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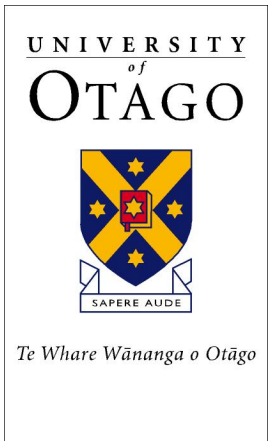


# RESEARCH REPORT

## Mucosal-associated invariant T cell regulation by bacterial signalling and immunometabolism

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# Abstract

Mucosal-associated invariant T (MAIT) cells are a subset of antibacterial innate-like T cells that are localised to mucosal surfaces. MAIT cells are characterised by the expression of a semi-invariant T cell receptor, specific to a bacterial antigen presented on the MHC class I-related protein, MR1. The bacterial ligand is derived from 5-amino-6-D-ribitylaminouracil (5-A-RU), produced as an intermediate in the bacterial riboflavin synthesis pathway. 5-A-RU undergoes non-enzymatic condensation with methylglyoxal (MG), a glycolysis by-product, to form the final ligand, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). Presentation of 5-OP-RU to MAIT cells stimulates a robust MAIT cell response. Given the abundance of MAIT cells at mucosal surfaces and the broad range of bacteria capable of activating them, it has been hypothesised that MAIT cell activation is tightly regulated to prevent hyperactivation and immunopathology. Understanding these regulatory mechanisms may enable modulation of MAIT cells to prevent or treat human disease. Here I show that both phagocytosis of bacteria by an antigen presenting cell (APC) and enhanced glycolysis regulate MAIT cell activation. To assess this, THP-1 cells, a monocytic cell line, served as APCs. THP-1 cells were incubated with glycolysis modulators, exogenous ligand, and non-ligand producing bacteria. Primary human MAIT cells were subsequently co-cultured with THP-1 cells and activation assessed by flow cytometry. Treatment with intact bacteria and 5-A-RU was found to activate MAIT cells to a greater extent than treatment with 5-A-RU alone or with lysed bacteria. Enhancement of THP-1 glycolysis augmented MAIT cell activation to 5-A-RU alone or 5-A-RU and lysed bacteria. In contrast, a reduction in activation was not observed when THP-1 cell glycolysis or PI3K, mTORC1, and mTORC2 signalling were inhibited. Furthermore, THP-1 cells did not exhibit increased glucose uptake upon stimulation with intact bacteria. These results suggest that phagocytosis of intact bacteria may enhance glycolysis,

resulting in increased production of MG and formation of 5-OP-RU. However, further research is required to confirm this. The process described provides a potential regulatory mechanism by which MAIT cell activation is regulated in response to intact bacteria but not to soluble bacteria-derived 5-A-RU.

# Acknowledgements

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# List of Abbreviations

| | |
|---|---|
| 2-DG | 2-deoxy-D-glucose |
| 5-A-RU | 5-amino-6-D-ribitylaminouracil |
| 5-OE-RU | 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil |
| 5-OP-RU | 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil |
| 6-FP | 6-formyl pterin |
| APC | Antigen presenting cell |
| BPC | Bacteria per cell |
| DCs | Dendritic cells |
| DN | Double negative |
| ER | Endoplasmic reticulum |
| F-1,6-BP | Fructose-1,6-bisphosphate |
| G | Glyoxal |
| Glo | Glyoxalase enzymes |
| GSH | Glutathione |
| IFNγ | Interferon γ |
| IL- | Interleukin- |
| iNKT cell | Invariant natural killer T cell |
| KU | KU0063794 |
| LY | LY294002 |
| MAIT cell | Mucosal-associated invariant T cell |
| MG | Methylglyoxal |
| MHC | Major histocompatibility complex |
| MR1 | MHC class I-related |

| | |
|---|---|
| PAMPs | Pathogen-associated molecular patterns |
| PBMC | Peripheral blood mononuclear cells |
| PRRs | Pattern recognition receptors |
| Rapa | Rapamycin |
| TCR | T cell receptor |
| Th-1 | T-helper-1 |
| Th-17 | T-helper-17 |
| TLRs | Toll like receptors |
| TNFα | Tumour Necrosis Factor α |

# 1 Introduction

## 1.1 Innate-like lymphocytes

Innate-like lymphocytes are a subset of immune cells redefining the field of immunology. Traditionally, immune cells have been classified as either innate or adaptive. The innate system is made up of rapid and broad-acting immune cells bearing cell surface pattern recognition receptors (PRRs) that detect highly conserved pathogen-associated molecular patterns (PAMPs) on invading pathogens.[1] In contrast, the adaptive immune system responds in a slower yet highly specific manner to variable antigens, made possible by the large variety of antigen receptors, known as T cell receptors (TCR) or B cell receptors.[1] The hypervariability of TCRs is made possible by V(D)J recombination of the TCRα and β chains, splicing together a range of V, D (in the β chain), and J segments to form unique TCR structures. The hypervariable TCR structure allows for the recognition of a wide range of antigens bound to variable Major Histocompatibility Complexes (MHC) at the population level. As our understanding of the immune system has expanded, the distinction between the two categories has blurred with the discovery of cell types that do not fit into either category. These cells, termed 'innate-like lymphocytes', include invariant natural killer T (iNKT) cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells, and have recombined TCRs that are restricted to highly conserved antigens.[2, 3, 4]

## 1.2 MAIT cell phenotype

MAIT cells are a key antibacterial immune cell first identified in 1993 and are primarily characterised by the presence of a semi-invariant αβ chain heterodimer TCR.[5, 6, 7] This receptor is fundamentally different than conventional TCRs which consist of highly variable α and β

chains. The process of V(D)J recombination allows for the production of a wide range of unique TCR structures. In contrast, the human MAIT cell TCR α chain is restricted to Vα7.2-Jα12/20/33.[5, 6] The sequence is highly conserved, with limited variability observed between humans, mice, and cattle.[6] More variability is found in the β chain, though bias is observed towards Vβ2 and Vβ13.[6] The TCR α chain is of vital importance in TCR binding to MHC class I-related, MR1, which presents the MAIT cell activating ligand on antigen presenting cells (APC). The interaction between the TCR and MR1 is mediated by six key residues on the α chain.[8] The major role of these TCR binding residues may explain the evolutionary conservation observed in the α chain, compared to the more variable β chain.

Human MAIT cells are further defined by their high expression of CD161 ($CD161^{++}$).[9, 10, 11] CD161, also known as NKR-P1A, is a C-type lectin receptor that is expressed by a range of T lymphocytes, including MAIT cells and iNKT cells.[11, 12] These cells have a shared innate-like phenotype, including high expression of IL-18Rα.[11] In humans, MAIT cells make up a small portion of $CD161^{++}CD8^{+}$ T cells at birth, with this proportion rising in adults.[11]

MAIT cells can be further classified into subtypes based on the expression of co-receptors CD4 and CD8. The frequencies of $CD4^{+}CD8^{-}$ and $CD4^{+}CD8^{+}$ MAIT cell subsets are significantly higher in the thymus than in circulating MAIT cell populations.[13] The composition of MAIT cell subsets in the blood is approximately 82.9% $CD4^{-}CD8^{+}$, 12.1% $CD4^{-}CD8^{-}$, known as double negative (DN), and 1.9% $CD4^{+}CD8^{-}$.[14] These MAIT cell subsets display some degree of plasticity, as stimulation of $CD4^{-}CD8^{+}$ MAIT cells can induce CD8 downregulation, producing a DN population.[15]

MAIT cells exhibit a mixed T-helper-1 (Th-1)/T-helper-17 (Th-17) phenotype, due to co-expression of the transcription factors T-Box Transcription Factor 21 (T-Bet) and RAR related orphan receptor C (RORγ).[16] This enables characteristic Th-1 and Th-17 polarisation,

and the production of the proinflammatory cytokines interferon (IFN)γ, tumour necrosis factor (TNF)α, and interleukin (IL)-17.[17] The $CD8^+$ MAIT cell subset tend to exhibit more Th1 polarisation, whereas DN subsets tend to show Th17 polarisation.[15]

MAIT cells are found in the peripheral blood, liver, and at mucosal sites, including the lungs, oral mucosa, and gastrointestinal tract.[17, 18, 19, 20] They are enriched in mucosal surfaces due to the expression of specific chemokine receptors, CCR2, CCR5 CCR6, CXCR6, and CCR9.[17, 21, 22, 23]

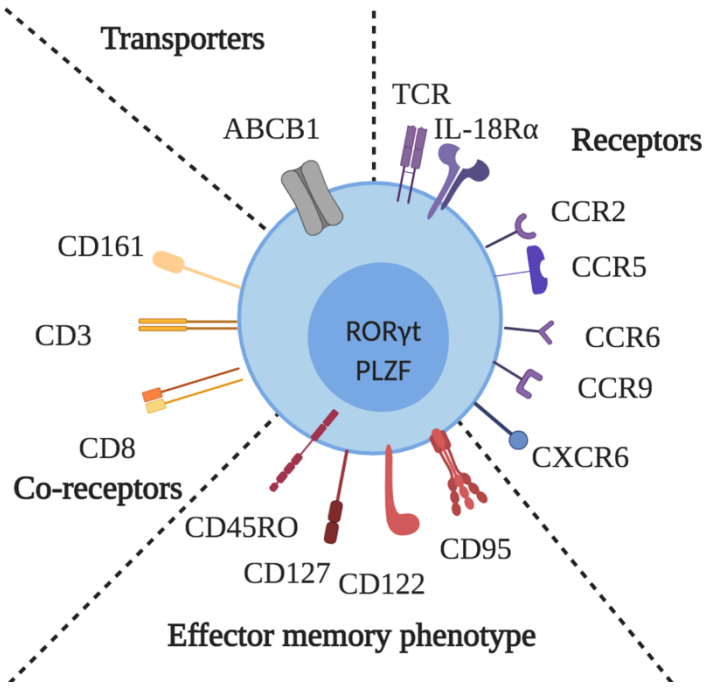



***Figure 1.1: Characteristic MAIT cell transcription factors and effector memory phenotype surface expression.*** *Created with BioRender.com.*

Overall, most human MAIT cells express an effector memory phenotype, defined as $CD45RO^+CD62L^{lo}CD122^+CD127^{++}CD95^+$ (Fig 1.1).[17] Experimentally, CD8, CD3, CD161, and the Vα7.2 component of the TCR are most commonly used to identify MAIT cells by flow cytometry. However, these are merely a small selection of characteristic markers of MAIT cells, including additional co-receptors, transporters, and transcription factors (Figure 1.1).[14, 17, 23, 24]

## 1.3 MR1 presentation of the MAIT cell activating ligand

### 1.3.1 MR1 function

The MR1 molecule on APCs exhibits remarkable evolutionary conservation across mammals, indicating a significant role of this protein.[25] MAIT cells were first shown to be restricted to MR1 by Treiner et al. who demonstrated that MR1 was capable of inducing cytokine production in MAIT cells.[18] Subsequent research revealed that MR1 binding and presentation of an unknown ligand was necessary for MAIT cell activation in response to a range of bacteria, including *Mycobacterium tuberculosis* and *Staphylococcus epidermidis*.[19, 26] Interestingly, non-riboflavin producing bacteria, including *Enterococcus faecalis* and *Streptococcus pyogenes*, do not activate MAIT cells in a TCR-dependent manner.[26] However, these pathogens are capable of activating MAIT cells in a cytokine dependent manner.[24]

