased from The Jackson Laboratory. Girdin floxed x LysMcre mice were generated by members of the Pradipta Ghosh laboratory before the beginning of this independent thesis and were maintained as homozygous floxed (fl/fl) and heterozygous LysMcre (Swanson et al., 2020). All mice studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee (IACUC). Both male and female mice (8-12 weeks) were used and maintained in an institutional animal care at the University of California San Diego animal facility on a 12-hour/12-hour light/dark cycle (humidity 30–70% and room temperature controlled between 68–75 °F) with free access to normal chow food and water.

Generation of stable cell lines.

The RAW 264.7 cell line was obtained from and cultured according to American Type Culture Collection (ATCC) guidelines. ShRNA control (ShC) and shRNA GIV (ShGIV) RAW 264.7 stable cells lines were generated by lentiviral transduction followed by selection with puromycin by members of the Pradipta Ghosh laboratory before the beginning of this independent thesis (Swanson et al., 2020). RAW264.7 macrophages were maintained in the DMEM media containing 10% FBS and 1% penicillin/streptomycin.

Macrophage Isolation

Thioglycolate-elicited murine peritoneal macrophages (TGPMs) were collected from peritoneal lavage of 8- to 12-wk-old WT and GIV-KO mice with ice cold RPMI + 2mM EDTA(10ml per mouse) 4 days after intraperitoneal injection of 3 ml of aged, sterile 3% thioglycolate broth, at appropriate times in accordance with the experiment. Cells were passed through 70 µm filter to remove possible tissue debris contamination during harvesting. Cells were counted, centrifuged, and resuspended in RPMI-1640 containing 10 % FBS and 1% penicillin/streptomycin. The appropriate concentration of cells, based on the specific experiment, were seeded and their viability was assessed using a confocal microscope.

Serum Shock

Serum shock treatment was performed on RAW 264.7 macrophages to reset their circadian rhythm (Heipertz et al., 2018; Oliva-Ramírez et al., 2014). ShC and ShGIV Raw 264.7 macrophages were starved overnight DMEM media with 1%FBS and plated on a 6cc dish at a concentration of 10^6 cells/ ml. The next day, RAW 264.7 macrophages were treated with DMEM media with 50% FBS (Serum Shock). After 2h, the media was aspirated and replaced with DMEM media with 10% FBS. Cells were collected in trizol at appropriate times for RNA isolation.

RNA isolation, cDNA synthesis and qPCR

Serum shocked ShC and ShGIV Raw 264.7 macrophages and Peritoneal macrophages isolated from WT and GIV-KO mice were pelted in 1.7ml Eppendorf

tubes. The cells were resuspended in cold PBS to remove excess FBS and centrifuged again at 1200rpm for 3 minutes. The supernatant was aspirated, and the cells were resuspended thoroughly in Trizol reagent. The sample is ready for RNA isolation. All RNA was isolated using Direct-zol RNA Miniprep Kit using the manufacturer's protocol from samples collected in Trizol reagent. RNA concentration and purity were quantified using a Nanodrop Microvolume Spectrophotometer. 500ng of RNA was used to synthesize cDNA for RT-PCR using qScript cDNA SuperMix kit in accordance with the manufacturer's protocol. cDNA was diluted 1:3.5 with ddH2O and qPCR was carried out using 2X PowerUp SYBR Green Master Mix (primer sequences listed in the appendix table). The cycle threshold (Ct) of target genes was normalized to 18S housekeeping gene, relative expression of mRNA was calculated using the DDCt method.

qPCR Primer Design

All the primers were designed using the NCBI website and were verified through BLAST. The primers were designed such that the product length was under 160 base pairs and the primer spanned exon junctions to prevent inaccurate amplification results due to genomic contamination.

Primers				
Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Length (BP)	Spanning
Per1	GAAACCTCTGGCTG TTCCTACC	AGGCTGAAGAGGC AGTGTAGGA	133	Exon 15 & Exon 16
Bmal	ACCTCGCAGAATGT CACAGGCA	CTGAACCATCGACT TCGTAGCG	115	Exon 14 & Exon 15
Clock	GGCTGAAAGACGG CGAGAACTT	GTGCTTCCTTGAGA CTCACTGTG	116	Exon 15 & Exon 16
Cry1	GGTTGCCTGTTTCC TGACTCGT	GACAGCCACATCCA ACTTCCAG	123	Exon 7 & Exon 8
Cry2	GGACAAGCACTTGG AACGGAAG	ACAAGTCCCACAGG CGGTAGTA	155	Exon 5 & Exon 6
Reverba	CAGGCTTCCGTGAC CTTTCTCA	TAGGTTGTGCGGCT CAGGAACA	136	Exon 6 & Exon 7
Reverbb	CAGTGAGAAGCTGA ATGCCCTC	TGCACGGATGAGTG TTTCCTGC	139	Exon 6 & Exon 8
Klf4	CTATGCAGGCTGTG GCAAAACC	TTGCGGTAG TGCCTGGTCAGTT	150	Exon 3 & Exon 4

Mcp-1	TGATCCCAATGAGTAGGCTGGAG	ATGTCTGGACCCATTCCTTCTTG	132	Exon 2 & Exon 3

Preparation of FITC labeled *E. coli* bacteria particles.

E. coli bacteria were grown overnight, counted, heat inactivated (heated to 75°C for 15 minutes) and washed once with PBS and centrifuged at 20,000 ×*g* for 2 min. Bacterial pellets were resuspended in 1 ml of 0.5 mg/ml FITC (Sigma-Aldrich, USA) in PBS in the dark at room temperature. After incubation of 30 min, the bacteria were washed three times with PBS to remove non-bound FITC and stored and -80 °C (Taddese et al., 2021).

Phagocytosis Assay

Peritoneal macrophages isolated from WT and GIV-KO mice and serum shocked Raw 264.7 and at different times were plated on 96 well black cell culture microplates (greiner bio-one) at a concentration of 100,000 cells/well for 2 hours. Culture medium was removed, and FITC labeled *E. coli* particles or RPMI media (negative control) was added. After 2 h, the supernatant was removed, and 100 µl of trypan blue was immediately added to each well for 1 min to quench extracellular fluorescence. Excess trypan blue dye was removed, and the plate was read on a Tecan Spark20M microplate reader (Molecular Devices; excitation of 480 nm and emission of 520 nm; bottom reading with 50 flashes per well). Data represented as phagocytosis activity relative to WT.

Monocyte infiltration assay

Mice were intraperitoneally injected with 2 ml of 3% thioglycolate broth, and the peritoneal cavity was flushed with 5 ml of PBS with 0.2mM EDTA 2 hours later. Isolated monocytes were filtered using a 70μm filter and centrifuged for 7 minutes at 3000 rpm. The cells are resuspended in 0.5% BSA at a concentration of 10^7 cells/ml and stained with appropriate antibodies. The cells were placed on ice for thirty minutes to allow for staining. After 30 minutes, the cells were resuspended in 700μl of red blood cell lysis buffer and placed on ice. After 15 minutes, the cells were filtered before flow cytometry was performed.