The identity of the MAIT cell activating ligand was originally unclear. Although compounds derived from vitamin B metabolism, such as 6-formyl pterin (6-FP), could bind and stabilise MR1, they were unable to activate MAIT cells.[27] Further study identified that derivatives of intermediates of riboflavin biosynthesis were able to bind MR1 and successfully activate Jurkat MAIT cell lines.[4, 27, 28] Mutagenesis studies of the riboflavin pathway performed by Corbett et al identified 5-amino-6-D-ribitylaminouracil (5-A-RU) as the specific component of the riboflavin synthesis pathway required for MAIT cell activation.[4] Mutation of genes encoding enzymes upstream of 5-A-RU production prevented MAIT cell activation, whereas disruption of those encoding downstream enzymes did not.[4] However, 5-A-RU itself does not bind MR1 causing MAIT cell activation. 5-A-RU reacts via an enzyme-independent condensation reaction with either glyoxal (G) or methylglyoxal (MG) to produce 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) or 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), respectively (Fig 1.2).[4] 5-OE-RU and 5-OP-RU are highly unstable pyrimidine

intermediates that rapidly undergo a non-enzymatic reaction to form lumazine structures if not stabilised (Figure 1.2).[4] 5-OE-RU and 5-OP-RU can be bound, stabilised, and presented by MR1 through the formation of a Schiff base to a lysine residue in the carbonyl group in MR1.[4] This process is vital in the preservation of the ligands for successful presentation to the MAIT cell TCR.[4]

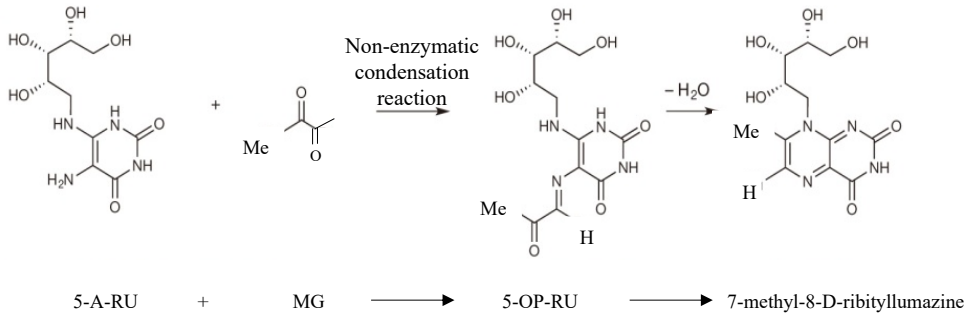



***Figure 1.2: Formation of the final MAIT cell activating ligand, 5-OP-RU****. 5-A-RU and MG substrates react to produce 5-OP-RU, which breaks down into the lumazine structure 7-methyl-8-D-ribityllumazine if not stabilised by MR1. Adapted from Corbett et al.*

### 1.3.2 MR1 loading

The exact pathway of MR1 loading and trafficking to the cell surface has not been fully established despite ongoing research in this area. In the absence of ligand, MR1 does not pass through the Golgi and unbound MR1 localises in the endoplasmic reticulum (ER).[29] Once bound, MR1 interacts with beta-2-microglobin and traffics through the Golgi to the cell surface.[29, 30, 31, 32] Ligand binding is thought to be necessary for MR1 folding. However, it is unclear how this soluble ligand, speculated to be either a exogenous or a low-affinity endogenous ligand, would access the ER.[29, 33] Potentially, MR1, either unbound or bound to soluble ligand, can reach the cell surface where the bacterial ligand would then bind.[29] As there are likely minimal amounts of unbound MR1 at the cell surface this would be a minor pathway.[29] Ligand loading may also occur in endosomes by displacement of the endogenous or exogenous ligand; MR1 was identified in endosomes after trafficking from the ER or

recycling from the cell surface.[29, 34] Finally, loading may occur when intracellular pathogens are recognised and sent to an endosomal compartment by xenophagy.[35]

### 1.4 The MAIT cell response

MAIT cells are capable of mounting both TCR- and cytokine-dependent responses. Early activation of MAIT cells is almost entirely dependent on TCR signalling following binding to ligand-bound MR1 occurring within 5 hours of infection *in vitro*.[24] Presentation of the final activating ligand to MAIT cells by MR1 results in the rapid production of effector functions, including proliferation and production of IFN-γ, TNF-α, and IL-17A.[17, 36, 37, 38] Furthermore, TCR signalling leads to the production of the chemokines CCL3, CCL4, and CCL20.[37] TCR signalling also enables production of cytotoxic molecules granzyme B and perforin by MAIT cells for direct killing of bacterially infected cells.[36]

At later stages of an infection, or in infections lacking riboflavin metabolising bacteria, cytokines act synergistically with TCR signalling for effective MAIT cell activation.[24] Cytokines IL-12 and IL-18 act on MAIT cells after 20 hours of infection *in vitro* leading to the production of IFNγ and the chemokines CCL3 and CCL4.[24, 37]

MAIT cell responses to each mode of stimulation differ. TCR activated MAIT cells produce higher levels of TNFα whereas cytokine stimulation results in higher production of IFNγ by MAIT cells.[37] Both cytokine-mediated and MR1-dependent responses play roles in mounting a robust MAIT cell response over the course of an infection.

Multiple factors are capable of influencing the major modes of MAIT cell activation. IL-7 and IL-15 both enhance weak bacterial stimulation of MAIT cells to produce a robust response.[39, 40, 41] IL-7 is capable of inducing perforin and granzyme A and B production.[40] As the IL-7 receptor is expressed highly on MAIT cells, it suggests that IL-7 plays an important role in

cytokine-mediated activation.[39] IL-15 activates MAIT cells indirectly by inducing IL-18 production in monocytes.[41] Furthermore, type 1 interferons are capable of enhancing the TCR dependent production of proinflammatory cytokines and cytotoxic molecules.[42] Different infective agents may also cause differential cytokine expression by MAIT cells.[43] In addition, opsonisation of bacteria influences TNF production by the APC, causing MAIT cells to increase expression of IFNγ and TNF.[44] More research is required to fully explore and characterise the range of factors capable of modulating a MAIT cell response.

## 1.5 Role of MAIT cells in health and disease

### 1.5.1 Role of MAIT cells in infectious disease

MAIT cells play a role in clearing a large range of bacterial infections. MAIT cell frequencies are decreased in the blood of patients with mucosal-associated bacterial infections, including *M. tuberculosis*, *Legionella longbeachae, E. coli*, and *Helicobacter pylori*. [26, 45, 46, 47] There is some evidence to suggest MAIT cells migrate to the site of infection. [26] Decreased blood MAIT cell frequencies correlated with increased time spent in hospital, persistently decreased frequencies with an increased risk of ICU acquired infection.[48, 49] Murine studies have demonstrated that MAIT cells produce proinflammatory cytokines and enable bacterial clearance in *L. longbeachae*, *Klebsiella pneumoniae,* and *Francisella tularensis* infections in an MR1-dependent manner.[38, 46, 50] Furthermore, in *F. tularensis* infection, a functional MAIT cell population aided in the recruitment of adaptive T cells to the site of infection.[38] In this way MAIT cells can link the innate and adaptive arms of the immune response, in order to aid bacterial clearance.[38]

Viruses activate MAIT cells independent of MR1 signalling. Originally, researchers concluded that viral infection does not result in MAIT cell activation.[26] However, more recent studies demonstrated that influenza and hepatitis C viruses activate MAIT cells in a cytokine-

dependent manner.[51, 52] In HIV infection, the frequency of MAIT cells in the blood is significantly reduced and, unlike $CD4^+$ T cell populations, does not recover with antiretroviral treatment.[40, 53] The remaining cells appear functionally impaired, producing less proinflammatory cytokines upon bacterial treatment.[40] This alteration of MAIT cell frequency and function may explain why HIV positive individuals are at a higher risk of opportunistic infections, even after antiretroviral treatment.[53]

### 1.5.2 Role of MAIT cells in non-communicable diseases

MAIT cells have been identified in multiple clinical settings, often related to their localisation to mucosal surfaces. MAIT cells are decreased in the blood of patients with colorectal, gastric, and lung cancers.[54, 55] Multiple studies have found MAIT cells infiltrating colorectal tumours.[54, 55, 56, 57] However, this is not universally accepted, as evidenced by the degree of discord in the literature.[54, 55, 56, 57] Their numbers are also decreased in the circulation of patients with autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, dermatomyositis, and inflammatory bowel disease.[58, 59, 60] MAIT cells may migrate to areas of inflammation in Crohn's disease as they are found at increased frequencies in inflamed tissues.[60]

The scope of the role of these cells in disease is rapidly increasing; recent studies have also implicated MAIT cells in both obesity and wound healing.[61, 62] More research is needed to fully understand how MAIT cells protect from or contribute to pathogenesis in specific diseases.

## 1.6 Regulation of MAIT cell activation

It is imperative that the mechanisms of MAIT cell activation and regulation are fully understood due to their localisation to mucosal sites as well as their implications in a range of disease states. Tight regulation of MAIT cells must occur to prevent hyperactivation in

response to commensal mucosal bacteria, many of which contain a riboflavin synthesis pathway. Previous work in the Ussher Laboratory has suggested this process may be mediated by both bacterial signalling, aided by Toll like receptors (TLRs), and immunometabolism within the APC.[63, 64]

### 1.6.1 Phagocytosis

Phagocytosis of bacteria by the APC has been implicated in MAIT cell activation. Inhibition of dendritic cell (DC) phagocytosis and endosomal acidification significantly decreases MAIT cell activation.[26] Furthermore, Ussher et al. found that treatment of APCs with whole bacteria caused greater MAIT cell activation than treatment with sonicated bacteria.[64] Phagocytosis may be an important regulatory component of MAIT cell activation by providing a method of 5-A-RU delivery into the APC in order to react with MG and bind MR1.[63]

### 1.6.2 Glycolysis and immunometabolism

Glycolysis plays an integral role in immunometabolism and is at the crossroads between cellular metabolism and immune function. Glycolysis has long been implicated in immune responses; a spike in glycolytic activity has been detected after activation of macrophages, mature T cells, and MAIT cells.[65, 66, 67, 68] Additionally, glycolysis is required to produce the characteristic effector phenotypes of T lymphocytes and MAIT cells.[67, 68] Glycolytic enzymes have been implicated in the regulation of IFNγ production in mouse $CD4^+$ T lymphocytes, with inhibition of glycolysis resulting in reduced IFNγ production.[67, 69] There appear to be both epigenetic and translational mechanisms of glycolysis regulation, potentially creating some level of functional redundancy.[67 69] Further, glycolysis appears to play an important role in the MAIT cell effector response. MAIT cells show similarities to other T lymphocytes; glycolysis is upregulated upon activation and is required for MAIT cell effector functions, such as

granzyme B and IFNγ expression.[62, 68] One key difference observed in MAIT cell activation is the absence of oxidative phosphorylation upregulation, as seen in T lymphocytes.[62, 67]

Glycolysis also appears to be an integral element of APC activation and in the stimulation of effector functions, however gaps in knowledge remain. Multiple studies have highlighted that the upregulation of glycolysis in macrophages induces a proinflammatory response, including the production of proinflammatory cytokines, reactive oxygen species, and caspase-1.[70, 71, 72] Regulation of this response is likely multifaceted, with multiple mechanisms of control identified.[70, 72] Multiple regulatory proteins have been implicated in this process including both mTOR induced glycolysis and glycolysis inhibition by carbohydrate kinase-like protein, CARKL.[70 72] The role of glycolysis may not be limited to a bacterial infection, as multiple viruses can stimulate glycolysis in plasmacytoid DC by an unknown mechanism.[73]

### 1.6.3 Role of TLRs in APC activation

TLRs allow APCs to quickly recognise and respond to potentially pathogenic bacteria. TLRs recognise highly conserved, broadly expressed microbial components, known as PAMPs or TLR-agonists. TLR signalling is crucial in the activation of multiple APCs, including macrophages and DCs.[74, 75] Stimulation of both endosomal and cell surface TLRs can lead to enhanced glycolysis of APCs.[74, 76] Increased glycolysis creates an excess of glycolytic by-products which are used as substrates in other metabolic pathways to produce lipids for ER and Golgi expansion, crucial aspects of DC activation.[74] Pre-treating THP-1 cells with TLR agonists before the addition of whole bacteria enhanced MAIT cell activation.[64] Endosomal TLRs may provide the connection between phagocytosis of potential pathogens APC immunometabolism.