Flow cytometry

Peritoneal monocytes isolated from WT and GIV-KO macrophages were stained with F4/80 – PE and Ly6C - FITC antibodies (detailed in materials table). Initially, the cells were gated using the F4/80 marker. Two populations of F4/80 cells were found: F4/80 low and F4/80 high monocytes. F4/80 high monocytes were identified as macrophages and Ly6C median fluorescence for these F4/80 high macrophages was determined. The data was analyzed using FlowJo™ Software and represented as Ly6C median fluorescence relative to the WT sample from each cohort.

Key resource table

Reagent or resource	Source	Identifier
Biological Samples & Cell Lines		
RAW 264.7 Macrophages	ATCC	TIB-71
LysMcre mice (B6.129P2-Lyz2tm1(cre)lfo/j)	The Jackson Laboratory	004781
Girdin flox mice	Asai et al. Biochem. Biophys. Res. Commun., 2012	NA
Chemicals and Reagents		
RPMI Media	Cytiva	SH30605.01
DMEM Media	Cytiva	SH30022.01
PowerUp SYBR Green Master Mix	Applied Biosciences	A25741
qScript cDNA SuperMix	QuantaBio	101414

Direct-zol RNA Miniprep Kit	Zymo Research	R1051
TRIzol Reagent	Invitrogen	15596018
Ultra cAMP Kit	Lance	TRF0262
PE anti-mouse F4/80 Antibody	Biolegend	123109
Ly6C antibody	Biolegend	128006
Ly6G Antibody	Biolegend	127614
RBC Lysis buffer	Biolegend	422401
Bovine Serum Albumin	Sigma-Aldrich	A7906-500G
Software		
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
ImageJ	NIH	https://imagej.nih.gov/ij/
Excel	Microsoft	https://www.microsoft.com/en-

		us/microsoft-365/excel
FlowJO	BD Biosciences	https://www.flowjo.com/solutions/flowjo

IV. RESULTS

Myeloid GIV displays circadian rhythmicity that is lost in aged mice

In our preliminary work, to investigate the potential links between GIV and the circadian rhythm of macrophages, we analyzed publicly available datasets on NCBI GEO to understand if the expression of GIV is under rhythmic cycling. From peritoneal macrophages isolated at different times of the day (Blacher et al., 2022) we observed that GIV expression is under strong rhythmic cycling, with expression peaking at ZT12 (Figure 3a). On further investigation, we saw that this rhythmic expression of GIV was completely abolished in peritoneal macrophages isolated from aged mice (Figure 3b).

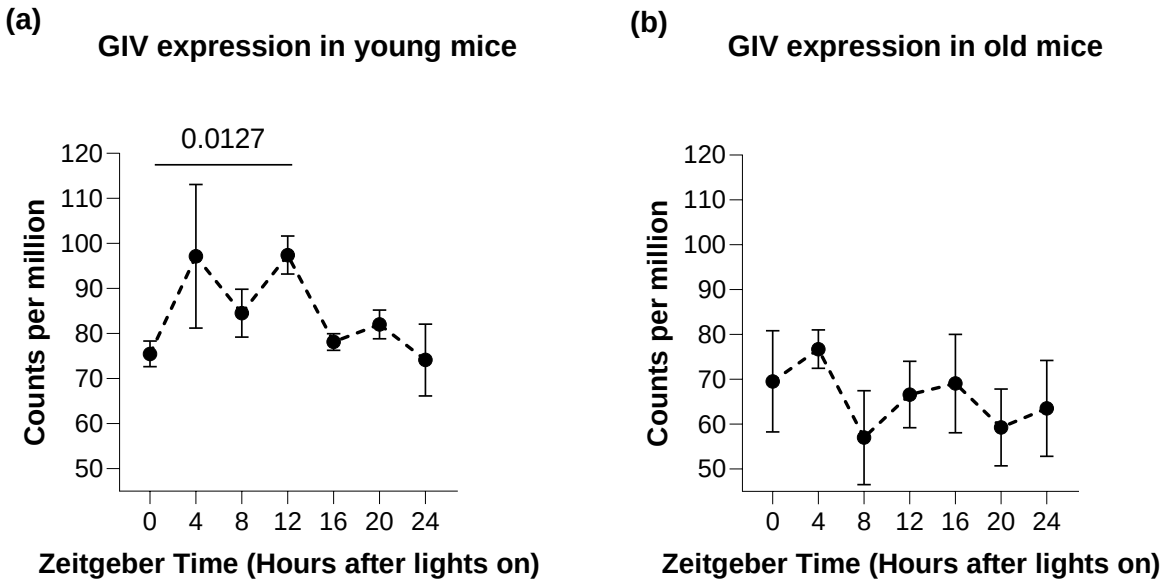


Figure 3: GIV expression displays rhythmic oscillations that are lost in aged mice. GIV mRNA rhythmicity in young (3a) and old (3b) macrophages isolated every 4h from ZT0 to ZT24; P= 0.0127 and P >0.05 for young and aged mice respectively. Data represented as mean ± S.E.M (n=3).