### 1.6.4 Potential mechanisms of MAIT cell regulation

As MAIT cells are largely found at mucosal surfaces, tight regulation must exist to prevent unnecessary activation in response to mucosal commensal bacteria.[18, 20] This hypothesis was first raised by Ussher et al., who reported that tight regulation through mechanisms such as phagocytosis of whole bacteria was necessary for MR1-dependent MAIT cell activation.[64] Unpublished work in the Ussher Laboratory used THP-1 cell lines to assess MAIT cell activation. MAIT cell activation by 5-A-RU was significantly enhanced when treated with fixed *E. coli* Δ*ribD*, a strain which cannot produce 5-A-RU, compared to sonicated *E. coli* Δ*ribD*.[63] This effect was abolished when THP-1 cells were treated with 5-OP-RU.[63] This raised the possibility that 5-OP-RU may bind directly within endosomes or at the cell surface, whereas 5-A-RU requires G or MG for formation of the final activating ligand.[63] MG is produced by the non-enzymatic break down of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, components of the glycolytic pathway.[77, 78] Additionally, G and MG can form in the breakdown of glycosylated proteins in early glyclation.[79] Therefore, it was hypothesised that the enhancement observed with the addition of whole bacteria to treated cells resulted from the induction of glycolysis in the APC and the associated increase in G and MG production.[63] Stimulation of APCs by TLR agonists present on bacteria induce a metabolic switch to glycolysis.[74, 75] A glycolytic enhancement may result in increased G and MG production, potentially leading to increased efficiency of 5-OP-RU formation.

Therefore, the Ussher Laboratory hypothesised that glycolysis may be an integral step in the successful formation of 5-OP-RU within APCs upon bacterial stimulation. Preliminary data obtained using peripheral blood mononuclear cell culture (PBMC) indicated a reduction of MAIT cell activation by 5-A-RU with inhibition of glycolysis, irrespective of *E. coli* Δ*ribD* treatment.[63] Furthermore, enhancement of PBMC glycolysis increased MAIT cell activation when exposed to 5-A-RU and *E. coli* Δ*ribD*.[63] These results suggest that phagocytosis of intact

bacteria may upregulate the APC glycolytic pathways, aiding ligand formation and resulting in the successful activation of MAIT cells (Fig 1.3). However, the experimental procedures investigating the role of glycolysis used PBMC cultures, which contain a mixture of immune cells. As glycolysis has been implicated in MAIT cell effector function it is not possible to attribute the results to glycolysis within the APC alone. There are also many unknowns in the mechanism occurring, such as whether increased uptake of glucose or increased expression of glycolytic enzymes occurs upon bacterial licensing. Continued investigation is required to confirm the hypothesis and to identify the exact mechanism occurring. In future, manipulation of this mechanism may enable modulation of MAIT cell responses to prevent or treat bacterial infections.

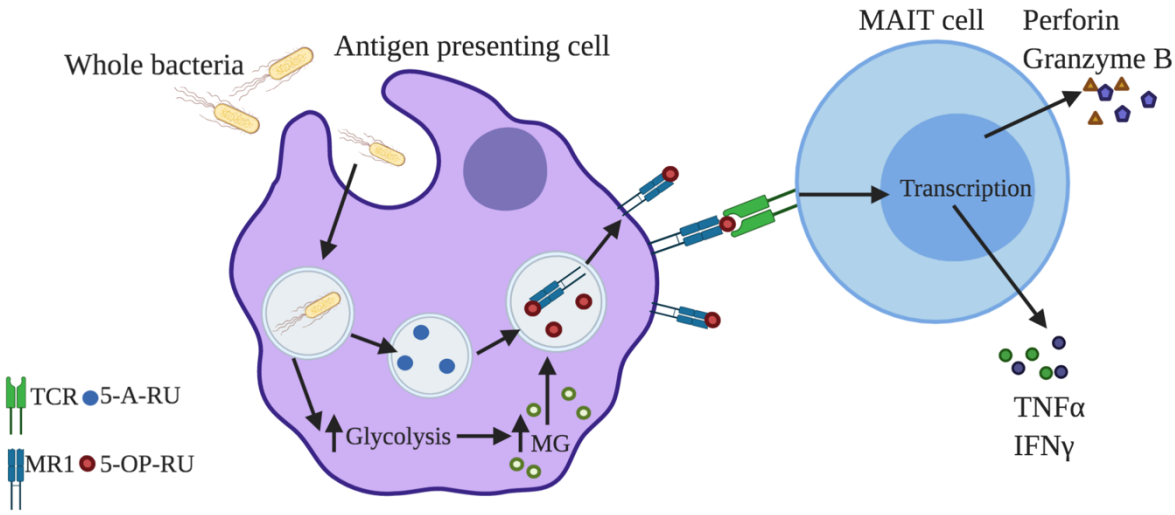



***Figure 1.3 Potential mechanism of MAIT cell activation***. *Phagocytosis increases the glycolytic capacity of the APC, increasing methylglyoxal (MG) and enabling efficient 5-OP-RU formation for binding and presentation to MAIT cells by MR1. Created with BioRender.com.*

## 1.7 Aims and hypothesis

MAIT cells are a subset of innate-like lymphocytes found at multiple mucosal surfaces which have been implicated in antibacterial immunity and multiple disease states.[26, 17, 18, 19, 20, 51, 58, 59, 60] MAIT cells must have strict regulatory mechanisms to prevent hyper-activation mediated immunopathologies in response to riboflavin producing bacteria at mucosal surfaces.[64] Previous evidence in the Ussher Laboratory has indicated that phagocytosis and glycolysis may provide the basis for one regulatory mechanism of MAIT cell activation.[63, 64] Therefore, I hypothesise that bacterial signalling, via phagocytosis of bacteria into APCs, enhances APC glycolysis leading to enhanced availability of MG for increased production of the final MAIT cell activating ligand, 5-OP-RU, for presentation by MR1 and activation of MAIT cells.

MAIT cell activation was assessed to address the following aims:

1. To investigate whether phagocytosis of intact bacteria is required for successful APC licensing for MAIT cell activation, and;

2. To determine if phagocytosis of intact bacteria effects APC glycolysis, leading to altered MAIT cell activation.

# 2 Materials

**Table 2.1: Cell lines**

| Cell line | Description | Source |
|---|---|---|
| THP-1 | Human monocytic cell line | Prof Alex McLellan, University Otago, NZ. |

**Table 2.2: Bacterial stocks**

| Bacteria name | Details | Culture conditions | Original Source | Local Source |
|---|---|---|---|---|
| *Escherichia coli* Δ*ribD* | Strain C93D, derived from strain BSV13 *ribD::Cm*$^{R}$, non-riboflavin producing. Genotype: *lacI*$^{q}$ *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ | LB broth + 20 mg/mL riboflavin at 37°C with shaking at 220 rpm. | Prof Olivier Lantz, Curie Institue, Paris, France. | |
| *E. coli* HB101 | Strain HB101, riboflavin producing. Genotype: F-, *araC14*, *leuB6*(Am), *Δ(gpt-proA)62*, *lacY1*, *glnX44*(AS), *galK2*(Oc), *λ*$^{-}$, *recA13*, *rpsL20*(strR), *xylA5*, *mtl-1*, *thiE1*, *[hsdS20]* | LB broth at 37°C with shaking at 220 rpm. | Prof Herbert Boyer, University of California. | Associate Prof Keith Ireton, University of Otago, NZ. |

**Table 2.3: Media and broth**

| Media or broth name | Components/Preparation |
|---|---|
| R10 | RPMI medium 1640 with L-glutamine (Life Technologies, CA, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS, 56°C for 30 minutes) (Life Technologies), 10,000 U/mL penicillin and 10,000 mg/mL streptomycin (Sigma-Aldrich, MO, USA). |
| R10 + human serum | R10 medium supplemented with 10% human serum (from male AB clotted whole blood, Sigma Aldrich). |
| Freezing media | 90% FBS and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich). |
| Glucose-free (GF) R10 | RPMI medium 1640, no glucose (Life Technologies), supplemented with 10% heat-inactivated FBS, 10,000 U/mL penicillin and 10,000 mg/mL streptomycin. |
| LB broth | 20 g LB broth (Invitrogen) per 1 L distilled water ($dH_2O$). Autoclave. |
| LB + riboflavin broth | 2X autoclaved LB broth mixed in a 1:10 ratio with 40 mg/mL filtered riboflavin (Sigma-Aldrich) to give a final concentration of 1X LB + 20 mg/mL riboflavin. |

**Table 2.4: Buffers**

| Media or broth name | Components/Preparation |
|---|---|
| 1X Permeabilisation buffer | 1 in 10 dilution of 10 X intracellular staining permeabilization wash buffer (BioLegend, San Diego, CA) in MilliQ water. |
| Phosphate buffered saline (PBS) | 1 PBS tablet (Oxoid, ThermoFisher, UK) per 100 mL $dH_2O$. Autoclaved. |
| MACS buffer | 1% FBS, 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), 1X PBS. |

**Table 2.5: Inhibitors and reagents**

| Reagent/inhibitor | Details | Working concentration | Source |
|---|---|---|---|
| 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) | Fluorescent glucose analogue. | 50 μM | Life technologies |
| 2-deoxy-D-glucose (2-DG) | Glucose analogue serving as glycolysis inhibitor. | 2 mM | Sigma-Aldrich |
| 5-amino6-Dribitylaminouracil (5-A-RU) | Synthetic precursor of 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), the MR1 ligand. Riboflavin synthesis pathway intermediate. | 1 μM | Dr Andrea Vernall (Department of Chemistry, University of Otago) |
| Brefeldin A | Inhibitor of protein transport between the endoplasmic reticulum (ER) and Golgi. | 3 μg/mL | BioLegend |
| D-glucose anhydrous | Monosaccharide energy source for cells. | 11 mM | Ajax Finechem Pty Ltd, Austrailia |
| Dimethyl sulfoxide (DMSO) | Solvent for reagent dilution and freezing media. | As stated | Sigma-Aldrich |
| Fructose 1,6-bisphosphate trisodium salt hydrate | Intermediate of glycolysis. Enhances the rate limiting step in glycolysis. | 1 mM | Sigma-Aldrich |
| KU-0063794 (KU) | Dual inhibitor of mTOR1 and mTOR2. | 1 μM | Sigma-Aldrich |
| Methylglyoxal (MG) | *In vivo* glycolysis by-product. Undergoes condensation with 5-A-RU to form 5-OP-RU. | 50X 5-A-RU concentration | Sigma-Aldrich |

| | | | |
|---|---|---|---|
| Paraformaldehyde (PFA) | Cellular fixative agent. | 2% | Sigma-Aldrich |
| LY294002 (LY) | Inhibitor of PI3 kinase p100a/d/b. | 10 μM | Selleck Chemicals |
| Rapamycin | Inhibitor of mTOR1. | 50 nM | Sigma-Aldrich |

**Table 2.6: Cell isolations**

| **Components** | **Details** | **Working concentration** | **Source** |
|---|---|---|---|
| Heparin sodium sulphate | Anticoagulant. | 1,000 IU/mL | Hospira Australia Pty Ltd, Melbourne, Australia |
| Lymphoprep | Density gradient for human PBMC isolation. | Undiluted | Axis-Shield, Dundee, UK |
| CD8 microbeads, human | Magnetic microbeads for $CD8^+$ T cell isolation. | 20 μL per $10^7$ cells (up to $2x10^8$ cells) | Miltenyi Biotec |
| MS column | Magnetic columns for separation of magnetic bead labelled cells. | N/A | Miltenyi Biotec |

**Table 2.7: Antibodies and dyes**

| Antibody/dye | Fluorophore | Clone | Working conc. | Source |
|---|---|---|---|---|
| Anti-CD69 | PerCP/Cy5.5 | FN50 | 2 μg/mL | BioLegend |
| Anti-CD107a | PE | H4A3 | 8 μg/mL | BioLegend |
| Anti-CD15 | PE/Cy7 | W6D3 | 8 μg/mL | BioLegend |
| Anti-CD161 | APC | 191B8 | 0.15 μg/mL | Miltenyl |
| Anti-CD3 | BV510 | OKT3 | 0.15 μg/mL | Biolegend |
| | PE/Cy7 | UCHT1 | 1.5 μg/mL | Biolegend |
| Anti-CD8a | eFluor450 (BV421) | RPA-T8 | 1 μg/mL | BioLegend |
| Anti-interferon-γ (IFNγ) | PerCP/Cy5.5 | 4S.B3 | 2.5 μg/mL | BioLegend |
| Anti-tumour necrosis factor-α (TNFα) | FITC | MAb11 | 4 μg/mL | BioLegend |
| Anti-Vα7.2 | PE/Cy7 | 3C10 | 4 μg/mL | BioLegend |
| | PE | 3C10 | 1 μg/mL | BioLegend |
| aMR1 | PE | 26.5 | 10 μg/mL | Medi'Ray |
| Glut1-AF488 | FITC | 202915 | 6 μg/mL | R&D systems |
| Live/Dead Fixable Near IR dye | N/A | N/A | 1μL of 1:1250 dilution of stock solution | Invitrogen |
| 123count eBeads | N/A | N/A | 1011 beads/μL | eBioscience San Diego, CO |

# 3 Methodology

## 3.1 Cell isolation and culture

### 3.1.1 PBMC isolation

Blood was drawn from healthy donors with informed consent, as approved by the University of Otago Human (Health) Ethics Committee, reference H14/046. 50 mL of heparinised blood (1000 IU/mL heparin sodium sulphate) was collected into sterile 50 mL tubes and diluted 1:1 with pre-warmed PBS. Diluted blood was layered onto a density gradient medium, Lymphoprep, and centrifuged at 800 xg for 30 minutes at room temperature (acceleration = 5; brake = 0). The enriched PBMC fraction was removed, transferred to a fresh tube, and cells were centrifuged at 250 xg for 10 minutes (acceleration and brake = 5). PBMC pellets were washed twice in 50 mL room temperature PBS with centrifugation for 10 minutes at 250 xg. Cells were prepared at $10^7$ cells/mL in cold freezing media (FBS + 10% dimethyl sulfoxide), aliquoted into 1 mL cryovials, and frozen at -80°C in a Mr. Frosty™ Freezing Container with isopropanol. The following day, samples were transferred into liquid nitrogen.

### 3.1.2 $CD8^+$ T cell isolation

Cryopreserved PBMCs were defrosted in a 37°C water bath and added to 10 mL R10 before centrifugation at 400 xg for 5 minutes. Thawed cells were resuspended 2 mL R10 per $1x10^7$ cells and rested at 37°C + 5% $CO_2$ overnight.

The following day, PBMCs were centrifuged at 400 xg for 5 minutes, resuspended in R10, and the cell count determined. PBMCs were then washed twice by centrifugation at 400 xg for 5 minutes and resuspended in 2 mL of MACS buffer. PBMCs were then resuspended in 20 µL CD8 microbeads and 80 µL MACS buffer per $10^7$ cells and incubated for 15 minutes at 4°C in the dark. After incubation cells were washed once in 2 mL of FACS per $10^7$ cells, and

resuspended in 500 µL MACS buffer. MS columns were attached to a MACS magnet and washed once with 500 µL MACS buffer. 500 µL of sample was then run through the MS columns, before washing the column three times with 500 µL MACS buffer. MS columns were transferred into sterile 15 ml tubes before adding 500 µL FACS and pushing a stopper through the column to displace the $CD8^+$ T cells. Isolated $CD8^+$ T cells were washed once and resuspended in 2 mL of R10, counted, and prepared to a final concentration of $10^5$ cells per 100 µL in R10. Cells were seeded at $10^5$ cells/well into a 96-well round-bottom plate containing THP-1 cells. A purity stain of isolated $CD8^+$ T cells was performed by staining with Live/Dead Fixable Near IR dye and ant-CD8 eFluor450. Purities 90% and above were achieved (see Figure 3.3 for gating strategy).

### 3.1.3 THP-1 cell culture

THP-1 cells were cultured in R10 media in 75 $cm^2$ Corning cell culture flasks. Cells were passaged every two days at a concentration of 2 x $10^5$ cells/mL. After 20 passages, cell cultures were discarded. Unless otherwise stated, cells were prepared for assays to a final concentration of $10^5$ cells per 100 µL in R10 and seeded into a 96-well round-bottom plate. The plate was incubated at 37°C + 5% $CO_2$ overnight.

## 3.2 Bacterial culture, fixation, and preparations

### 3.2.1 Preparation of fixed bacterial stocks

Four 10 mL LB or LB + riboflavin broths (Table 2.3) were each inoculated with *E. coli* HB101 or *E. coli* Δ*ribD*, respectively, and incubated overnight at 37°C with shaking at 200 - 220 rpm. The following day, broth cultures were pooled, centrifuged for 8 minutes at 3220 xg, and washed twice in 40 mL PBS. Fixation was performed by resuspension of bacteria in 2 mL 2% paraformaldehyde (PFA) for 20 minutes at 4°C. Following fixation, bacteria were washed twice in PBS, as described above. Following the final centrifugation, bacteria were

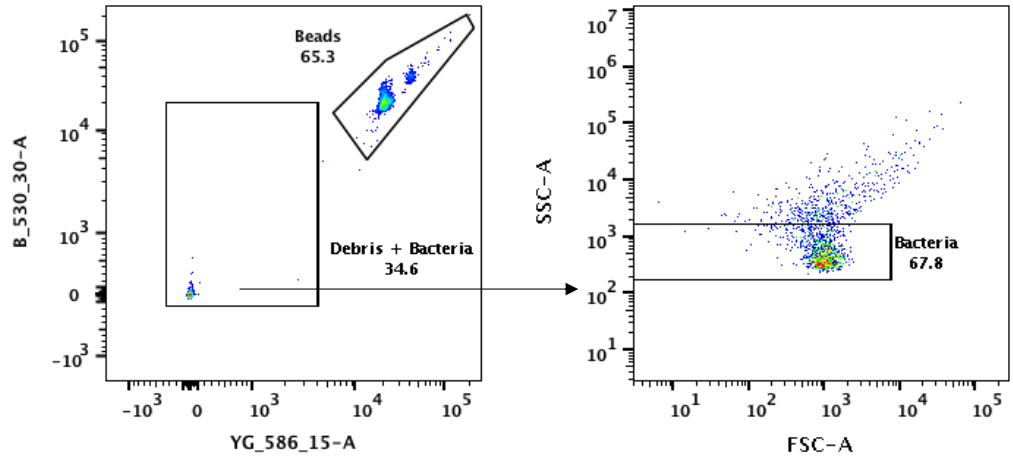
resuspended in 1 mL PBS and stored at 4°C for up to 1 month. The concentration of bacterial stock was determined by flow cytometry using 123count eBeads (Figure 3.1, 3.2).

$$Average\left(\frac{bacteria\ count\ in\ 1\ in\ 1{,}000\ dilution - 'bacteria'\ count\ in\ beads\ only\ sample}{1\ in\ 1{,}000\ bead\ count * 1011 * 1{,}000}\right.$$

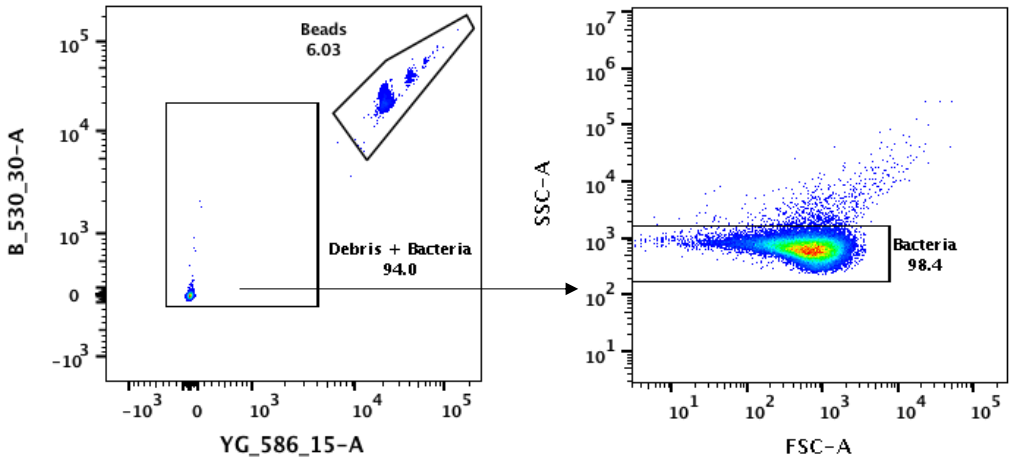
$$\left. + \frac{bacteria\ count\ in\ 1\ in\ 10{,}000\ dilution - 'bacteria'\ count\ in\ beads\ only\ sample}{1\ in\ 10{,}000\ bead\ count * 1011 * 10{,}000}\right)$$

***Figure 3.1 Equation for counting of <u>E. coli</u> bacterial stocks (bacteria/ µL).*** *The concentration of bacterial stock was calculated using two ten-fold dilutions and 123count eBeads (1011 beads/µL).*

A




B




C

| | Beads count | Debris + Bacteria count | Bacteria count - beads | *E. coli* HB101 count | Dilution corrected count (Bacteria/ µL) | Mean (Bacteria/ µL) |
|---|---|---|---|---|---|---|
| **Beads only** | 3000 | 2082 | | | | |
| ***E. coli* HB101 1/1000 + Beads** | 2999 | 292789 | 290716 | 98003.960 | 98003960.0 | $9.21 \times 10^7$ |
| ***E. coli* HB101 1/10,000 + Beads** | 3002 | 27700 | 25618 | 8627.51432 | 8625143.2 | |

***Figure 3.2:Gating strategy for calculation of fixed E. coli ΔribD and E. coli HB101 stock concentrations.*** *Representative plots of (A) a beads only control or (B) E. coli HB101 at 1:1,000 dilution in PBS. (C) Example calculation of E. coli HB101 stock concentration.*

### 3.2.2 Sonication of fixed bacteria

1 mL of fixed bacteria for sonication was prepared in a 15 mL tube at the same concentration as was used for treatment with whole fixed bacteria and kept on ice. Bacteria were sonicated using a SONICS Vibra- cell CV33 for one minute at 20% amplitude, followed by two 30 second pulses at 20% amplitude. Samples were placed on ice between sonication intervals to prevent overheating. Once sonicated, lysates were filtered through a 0.2 μM filter. Bacterial lysates were prepared fresh for each experiment.

### 3.2.3 Opsonisation of bacteria

Fixed bacteria stocks were diluted 1/10 for a final volume of 500 μL and centrifuged for 5 minutes at 1500 xg. The supernatant was removed and bacteria were resuspended in 500 μL R10 + 10% human serum (Sigma Aldrich). Bacteria were incubated at 37°C for 20 minutes with rotation (20 rpm). After incubation, bacteria were centrifuged for 5 minutes at 1500 xg, the supernatant removed, and resuspended in 500 μL R10. Opsonised bacteria were prepared fresh for each experiment.

### 3.2.4 Bacterial treatment of THP-1 cells

Fixed whole and fixed opsonised bacteria were added at a concentration of 10 bacteria per cell (BPC). An equivalent volume of sonicated fixed bacteria to the fixed whole bacteria was added for all assays.

## 3.3 Preparation of 5-A-RU and 5-OP-RU

The synthesis of 5-A-RU has been described previously.[80] Ligand treatment consisted of 1 or 10 nM 5-OP-RU or 1 μM 5-A-RU.[37] Fresh aliquots of 5-A-RU, stored at -80°C, were defrosted for each experiment. 5-OP-RU was prepared by mixing 5-A-RU with MG at a 1:50 molar ratio until a colour change from clear to yellow was observed (1 - 2 minutes). 5-OP-RU was

subsequently diluted to 1 nM and/or 10 nM, based on input concentrations of 5-A-RU. All ligand dilutions were performed using MilliQ water.