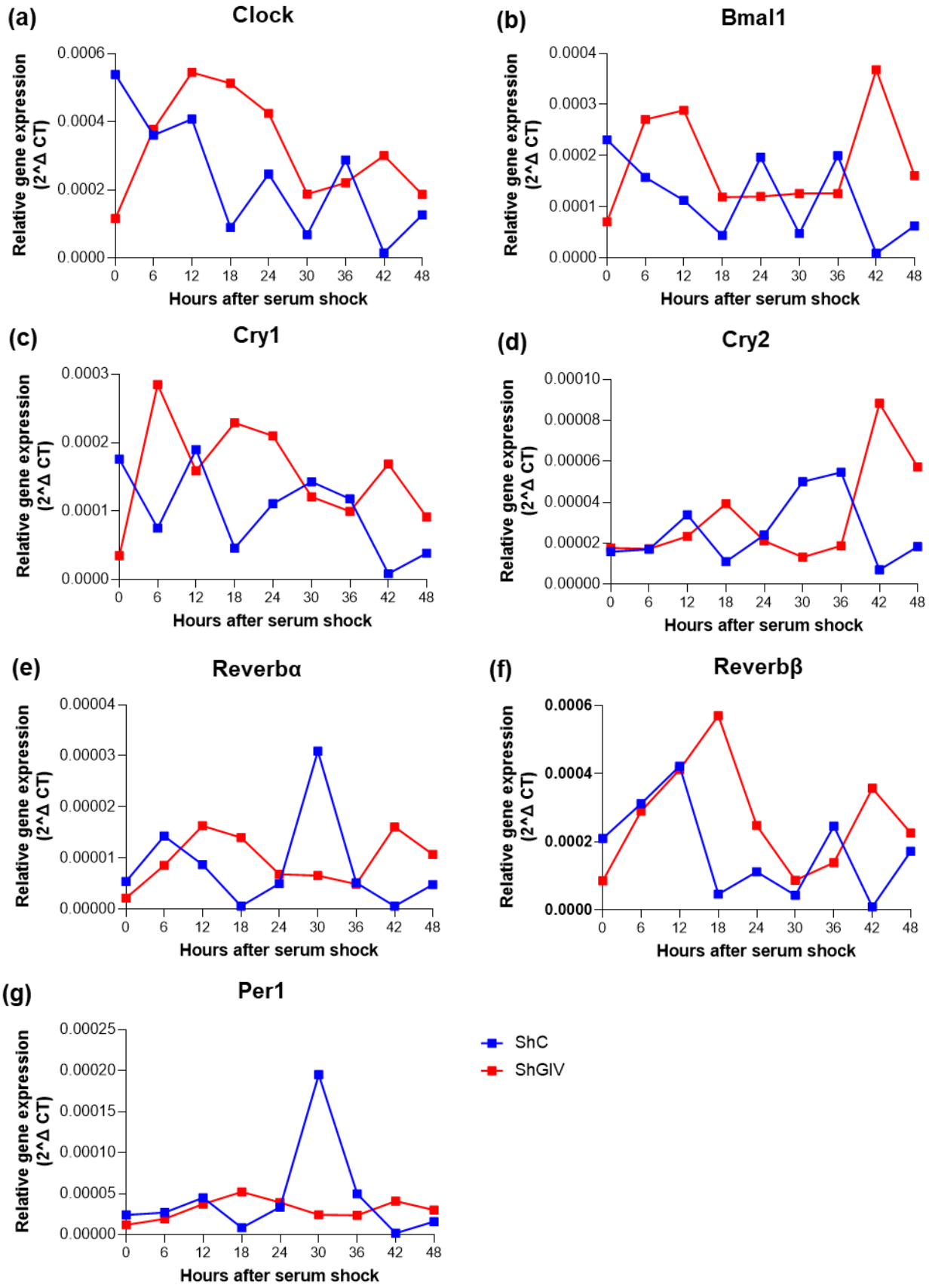
The extensive influence of circadian rhythms on tissue homeostasis, sleep regulation and immune activity has been well established; with direct links to aging. Appropriately timed and high-amplitude circadian rhythms correlate with increased lifespan, whereas aging is associated with decreased circadian rhythmicity and as a consequence, a loss in the ability of the immune system to mount an effective immune response and clear pathogens (Dubrovsky et al., 2010; Froy, 2013; B. He et al., 2016; Hood & Amir, 2017). It is also important to note that the immune-active phase of mice commences at ZT12, and that this time is associated with increased expression of various genes involved in pathogenic clearance and inflammatory responses (Blacher et al., 2022),(Gagnidze et al., 2016; Mattis & Sehgal, 2016; Melillo et al., n.d.). Given that GIV expression also peaks at ZT12, and the rhythmicity of GIV expression is lost in the immunosenescence phase seen during aging, we hypothesized that the loss of GIV may influence the timely expression of various peripheral clock genes and the ability of macrophages to mount a time appropriate immune response.

Loss of GIV disrupts rhythmic expression of peripheral circadian genes in Raw 264.7 macrophages.

To understand if GIV influences the ability of macrophages to establish a proper circadian rhythm, we assessed if the rhythmic expression of core peripheral clock genes were disrupted in the absence of GIV. qPCR analysis was performed on GIV depleted and control Raw 264.7 macrophages over 48 hours at different times

post serum shock, a treatment known to reset the circadian rhythm of macrophages in-vitro (Heipertz et al., 2018; Oliva-Ramírez et al., 2014).

Figure 4:Loss of GIV disrupts peripheral clock gene expression. Peripheral clock genes mRNA rhythmicity in control (ShC) and GIV depleted (ShGIV) Raw 264.7 macrophages. mRNA was isolated every 6h after serum shock. The loss of GIV resulted in dysrhythmic expression of Clock (4a), Bmal1 (4b), Cry1 (4c), Cry2 (4d), Reverbα (4e), Reverβ (4f) and Per1 (4g)


(a)
Clock
Relative gene expression (2^Δ CT)
0.0006
0.0004
0.0002
0.0000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(b)
Bmal1
Relative gene expression (2^Δ CT)
0.0004
0.0003
0.0002
0.0001
0.0000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(c)
Cry1
Relative gene expression (2^Δ CT)
0.0003
0.0002
0.0001
0.0000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(d)
Cry2
Relative gene expression (2^Δ CT)
0.00010
0.00008
0.00006
0.00004
0.00002
0.00000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(e)
Reverbα
Relative gene expression (2^Δ CT)
0.00004
0.00003
0.00002
0.00001
0.00000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(f)
Reverbβ
Relative gene expression (2^Δ CT)
0.0006
0.0004
0.0002
0.0000
0 6 12 18 24 30 36 42 48
Hours after serum shock
(g)
Per1
Relative gene expression (2^Δ CT)
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
0 6 12 18 24 30 36 42 48
Hours after serum shock
ShC
ShGIV

We found that the loss of GIV in Raw 264.7 macrophages completely disrupted the circadian expression of peripheral clock genes over a period of 48 hours post serum shock treatment. The loss of GIV resulted in dysregulated expression of the core clock genes, Bmal1 and Clock, as well as the following genes involved in their negative regulatory networks: Cry1, Cry2, Reverbα, Reverβ and Per1 (Figure 4). This indicated to us that GIV may play a crucial role in establishing a proper circadian rhythm in immune cells such as macrophages and that the loss of GIV may result in chronically altered immune environments that do not follow the conventional pattern of immune-active and immunosuppressive phases in their daily circadian cycles.

Loss of GIV significantly disrupted the diurnal expression of Cry1 in Peritoneal macrophages

To understand how the absence of GIV changed baseline diurnal expression of peripheral clock genes of macrophages in-vivo, we developed a new model in which peritoneal macrophages were isolated from Wild Type (WT) and myeloid specific GIV Knock Out (GIV-KO) mice at different times of the day. The diurnal expression of peripheral circadian genes was assessed using qPCR performed on peritoneal macrophages isolated at ZT0 and ZT12. From our analysis, we found that the loss of GIV significantly disrupted the diurnal rhythmicity of Cry1 in peritoneal macrophages.