### 3.4 MAIT cell activation assays

Unless otherwise stated, $10^5$ THP-1 cells were treated with bacteria (fixed whole, fixed sonicated, or fixed opsonised) and/or ligand (5-A-RU or 5-OP-RU) for 5 hours before three wash steps in 200 µL PBS (centrifugation = 400 xg, 3 min). Following the final centrifugation, THP-1 cells were resuspended in R10 before addition of $10^5$ $CD8^+$ T cells for the final 4 hour incubation. $CD8^+$ T cells were suspended in R10 before addition to the THP-1 cells (as described in figure legends). After 1 hour of THP-1 and $CD8^+$ co-culture, brefeldin A (3 µg/mL) was added to required wells for assessment of cytokine production. Where CD107a expression was assessed, an anti-CD107a-PE antibody was added at the same time as $CD8^+$ T cell seeding. Cells were stored overnight at 4°C following assay completion prior to staining.

In glycolysis enhancement experiments, THP-1 cells were pre-treated with 1 mM fructose 1,6-bisphosphate (F-1,6-BP) in R10 for 1 hour at 37°C + 5% $CO_2$ before the addition of further treatments.

In glycolysis inhibition experiments, THP-1 cells were washed twice in PBS before resuspension in 100 µL of GF-R10. Cells were pre-incubated for 2 hours at 37°C + 5% $CO_2$ before addition of 2-deoxy-D-glucose (2-DG) or D-glucose at a final concentration of 2 $mmolL^{-1}$ or 11 $mmolL^{-1}$ per well, respectively. Cells were incubated for 1 hour before the addition of further treatments. 2-DG treatment concentration was optimised by assessment of MAIT cell activation by THP-1 cells treated with 2, 4, 10, 20, 50, 100, or 150 $mmolL^{-1}$ 2-DG per well. Inhibition of glycolysis was alternatively assessed by pre-treatment of THP-1 cells

for one hour with either 10 μM LY294002 (LY), 50 nM rapamycin (Rapa), or 1 μM KU 0063794 (KU), in glucose-containing R10 before the addition of bacterial treatments.

### 3.5 Assessment of 2-NBDG uptake by THP-1 cells

To assess glucose uptake following bacterial treatment, THP-1 cells were treated with 50 μM 2-NBDG with or without fixed whole *E. coli* Δ*ribD* for between 10 minutes and 1 hour. A second matched set of cells lacking 2-NBDG treatment were prepared simultaneously for normalisation. After treatment cells were washed twice with cold PBS (4°C). Cells were fixed in 100 μL 2% PFA for 25 minutes at 4°C in the dark. The THP-1 cells were washed twice in cold PBS (4°C) and resuspended in 200 μL PBS, transferred into FACS tubes, and run immediately on the flow cytometer.

### 3.6 Assessment of THP-1 cell GLUT-1 expression

To assess GLUT-1 expression, THP-1 cells were treated with fixed whole *E. coli* Δ*ribD* at the following timepoints: 0, 30 minutes, and hourly for up to 4 hours. Following treatment, the cells were washed twice with PBS and stained with anti-GLUT-1-AF488 and Live/Dead fixable near IR dye. A second set of cells lacking staining with anti-GLUT-1-AF488 were prepared simultaneously. After fixation, the THP-1 cells were suspended in 200 μL PBS and transferred into FACS tubes for flow cytometry.

### 3.7 Immunostaining

All wash steps consisted of centrifugation of sample plates (400 xg, 3 min), removal of supernatant, and resuspension in 200 μL PBS, unless otherwise stated. Treated cells were washed twice and resuspended in 50 μL of surface antibody master mix consisting of: anti-CD161 APC, anti-CD69 PerCP/Cy5.5 (when required), and Live/Dead fixable near IR dye in PBS. Cells were incubated for 25 minutes at 4°C in the dark. After incubation, cells were

washed twice in PBS and fixed in 100 µL of 2% PFA for 10 minutes at room temperature (RT) in the dark, unless otherwise indicated. The cells were washed twice in PBS, then once in 1X Permeabilisation Wash Buffer (PWB). Cells were resuspended in 50 µL of intracellular antibody master mix prepared in PWB, consisting of: Vα7.2 PE or PE/Cy7, CD3 BV510 or PE/Cy7, CD8 eFluor450, and when required IFNγ PerCP/Cy5.5 and TNFα FITC. Cells were incubated for 25 minutes at RT in the dark. After incubation, the cells were washed once in PWB and once in PBS, before resuspension in 200 µL PBS and transfer into FACS tubes for sample collection.

### 3.8 Analytical flow cytometry

Stained cells were transferred into FACS tubes for data collection using either a BD FACSCanto II or BD LSRFortessa flow cytometer. Analysis of FCS files was performed on FlowJo version 10.6.2 (TreeStar, USA). Positive gates were set on untreated cells for proinflammatory cytokines, IFNγ and TNFα, as well as cytotoxic marker CD107a. CD69 gating was determined by gating between the two cell populations observed in untreated samples. Gating strategies are depicted in Figures 3.2 to 3.6, inclusive, below.

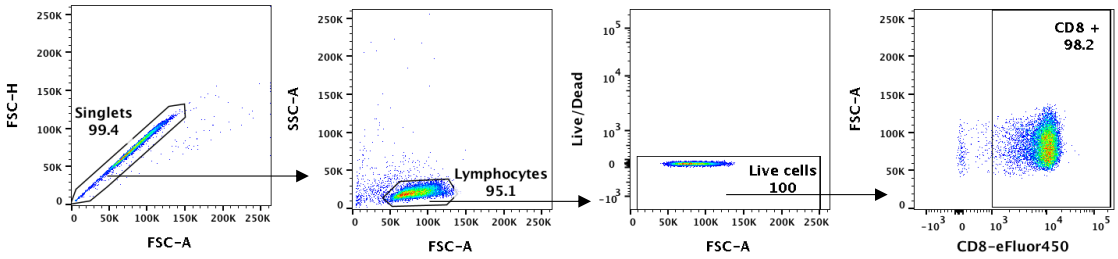



***Figure 3.3 Gating strategy to assess CD8⁺ T cell purity****. CD8⁺ T cells were defined as single cells (singlets)/lymphocytes/live/CD8⁺. Forward Scatter Height = FSC-H, Forward Scatter Area = FSC-A.*

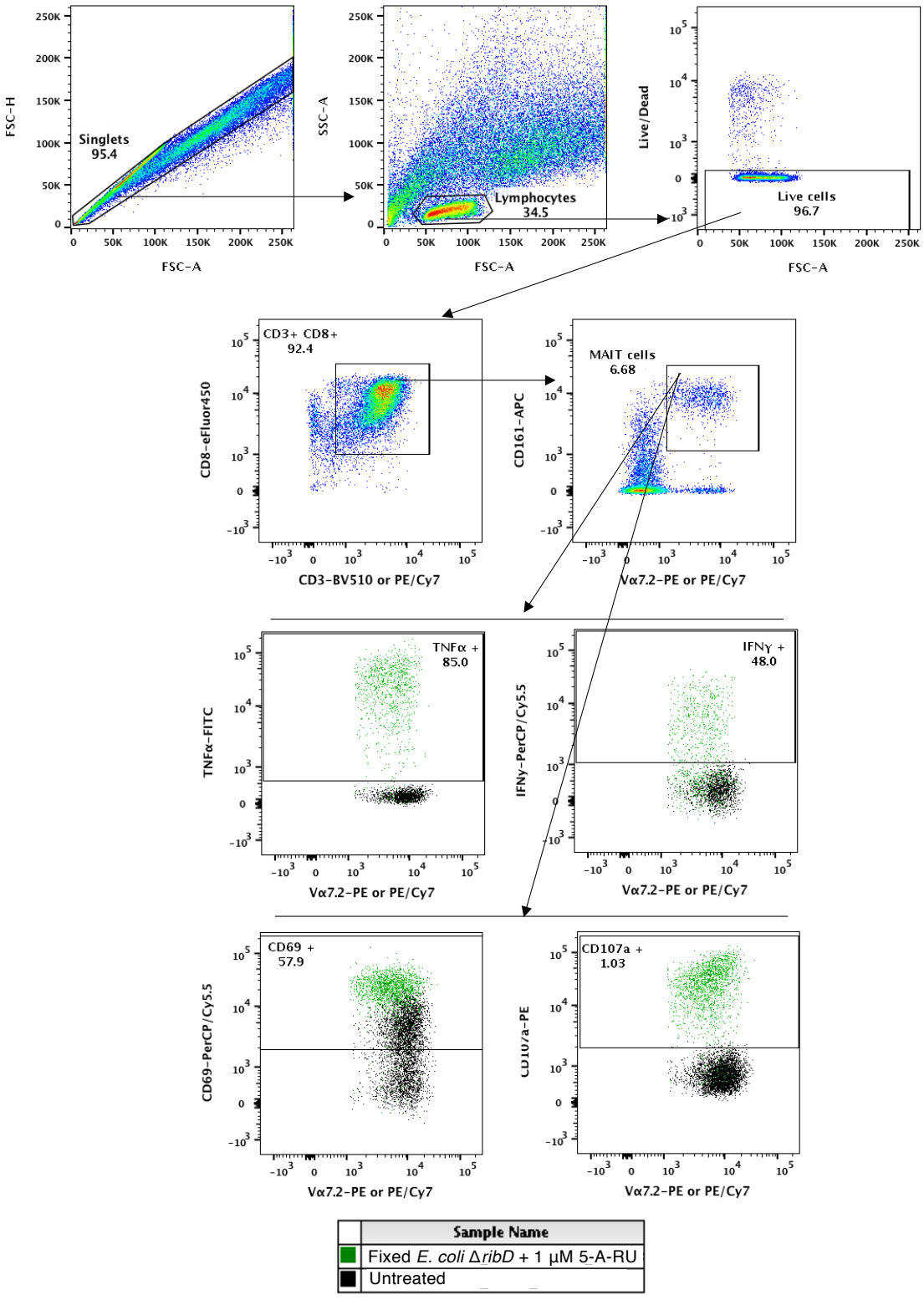



***Figure 3.4 Gating strategy for the identification and characterisation of MAIT cells.*** *Cells were first gated for single cells (singlets) prior to identification of lymphocytes by size and granularity (FSC and SSC, respectively). Dead cells were excluded from assessment and MAIT cells identified as $CD3^{+}/CD8^{+}/CD161^{++}/Vα7.2^{+}$. Gating for assessment of MAIT cell activation is shown (IFNγ, TNFα, CD69 and CD107a). Representative pseudoplots from one*

*donor left untreated or treated with fixed E. coli ΔribD + 1 μM 5-A-RU are shown. Forward Scatter Area (FSC-A), Side Scatter Area (SSC-A), and Forward Scatter Height (FSC-H).*