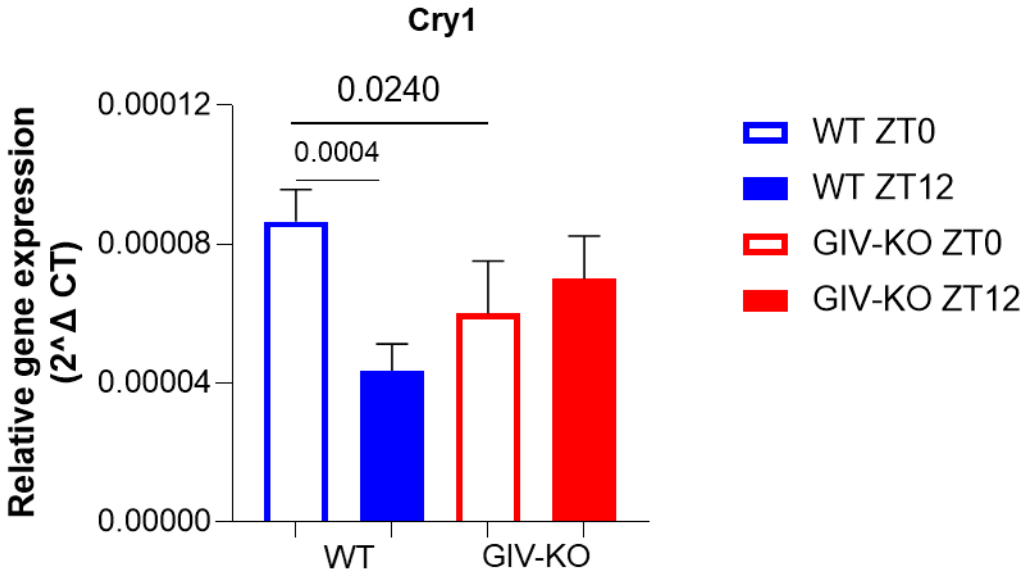


Figure 5: Loss of GIV disrupts Cry1 expression. Diurnal variations in Cry1 mRNA levels in WT and GIV KO peritoneal macrophages isolated at ZT0 and ZT12; P = 0.0004 for WT ZT0 vs ZT12 and P = 0.0240 for WT ZT0 vs GIV-KO ZT0. Data represented as mean ± S.D (n=4).

Consistent with previously published data (Keller et al., 2009), we observed that Cry1 displayed high expression at ZT0, which significantly dropped at ZT12 for macrophages from WT mice. In stark contrast, macrophages from GIV-KO mice did not display diurnal changes in Cry1 levels, with Cry1 being expressed at intermediate levels at both ZT0 and ZT12 in GIV-KO macrophages, highlighting the chronically altered circadian expression of Cry1 (Figure 5). Cry1 expression plays a crucial role in the innate immune function of macrophages. Loss of Cry1 creates a hyper-immune environment, resulting in the increased production of pro-inflammatory cytokines via the constant activation of the NFkB signaling pathway (Narasimamurthy et al., 2012). In general, NFκB signaling is activated by numerous discrete stimuli, such as ligands of pathogen recognition receptors (PRR) and cytokines. NFkB the master regulator of the inflammatory response against pathogens and cancerous cells, as well as a key

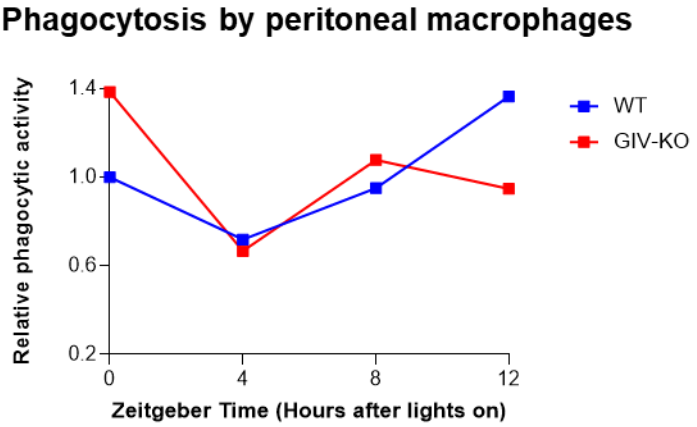
regulator of autoimmune diseases (Dorrington & Fraser, 2019; Medzhitov & Horng, 2009). With several recent lines of evidence supporting the circadian control of NFkB activity (Lee & Sancar, 2011; Spengler et al., 2012), we believe that the loss of circadian Cry1 variation and constant moderate levels of Cry1 expression in GIV-KO macrophages may cause chronic disruption in NFkB production.

In addition to regulating NF-kB production, Cry1 overexpression has been shown to increase cAMP levels (Narasimamurthy et al., 2012), a second messenger that has been long recognized as a potent inducer of anti-inflammatory responses and is an important player in the resolution of inflammation that is often targeted pharmacologically (Erdogan et al., 2008; Lima et al., 2017; Raker et al., 2016; Spengler et al., 2012; Tavares et al., 2020). Previously our group has demonstrated that GIV inhibits the cAMP→PKA→cAMP response element-binding protein signaling axis by serving as a bifunctional modulator of G proteins; GIV serves as a guanine nucleotide dissociation inhibitor (GDI) for Gαs using the same motif that allows it to serve as a GEF for Gαi (Reynoso et al., 2021). These reports are consistent with the finding that the loss of GIV disrupts Cry1 rhythmic expression and more importantly that the expression of Cry1 does not sharply drop at ZT12, correlating with systemically increased levels of cAMP. As the loss of GIV resulted in the rhythmic loss of Cry1 expression, with implications in disruption of the NFkB and cAMP signaling axis, we hypothesized that the loss of GIV disrupts the ability of macrophages to respond to and clear pathogenic stimuli in a circadian manner.

The loss of GIV disrupted rhythmic phagocytosis activity in Raw 264.7 and Peritoneal macrophages.

As a key part of their innate immune functions, macrophages are responsible for recognizing, ingesting, and clearing up pathogens in the early stages of an infection via phagocytosis (Hirayama et al., 2017). Dysfunction in macrophage phagocytic activity results in the accumulation of un-phagocytosed debris, chronic sterile inflammation and exacerbation of tissue aging and damage. In-vivo and in-vitro studies have revealed that macrophage display time of day dependent phagocytosis activity for different bacterial ligands (A-Gonzalez et al., 2017; Hriscu et al., n.d.; Kitchen et al., 2020) a rhythmic response that is completely lost on aging (Blacher et al., 2022). To assess whether the loss of GIV resulted in impaired immune environments and the ability of macrophages to respond to and clear pathogenic stimuli, we developed assays to test the rhythmic phagocytic activity of Raw 264.7 and peritoneal macrophages.

(a)



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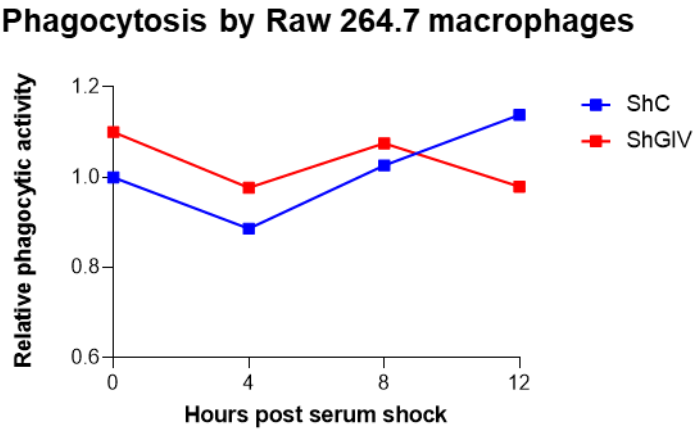

(c)

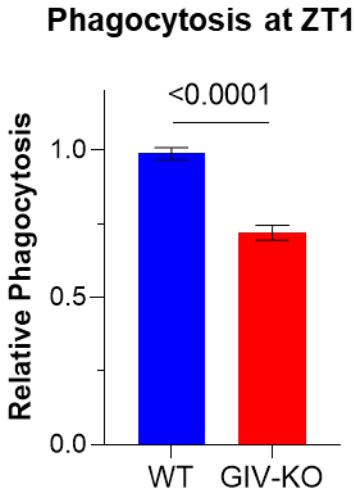

Figure 6: Loss of GIV disrupts rhythmic phagocytosis. Circadian variation in phagocytic activity against *E.coli* for Raw 264.7 at 0 hours, 4 hours, 8 hours and 12 hours post serum shock, and peritoneal macrophages (6b) isolated at ZT0, ZT4, ZT8, ZT12. Data represented as phagocytic activity relative to ShC at 0 hours (6a) and WT ZT0 (6b). The loss of GIV resulted in a 27% decrease in relative phagocytic activity at ZT12 in peritoneal macrophages (6c); $P < 0.0001$. Data represented as relative mean ± S.D (n=3).

Consistent with previously published data, we found that time dependent phagocytic activity peaked at ZT12 for WT peritoneal macrophages and control Raw 264.7 macrophages. The rhythmic expression of phagocytic activity was completely lost in GIV depleted Raw 264.7 macrophages with phagocytic activity dropping at ZT12 and peaking at ZT0 (Figure 6a). Comparably, the diurnal expression of phagocytic activity was completely flipped in peritoneal macrophages isolated from GIV-KO mice (Figure 6b), with a strong peak occurring at ZT0 and relative phagocytic activity significantly dropping by 27% at ZT12 (Figure 6c).

From these results, we conclude that GIV is essential for the diurnal phagocytic response and bacterial clearance by macrophages. More importantly, it can be inferred that the loss of GIV causes the conventional immune environments of macrophages to be flipped, with the immune active phase starting at ZT0 and ending at ZT12 in GIV KO macrophages.

Inversion of time-of-day phagocytic activity on loss of GIV is due to a flip in Klf-4 diurnal expression.

The ability of macrophages to perform phagocytosis is controlled in a circadian manner by a distinct repertoire of CCGs, receptors, opsonins, and transcription factors in macrophages (A-Gonzalez et al., 2017). Of these phagocytic regulators, we hypothesized that the rhythmic expression of transcription factor Gut Krüppel-like factor 4 (Klf-4) may be disrupted in the absence of GIV. We chose Klf-4 as our candidate gene based on the following four factors: (1) Klf-4 promotes rhythmic transcription in macrophages, (2) The time of day dependent expression of Klf-4 in

peritoneal macrophages strongly correlates with phagocytic activity, (3) Klf-4 time of day dependent expression levels strongly correlates with that of GIV, with both peaking at ZT12 (Blacher et al., 2022; Geiger et al., 2019; Tsoyi et al., 2015; Xia et al., 2022) and (4) Klf-4 has been shown to interact with Cyclic AMP Response Element Binding Protein (CREB), a downstream element of the cAMP -> PKA pathway that we have previously shown to be upregulated in the absence of GIV (Getz et al., 2019; Reynoso et al., 2021).

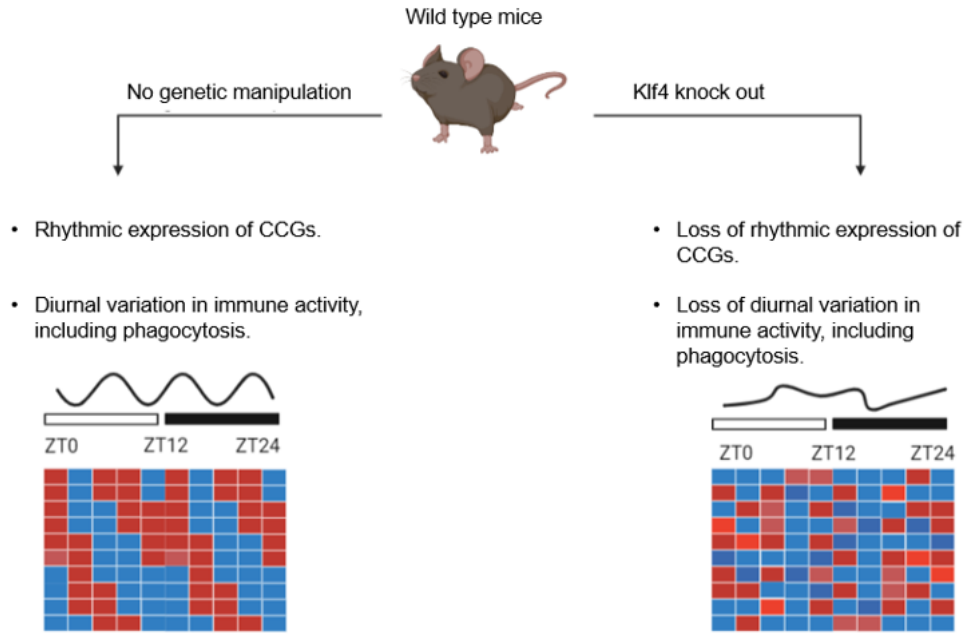

Figure 7: Klf-4 regulates rhythmic transcription and immune functions in mice. Graphical representation of the effect of loss of Klf-4 on the macrophage circadian rhythms. The loss of Klf-4 abolishes diurnal variation of clock-controlled genes and phagocytic activity of macrophages.

To determine if GIV influences the diurnal variation in Klf-4 expression, qPCR analysis was performed on peritoneal macrophages harvested from wild type and GIV-KO mice at ZT0 and ZT12.