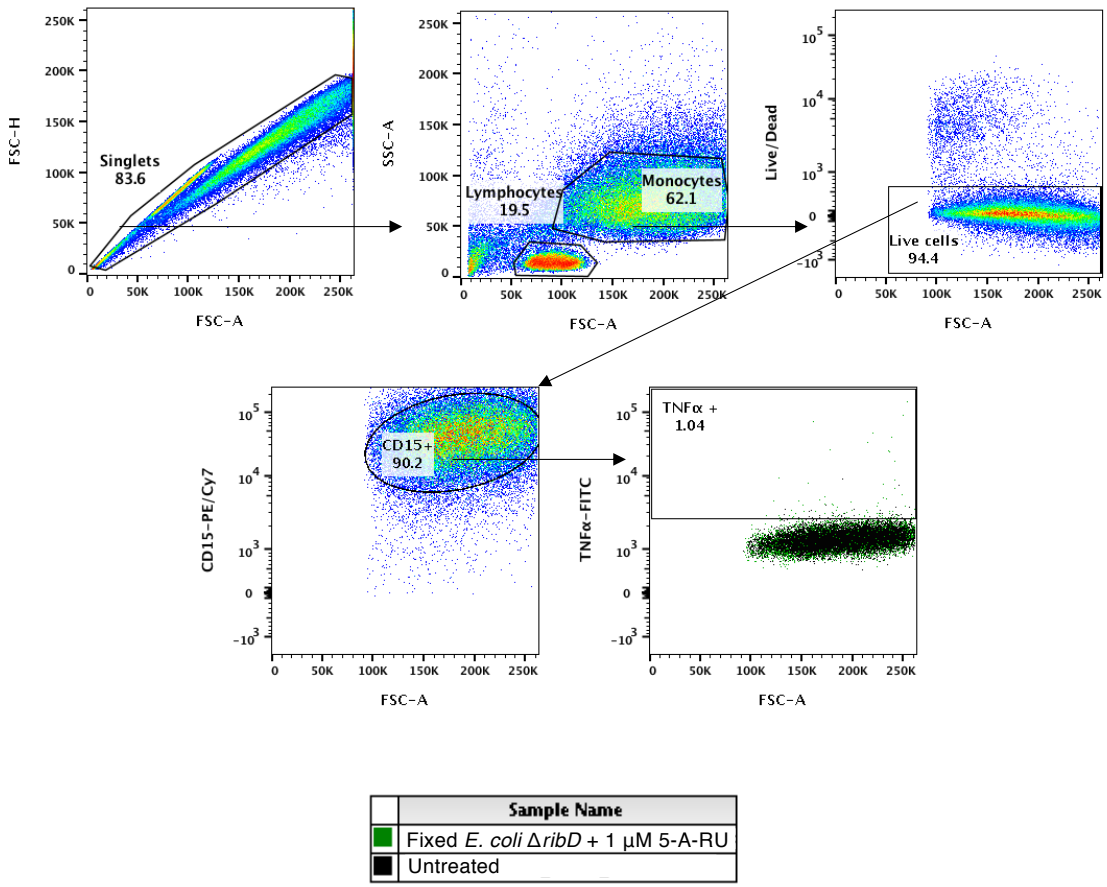



***Figure 3.5: Gating strategy for assessment of TNFα expression by THP-1 cells****. Cells were first gated for single cells (singlets) prior to identification of monocytes by size and granularity (FSC and SSC, respectively). THP-1 cells were identified as CD15$^{+}$ monocytes. Representative pseudoplots from one donor left untreated and/or treated with fixed E. coli ΔribD + 1 µM 5-A-RU are shown. Forward Scatter Area (FSC-A), Side Scatter Area (SSC-A), and Forward Scatter Height (FSC-H).*

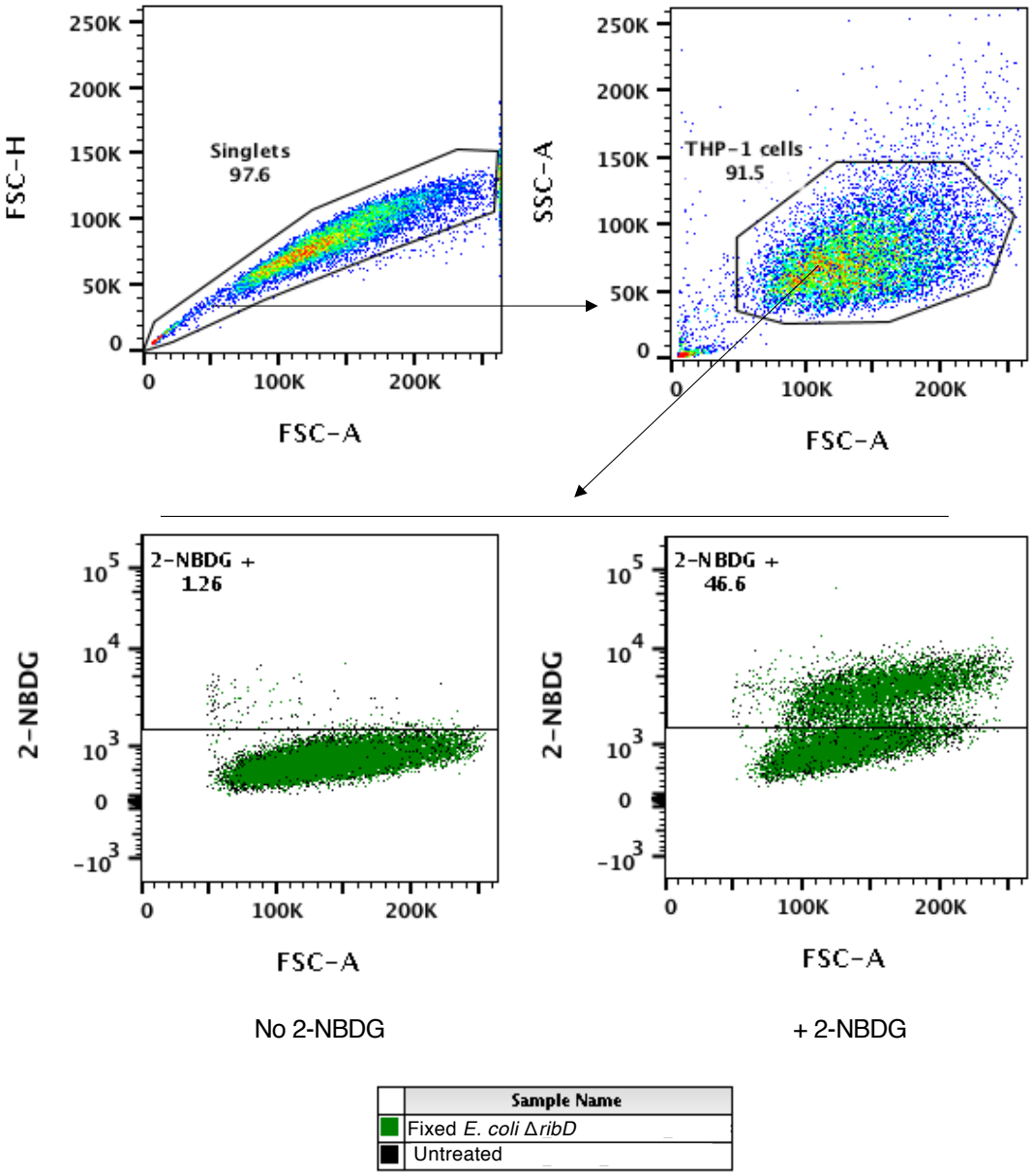



***Figure 3.6: Gating strategy used to determine 2-NBDG uptake by THP-1 cells.*** *THP-1 cells were defined as singlets/THP-1 cells. Representative pseudoplots from one biological replicate left untreated or treated with fixed E. coli ΔribD are shown. Cells were incubated with (right) or without (left) 50 µM 2-NBDG. Forward Scatter Area (FSC-A), Side Scatter Area (SSC-A), and Forward Scatter Height (FSC-H).*

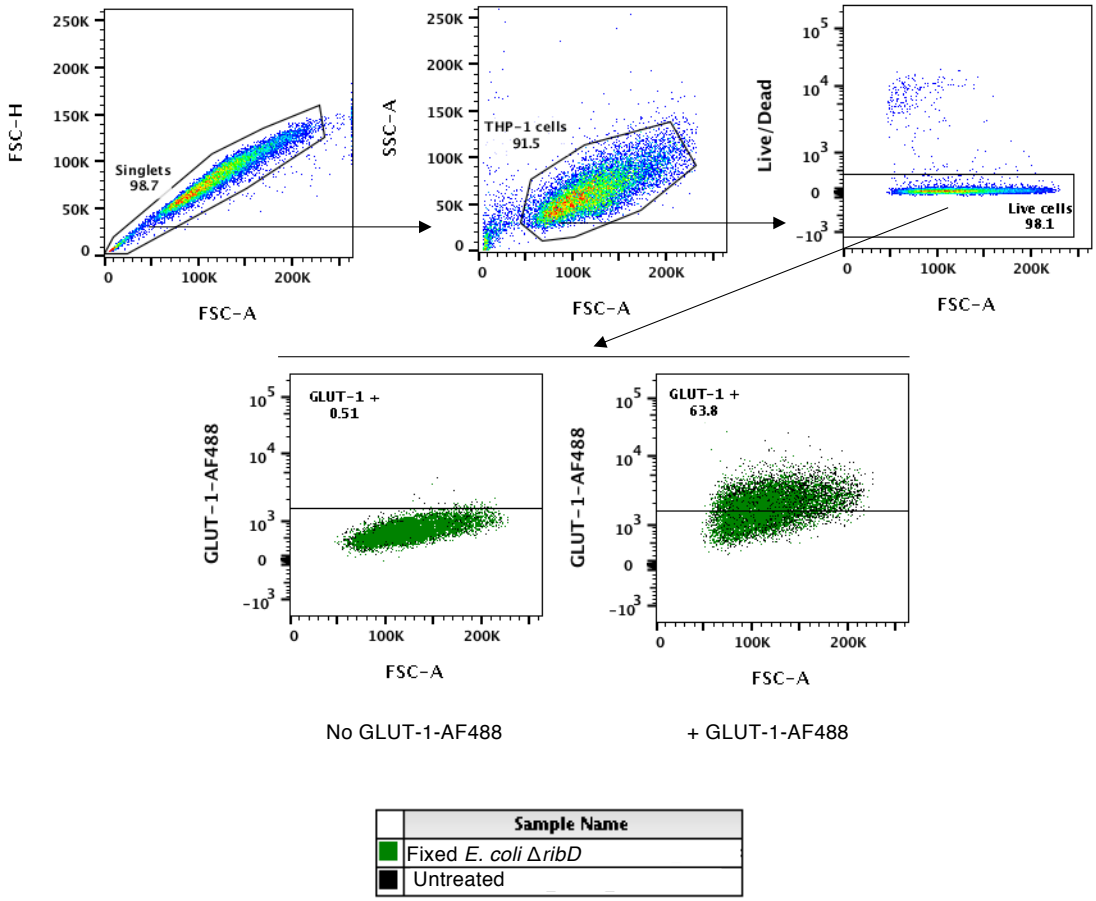



***Figure 3.7: Gating strategy used to determine GLUT-1 expression by THP-1 cells.*** *Cells were first gated for single cells (singlets) prior to identification of THP-1 monocytes by size and granularity (FSC and SSC, respectively). Dead cells were excluded from assessment by live/dead staining. Representative pseudoplots from one biological replicate left untreated or treated with fixed E. coli ΔribD are shown. Cells were stained with (right) or without (left) anti-GLUT-1-AF488. Forward Scatter Area (FSC-A), Side Scatter Area (SSC-A), and Forward Scatter Height (FSC-H).*

## 3.9 Statistical analysis

Data was analysed using GraphPad Prism software, version 8.4.0. Data are presented as mean with the standard error of the mean (SEM). Statistical analyses used for specific experiments are described in figure legends. Normality of the data was assessed using a Shapiro-Wilk test. If the data was normally distributed, multiple groups were compared by repeated measures one-way ANOVA, with comparisons made between groups using the Sidak's multiple

comparisons test. If comparisons between two treatments were made, two-tailed, repeated measures two-way ANOVA was used, with multiple comparisons made using Bonferroni's multiple comparisons test.

If the data in an experiment comparing multiple groups was deduced to be non-normal, a Friedman's test was used to compare multiple groups. When two treatment groups were being simultaneously assessed for non-normal data, an arcsine transformation was performed. Normality was then re-assessed, and a two-tailed, repeated measures two-way ANOVA was performed, with multiple comparisons made using Bonferroni's multiple comparisons test. Statistical significance was defined as a two-tailed p value of 0.05 or below. Statistical guidance was sought from a statistician, Dr Matthew Parry, before data analysis.