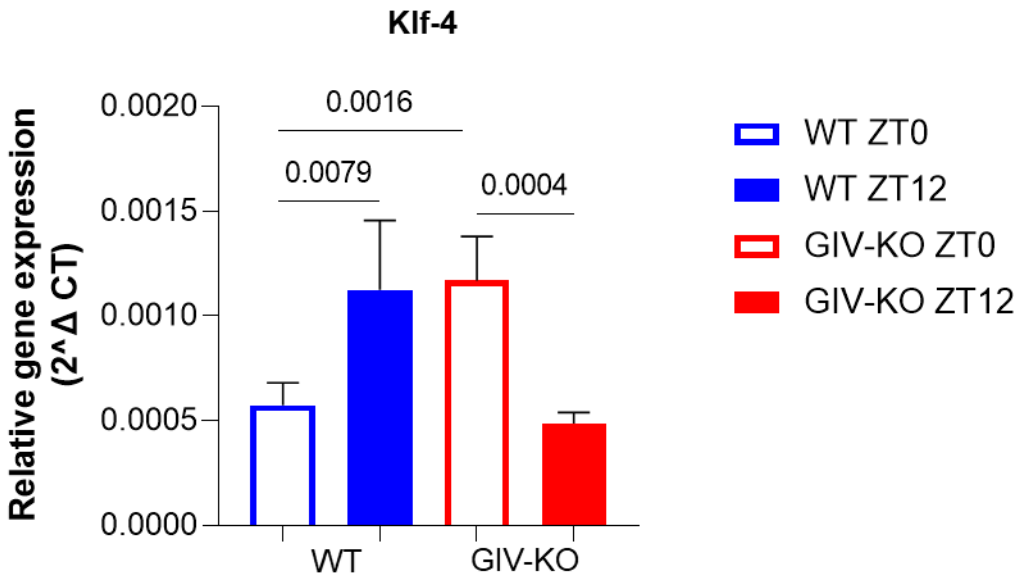

Figure 8: Loss of GIV disrupts Klf-4 expression. Diurnal variations in Klf-4 mRNA levels in WT and GIV KO peritoneal macrophages isolated at ZT0 and ZT12; P = 0.0079 for WT ZT0 vs ZT12, P = 0.0016 for WT ZT0 vs GIV-KO ZT0, and P = 0.0013 for GIV KO ZT0 vs GIV KO ZT12. Data represented as mean ± S.D (n=3-5).

Consistent with previously published data, Klf-4 expression was low at ZT0 and strongly spiked at ZT12 in macrophages isolated from WT mice (Blacher et al., 2022). However, it was seen that this diurnal pattern of Klf-4 expression was completely flipped in peritoneal macrophages from GIV-KO mice, with the peak of Klf-4 expression occurring at ZT0 and a significant drop occurring at ZT12 (Figure 8). This is consistent with the drastic change seen in the rhythmic phagocytic activity of peritoneal macrophages isolated from GIV KO mice observed in Figure 6b, with phagocytic responses peaking at ZT0 and dropping at ZT12.

The flip in diurnal Klf-4 expression seen on the loss of GIV further bolsters our claim that GIV is a crucial player in establishing the proper time of day dependent

immune environment seen in macrophages and that the loss of GIV may result in a functional switch between the immune active and immunosuppressive phases, with diminished Klf-4 expression and phagocytic response during the conventional immune active phase.

The loss of GIV disrupts cycling monocyte infiltration to sites of inflammation

In addition to mounting an appropriate phagocytic response, the diurnal circulation of inflammatory monocytes is under strong circadian control, which regulates their trafficking to sites of inflammation, a myeloid process critical to antimicrobial immune defense and appropriate resolution of inflammation (Blacher et al., 2022; Hayashi et al., 2007; Huo et al., 2017; Lang et al., 2021; Nguyen et al., 2013). During both homeostasis and inflammation, circulating monocytes migrate into tissues from the bloodstream where following stimulation by local growth factors, inflammatory cytokines and damage associated molecular patterns (DAMPS), they differentiate into appropriate neutrophil, macrophage or dendritic cell populations (Auffray et al., 2009). Proper monocyte recruitment is pivotal for effective control and clearance of pathogens (Serbina et al., 2008; Shi & Pamer, 2011), with the dysregulated recruitment contributing to the pathogenesis of diseases by creating hyper-immune and hypo-immune immune responses (Peranzoni et al., 2010; Woollard & Geissmann, 2010). Monocyte chemoattractant protein-1 (Mcp-1/Ccl2) is one of the key chemokines that regulates the infiltration of monocytes to sites of infection and inflammation (Gschwandtner et al., 2019; Shi & Pamer, 2011). Mcp-1 is a member of the C-C chemokine family that binds to its receptor, Ccr2, and mediates

the recruitment of Ly6C high monocytes, an inflammatory monocyte subset (Tsou et al., 2007). As previously mentioned, GIV is a potent inhibitor of cyclic AMP (Getz et al., 2019; Gupta et al., 2016), and cyclic AMP is a well-documented inhibitor of Mcp-1, with increase in intracellular cAMP preventing Ccl2 secretion in macrophages (Miller et al., 2005; Talvani et al., 2009). Therefore, we hypothesized that the loss of GIV would result in reduced macrophage Mcp-1 levels.

On analysis of previously generated in-house macrophage sequencing data, we found that GIV depleted Raw 264.7 macrophages displayed reduced expression of Mcp-1 levels in comparison to control Raw 264.7 macrophages when treated with LPS, a major PAMP present on the outer surface of almost all Gram-negative bacteria (Alexander & Rietschel, 2001).

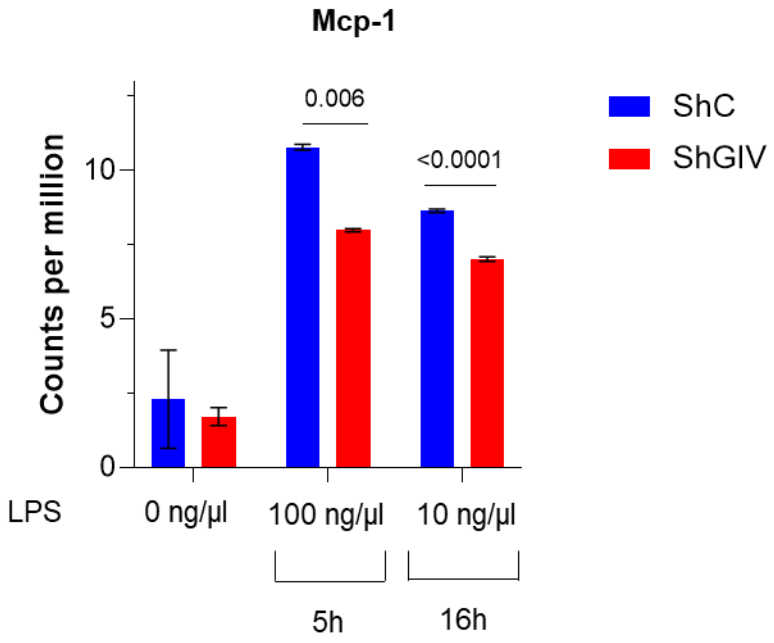

Figure 9: Loss of GIV reduces Mcp-1 expression. Mcp-1 mRNA levels in control and GIV depleted Raw 264.7 macrophages when unstimulated, treated 100ng/µl of LPS for 5 hours, and 10ng/µl of LPS for 16h. The loss of GIV resulted in a 25.9% and 18.6% reduction in Mcp-1 mRNA levels upon LPS stimulation respectively. Data represented as mean ± S.D (n=3).