# 4 Results

## 4.1 Bacteria differentially regulate MAIT cell activation in response to 5-A-RU or 5-OP-RU

Past data (unpublished, Ussher Laboratory) using PBMC cultures observed enhanced MAIT cell activation upon treatment with 5-A-RU, but not 5-OP-RU, in conjunction with fixed *E. coli* Δ*ribD*.[63] This effect was mitigated in the presence of sonicated *E. coli* Δ*ribD.*[63] To confirm the past data, THP-1 cells were treated with 5-A-RU or 5-OP-RU and either fixed or sonicated *E. coli* Δ*ribD* for ten hours (Figure 4.1A). $CD8^+$ T cells were added for the final four hours. *E. coli* HB101 treatment was included as a control (Figure 4.1A). MAIT cell activation was determined by measurement of production of proinflammatory cytokines, IFNγ and TNFα.

No change was observed in MAIT cell IFNγ or TNFα production upon THP-1 cell treatment with 1 nM 5-OP-RU and fixed or sonicated *E. coli* Δ*ribD,* compared to cells treated with 1 nM 5-OP-RU alone (Figure 4.1B, E). The production of IFNγ and TNFα by MAIT cells increased across all treatments with increased 5-OP-RU concentration (Figure 4.1B, E). Furthermore, no change was seen in IFNγ production upon treatment with 10 nM 5-OP-RU, irrespective of bacterial treatment (Figure 4.1B, E). MAIT cell TNFα production increased significantly when THP-1 cells were treated with 10 nM 5-OP-RU and fixed *E. coli* Δ*ribD,* compared to 10 nM 5-OP-RU alone (Figure 4.1 E).

Differences in MAIT cell activation were observed when THP-1 cells were treated with 5-A-RU and fixed or sonicated *E. coli* Δ*ribD*. There was a non-significant trend towards increased MAIT cell IFNγ production in response to 1 μM 5-A-RU and fixed *E. coli* Δ*ribD*, compared to 5-A-RU alone (Figure 4.1B). No difference in MAIT cell IFNγ production was observed between the 5-A-RU alone and 5-A-RU plus sonicated *E. coli* Δ*ribD* treatments

(Figure 4.1B). A significant increase in TNFα production was observed with the addition of *E. coli* Δ*ribD* and 5-A-RU treatment compared to 5-A-RU alone (Figure 4.1E). This effect was mitigated when THP-1 cells were treated with sonicated *E. coli* Δ*ribD* and 5-A-RU (Figure 4.1E). MAIT cell activation was prevented with the addition of αMR1 for all treatments, indicating that MAIT cell activation occurred in an MR1-dependent manner (Figure 4.1B, E).

The ratios of MAIT cell IFNγ and TNFα production altered in response 5-A-RU in conjunction with fixed or sonicated *E. coli* Δ*ribD*. IFNγ and TNFα production was significantly higher with fixed *E. coli* Δ*ribD* than with sonicated *E. coli* Δ*ribD* (Figure 4.1C, F). Conversely, no statistically significant changes were observed between the ratios of MAIT cell IFNγ and TNFα production upon 5-OP-RU treatment in conjunction with fixed and sonicated *E. coli* Δ*ribD* (Figure 4.1D, G). Overall, these results indicated that effective MAIT cell activation by 5-A-RU was increased with fixed bacteria, whereas activation with 5-OP-RU was less affected by bacteria.

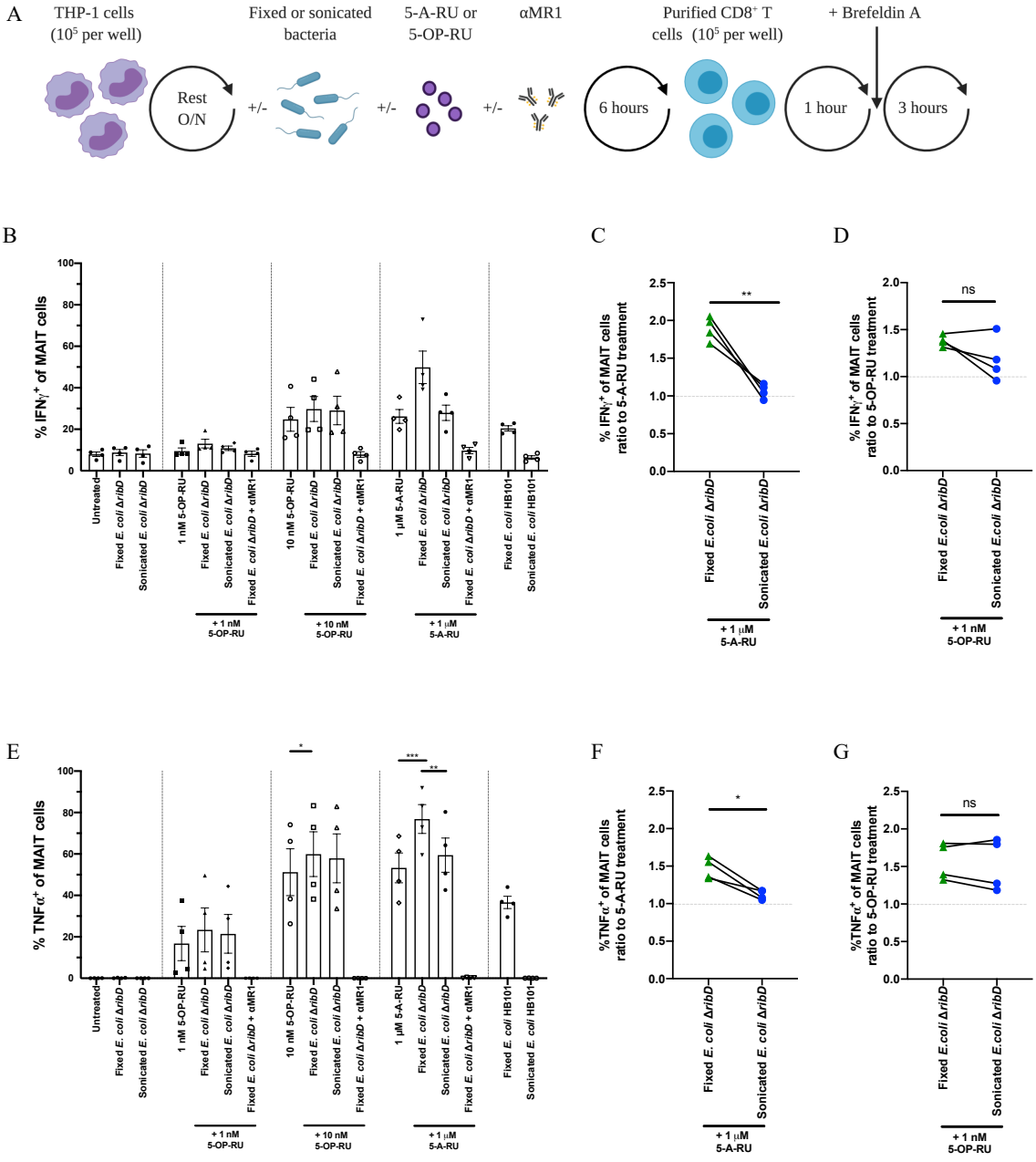



***Figure 4.1: Sonication of bacteria disrupts the response to 5-A-RU stimulation***. *(A) Schematic depicting the MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. THP-1 cells were treated for 6 hours with bacteria ± 5-A-RU or 5-OP-RU ± αMR1, as indicated. CD8⁺ T cells were added to the co-culture and incubated for 1 hour. Brefeldin A (3 μg/mL) was then added for the final 3 hours. Schematic created with BioRender.com. (B - E) THP-1 cells were treated with 10 bacteria per cell (BPC) fixed or sonicated E. coli HB101 or E. coli ΔribD ± 1 nM or 10 nM 5-OP-RU, or 1 μM 5-A-RU ± 10 μg/mL αMR1. The production of (B) IFNγ and (E) TNFα by MAIT cells was assessed by intracellular staining and flow cytometry (n = 4). MAIT cell production of (C) IFNγ or (F) TNFα in response to 5-A-RU and fixed or sonicated E. coli ΔribD normalised to 5-A-RU treatment alone. MAIT cell production of (D) IFNγ or (G) TNFα in response to 5-OP-RU and fixed or sonicated E. coli ΔribD normalised to 5-OP-RU treatment alone. Data points represent individual blood donors tested in three independent experiments. Statistical*

*significance was calculated by a Friedman test with Dunn's multiple comparisons test (B), one-way ANOVA with Sidak's multiple comparisons test (E), or a paired T test (C, D, F, and G). ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.001.*

### 4.2 Magnitude of MAIT cell activation by THP-1 cells varies based on treatment time

Previous experiments performed in the Ussher Laboratory incubated THP-1 cells with bacteria and 5-A-RU for a total of 10 hours in order to assess MAIT cell activation. The incubation time was based on past data (unpublished, Ussher Laboratory) which found the highest levels of MR1 expression on THP-1 cells occurred after 10 hours.[81] However, more recent experiments have suggested that the abundance of MR1 at the cell surface may not correlate with the level of MAIT cell activation (unpublished, Ussher Laboratory). Therefore, a time course was performed to determine the optimal pre-incubation time for THP-1 cells before MAIT cell addition.

The optimal assay incubation time was assessed by treatment of THP-1 cells hourly over 6 hours before the addition of $CD8^+$ T cells. THP-1 cells were treated with fixed *E. coli* Δ*ribD* and either 5-OP-RU, 5-A-RU, or fixed *E. coli* HB101 and incubated for 2 - 6 hours (Figure 4.2A). THP-1 cells were treated with fixed *E. coli* Δ*ribD* alone for either 2 or 6 hours (Figure 4.2A). $CD8^+$ T cells were then co-cultured with the THP-1 cells for a further four hours and production of proinflammatory cytokines assessed. The percentage of MAIT cells producing IFNγ or TNFα, as well as production of TNFα by THP-1 cells, was then determined.

MAIT cell production of both IFNγ and TNFα gradually increased with time across samples treated with 5-A-RU or *E. coli* HB101 (Figure 4.2B, C). Where samples were treated with 5-A-RU and *E. coli* Δ*ribD*, no marked difference in cytokine production was observed between 8 - 10 hour treatment times (Figure 4.2B, C). Treatment with 5-OP-RU, with or without *E. coli* Δ*ribD,* showed minimal variation in cytokine production between time points (Figure

4.2B, C). Minimal production of TNFα by THP-1 cells (monocytes) was detected; this was highest in samples treated with bacteria and peaked at early time points, and decreased over time (Figure 4.2D). As expected, no MAIT cell activation was observed at 6 or 10 hours with *E. coli* Δ*ribD* treatment alone (Figure 4.2B, C).