The loss of GIV resulted in decreased Mcp-1 expression when Raw 264.7 macrophages were stimulated with both chronic-low and acute-high dosage of LPS. When treated with 100ng/ul of LPS for 5 hours and 10ng/ul of LPS for 16 hours, the loss of GIV amounted to a 25.9% and 18.6% decline in Mcp-1 expression respectively. This suggests that in the absence of GIV, there is in an extensive loss in monocyte chemoattractants under different doses of inflammatory stimulants. Therefore, we hypothesized that the loss of GIV may result in the dysregulated time of day dependent circulation of monocytes and their infiltration to sites of inflammation. To test this, we initially performed a qPCR to analyze Mcp-1 expression of peritoneal macrophages isolated from wild type and GIV-KO mice at ZT0 and ZT12.

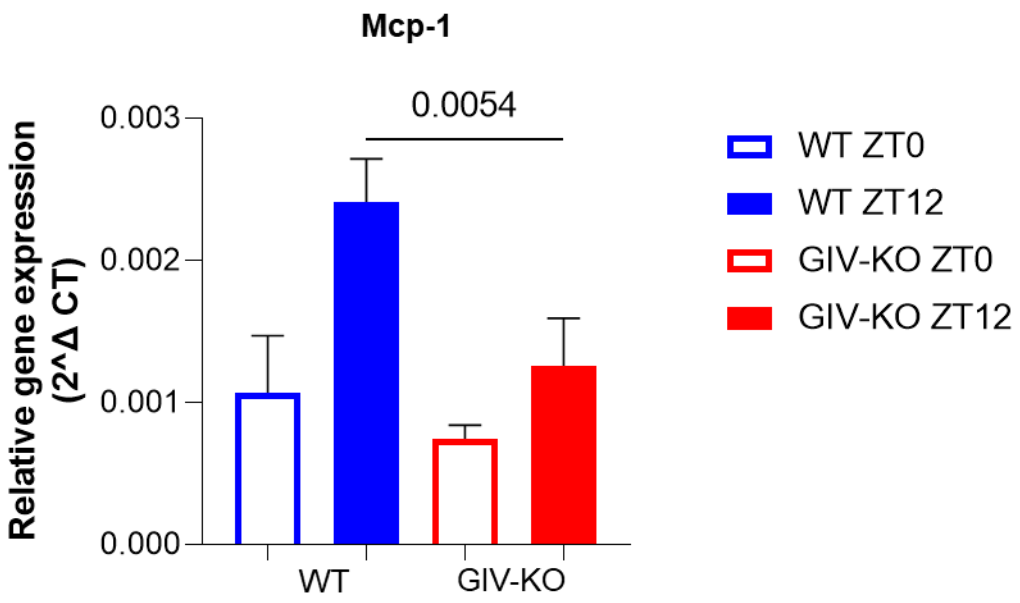

Figure 10: Loss of GIV reduced Mcp-1 expression at ZT12. Diurnal variations in Mcp-1 mRNA levels in WT and GIV KO peritoneal macrophages isolated at ZT0 and ZT12. The loss of GIV resulted in a 47.9% reduction in Mcp-1 gene expression; P = 0.0054. Data represented as mean ± S.D (n=3/4).

From our qPCR analysis, we saw that Mcp-1 expression significantly increased from ZT0 to ZT12 in wild type macrophages. However, the loss of GIV resulted in a 50.6% drop in Mcp-1 gene expression at ZT12, the immune active phase of mice (Figure 10). Based on this, we hypothesized that the loss of GIV would reduce monocyte infiltration to sites of inflammation. To test this hypothesis, we investigated the infiltration of Ly6C high macrophages to sites of inflammation using a thioglycolate elicited in-vivo model of sterile peritonitis. For this model we chose the time points of ZT8 and ZT12. The ZT8 time point was chosen as monocyte infiltration has been observed to peak at this time following peritoneal injury (Nguyen et al., 2013) and the time point of ZT12 was chosen as it is not only the beginning of the immune-active phage in mice but also is the time during which GIV and Klf4 diurnal gene expression peaks.

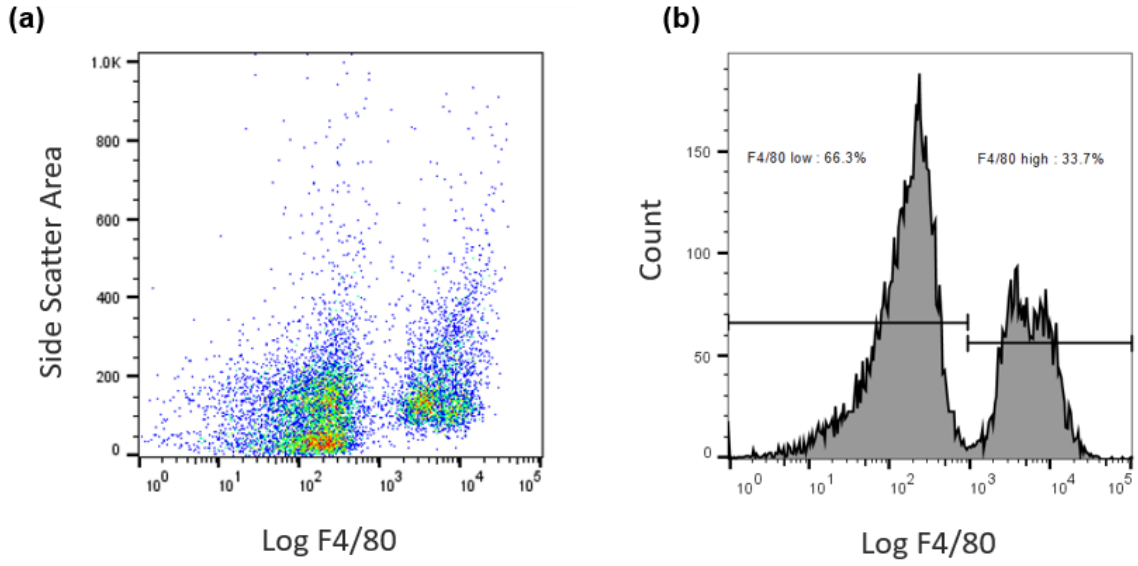

Figure 11: Population of monocytes recruited to sites of peritoneal injury. Monocytes were stained with F4/80_PE antibody. Two distinct population of monocytes were found based on F4/80 expression; F4/80 low and F4/80 high monocytes, that accounted for 66.3% and 33.7% of the population respectively.

From our flow cytometric analysis, we observed two distinct populations of cells based on their expression of the F4/80 surface markers: the F4/80 low and the F4/80 high cells (Figure 11a). The F4/80 high cells comprised of approximately 34% of the total population of cells (Figure 11b) and were identified as macrophages. The Ly6C median fluorescence of these cells was determined as monocytes with high expression of F4/80 and Ly6C have been determined to possess proinflammatory and antimicrobial functions (Kratofil et al., 2017). Ly6C high monocytes are crucial for mounting an appropriate immune response following injury, with their infiltration depending on the activation of the Mcp-1/Ccl2-Ccr2 signaling axis (Geissmann et al., 2003). These monocytes accumulate at sites of injury where they express high levels of inflammatory cytokines ILβ and TNFα (Glezeva et al., 2015; Mukherjee et al., 2015). The infiltration of Ly6C high inflammatory monocytes is also an integral aspect

of defense against pathogenic bacteria and is paramount in bacterial clearance during early innate and late adaptive phases of an immune response (Shi et al., 2011).

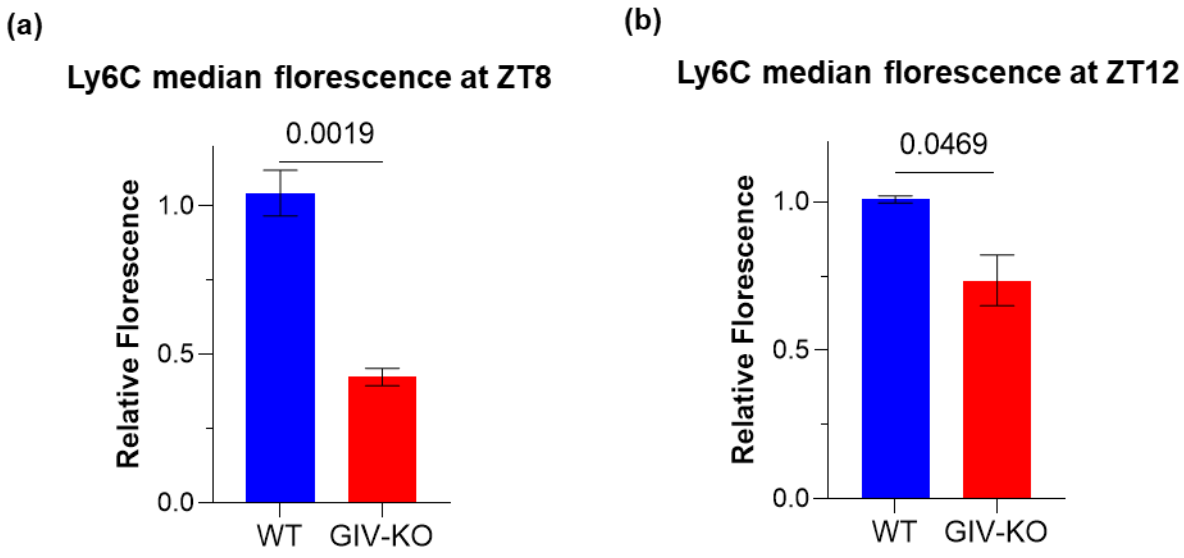

Figure 12: Loss of GIV reduces macrophage infiltration at ZT8 and ZT12. Ly6C median florescence for F4/80 positive macrophages isolated post peritoneal injury at ZT8 and ZT12. The loss of GIV resulted in a 59.4% (12a) and a 26.4% (12b) reduction in median florescence respectively. Data represented as mean ± S.D (n = 2/3).

We observed that the loss of GIV significantly reduced the median Ly6C florescence of macrophages during ZT8 and ZT12. The loss of GIV resulted in an 59.3% (Figure 12A) and a 26.4% (Figure 12B) reduction in Ly6C median fluorescence at ZT8 and ZT12 respectively. Based on this, we conclude that the loss of GIV significantly reduces the ability of inflammatory macrophages to be recruited to sites of inflammation following peritoneal injury at ZT8 and ZT12. This further highlights how the loss of GIV disrupts the circadian immune function of macrophages resulting in inadequate bacterial clearance and poor resolution of inflammation.

V. DISCUSSION

The circadian rhythm regulates overt behavioral and fundamental cellular responses of an organism. The circadian rhythm is a prominent mediator of various essential physiological functions with an unhealthy or disturbed circadian rhythm is a key risk factor for infection related mortalities, cardiovascular diseases, cancer, inflammatory bowel diseases, obesity, mood disorders and an overall decline in physical and mental health of an individual. Particularly, the circadian rhythm is strongly expressed in immune cells such as macrophages, where it regulates their effector capabilities. Anticipating certain time of day dependent immune challenges, a functional macrophage circadian rhythm is paramount in eliciting an appropriate response against various pathogens. In this thesis, we demonstrate that GIV, a non-canonical guanine -nucleotide exchange modulator of heterotrimeric G proteins (GEM), maintains the circadian rhythm and time dependent immune response of macrophages. The loss of GIV significantly alters the diurnal expression of Cry1 in-vivo and the in-vitro circadian expression of core peripheral clock genes Bmal1, Clock, Cry1, Cry2, Per1, Per2, Reverb, and Reverbb. GIV promotes the rhythmic phagocytic response of Raw 264.7 and peritoneal macrophages against *E.coli*, by regulating the diurnal expression of Klf-4, a transcription factor pivotal for circadian gene expression and immune function in macrophages. The loss of GIV flips diurnal Klf-4 gene expression and functionally reduces the phagocytic activity of macrophages at ZT12, the time during which phagocytic activity has been demonstrated to naturally peak. In addition to regulating the phagocytic effector function of macrophages, GIV also regulates the diurnal expression of Mcp-1/Ccl2,

one of the key chemokines that regulate monocyte migration. In the absence of GIV, Mcp-1 expression is significantly reduced in Raw 264.7 macrophages stimulated with different doses of LPS as well as peritoneal macrophages isolated at ZT12. The regulation of diurnal Mcp-1 expression by GIV mediates the time-of-day dependent variation in the infiltration of inflammatory macrophages to sites of peritoneal injury, with a loss of GIV resulting in significantly reduced L6C median fluorescence of macrophages at ZT8 and ZT12.

VI. CONCLUSION AND FUTURE DIRECTIONS

The establishment and maintenance of the circadian rhythm is key in generating a proper immune response to effectively clear pathogens and resolve inflammation. Based on all the studies conducted in this thesis, it seems highly likely that GIV plays a central role in regulating the circadian rhythm and time of day dependent effector functions of macrophages. Going forward, we wish to 1) Include more time points in our in-vivo studies to develop increased granularity and understand how GIV influences expression of peripheral clock genes and variations in macrophage infiltration to sites of injury at different times of the day, 2) Study if GIV influences the diurnal variation in LPS induced mortality to elucidate the role of GIV in modulating the time-of-day dependent ability to recover from TLR4 ligand challenge, and 3) Generate and analyze RNA sequencing data from peritoneal macrophages isolated from WT and GIV-KO mice isolated every 4 hours from ZT0 to ZT24 as this

may help identify other aspects of the circadian immune responses that are modulated by GIV.

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