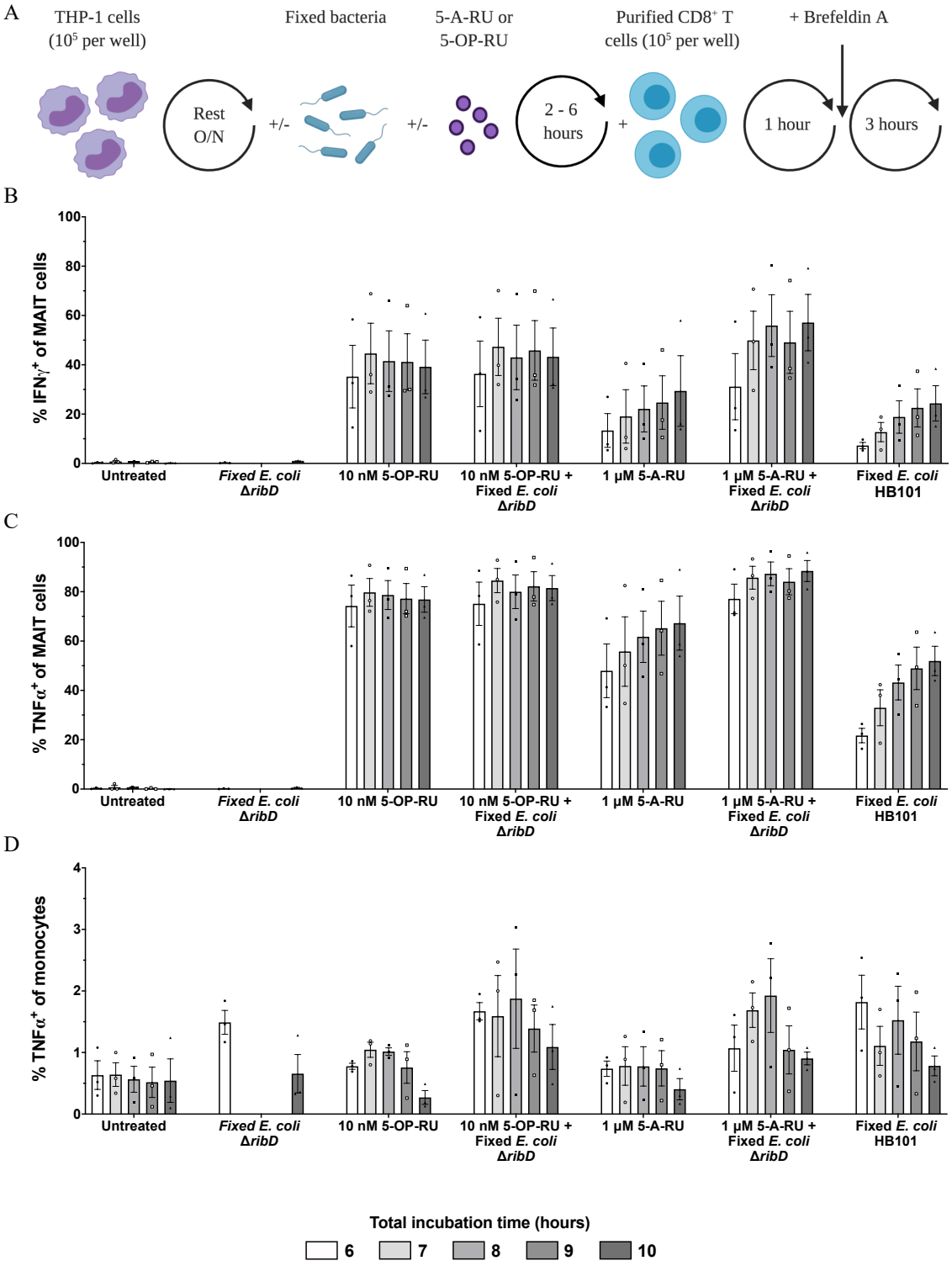



***Figure 4.2: MAIT cell activation enhanced with increased THP-1 cell exposure to treatment: no wash step.*** *(A) Schematic diagram depicting the MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B-D) THP-1 cells were treated for 2 - 6 hours with 10 BPC fixed E. coli HB101 or E. coli ΔribD ± 10 nM 5-OP-RU or 1 μM 5-A-RU, as indicated. Isolated CD8⁺ T cells were added for a further 4 hours, with addition of brefeldin A (3 μg/mL) for the final 3 hours. MAIT cell IFNγ (B) and TNFα (C) production and THP-1 cell TNFα production*

*(D) was assessed by intracellular staining and flow cytometry. Data points represent individual blood donors (n = 3) tested in three independent experiments. Due to the COVID-19 lockdown, testing was limited to three donors, therefore statistical comparisons were not made.*

Subsequent experiments require washing of THP-1 cells prior to the addition of $CD8^+$ T cells, removing potentially important secreted co-stimulatory molecules produced by the THP-1 cells. Therefore, a second time course was performed to deduce whether washing THP-1 cells would alter the magnitude of MAIT cell activation. To achieve this THP-1 cells were treated with the same treatments as seen in Figure 4.2 every hour for a total of 6 hours. After 6 hours the THP-1 cells were washed three times in PBS before the addition of $CD8^+$ T cells for a further four hours (Figure 4.3A). $CD8^+$ T cells were then stained for production of proinflammatory cytokines. The percentage of MAIT cells producing IFNγ or TNFα, as well as production of TNFα by THP-1 cells, was then determined.

MAIT cell activation was similar when THP-1 cells were washed (Figure 4.3), compared to when they were not washed prior to the addition of $CD8^+$ T cells (Figure 4.2). MAIT cell activation increased over time with 5-A-RU, *E. coli* HB101 and, for IFNγ production, 5-A-RU plus *E. coli* Δ*ribD,* reaching peak activation at nine hours (Figure 4.3B, C). Minimal production of TNFα by THP-1 cells was seen; increased production occurred with addition of whole bacteria, peaking at 8 hours total incubation time (Figure 4.3D). Conversely, TNFα by THP-1 cells remained unchanged in ligand only treatments (Figure 4.3D). Therefore, subsequent assays were incubated for nine hours total.

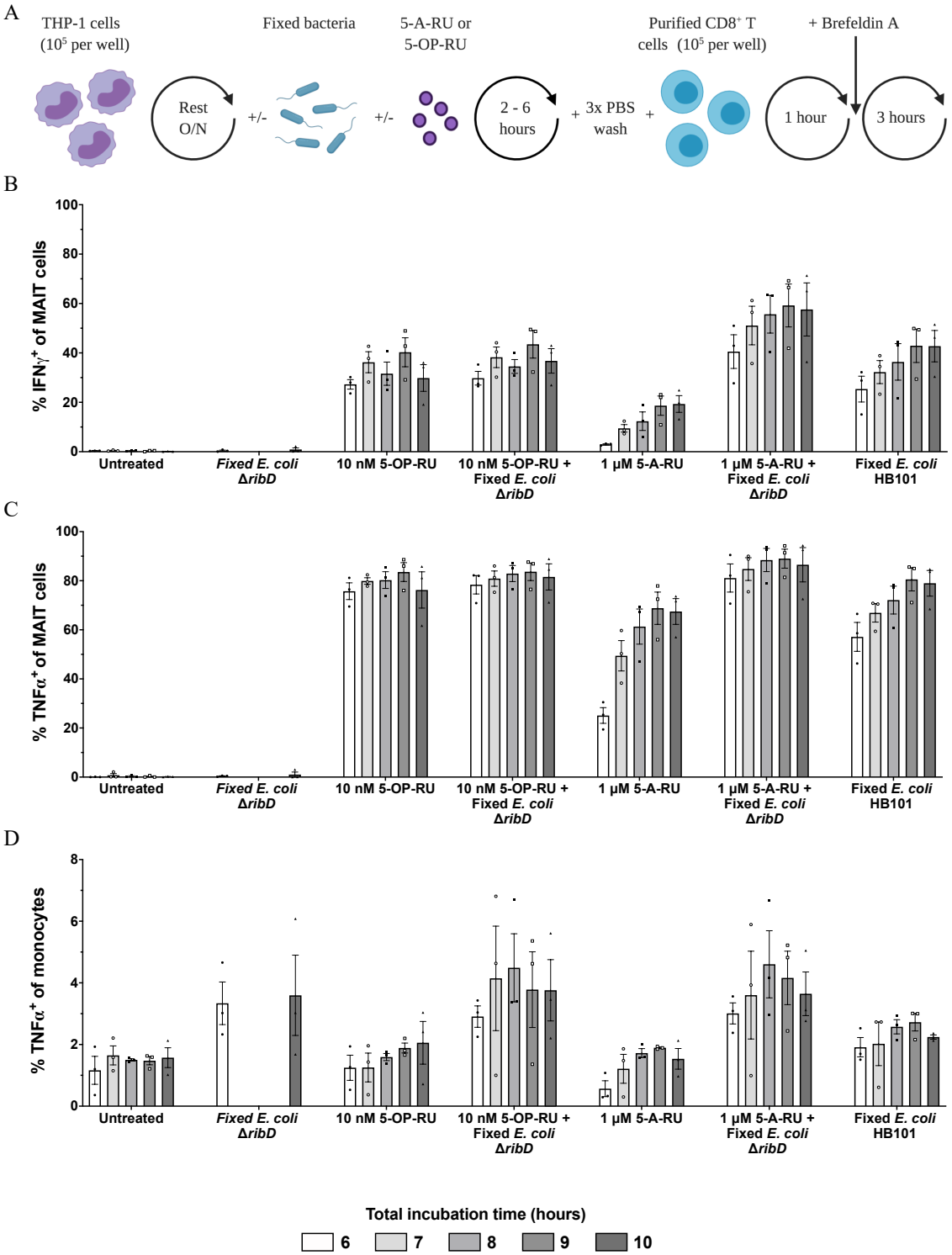



***Figure 4.3: MAIT cell activation enhanced with increased THP-1 cell exposure to treatment: with PBS wash step.*** *(A) Schematic diagram depicting the MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B - D) THP-1 cells were treated for 2 - 6 hours with 10 BPC fixed E. coli HB101 or E. coli ΔribD ± 10 nM 5-OP-RU or 1 μM 5-A-RU, as indicated. Three PBS washes of THP-1 cells occurred prior to the addition of CD8⁺ T cells. Isolated CD8⁺ T cells were added and the culture incubated for a further 4 hours, with addition of brefeldin A*

*(3 μg/mL) for the final 3 hours. MAIT cell IFNγ (B) and TNFα (C) production and THP-1 cell TNFα production (D) was assessed by intracellular staining and flow cytometry. Data points represent individual blood donors (n = 3) tested in two independent experiments. Due to the COVID-19 lockdown, testing was limited to three donors, therefore statistical comparisons were not made.*

## 4.3 Modulation of THP-1 cell glycolysis affects MAIT cell activation

The differences observed in Figure 4.1 indicated that THP-1 cell treatment with whole bacteria enhanced the MAIT cell response to 5-A-RU but not to 5-OP-RU. MG, a glycolytic by-product, reacts with 5-A-RU to produce the MAIT cell activating ligand, 5-OP-RU.[4] Additionally, activation of endosomal TLRs, typically activated upon phagocytosis of microorganisms, leads to an increase in APC glycolysis.[74] Therefore, I hypothesised treatment with whole bacteria was enhancing the conversion of 5-A-RU to 5-OP-RU through enhanced glycolysis within THP-1 cells. Previous experiments performed in PBMCs indicated that glycolysis modulation, either enhancement or inhibition, was able to alter MAIT cell actvation.[63] However, a secondary effect of the modulators on MAIT cells could not be excluded in the PBMC system. Therefore, to investigate the role of glycolysis specifically within APCs, THP-1 cells were pre-treated with glycolysis modulators before bacterial treatments and subsequent $CD8^+$ T cell co-culture.

### 4.3.1 Enhancing THP-1 cell glycolysis augments MAIT cell activation in the absence of whole bacteria

Fructose-1,6-bisphosphate (F-1,6-BP), the substrate for aldolase, a rate limiting enzyme of the glycolytic pathway, was used to enhance glycolysis within the THP-1 cells.[82] THP-1 cells were pre-treated with F-1,6-BP for one hour before treatment with 5-A-RU and fixed, sonicated, or opsonised *E. coli* Δ*ribD*, or 5-OP-RU for 5 hours (Figure 4.4A). Opsonisation of *E. coli* Δ*ribD* enhances phagocytosis of intact bacteria by APCs, and therefore was used to investigate if enhancement of phagocytosis further modulates the MAIT cell response. $CD8^+$ T cells were co-cultured with THP-1 cells for 4 hours. MAIT cell activation was assessed by staining for

IFNγ and TNFα production, as well as the degranulation marker, CD107a, and activation marker, CD69.

Prior treatment of THP-1 cells with F-1,6-BP that were subsequently treated with 5-A-RU alone or with sonicated *E. coli* Δ*ribD* significantly increased MAIT cell production of IFNγ and TNFα, compared to the equivalent samples that were not pre-treated with F-1,6-BP (Figure 4.4B, C). This change was also observed in the CD107a geometric mean florescence intensity (geoMFI), but not CD69 geoMFI (Figure 4.4D, E). No change in IFNγ, TNFα, CD107a or CD69 was observed with F-1,6-BP pre-treatment with 5-A-RU in conjunction with fixed or opsonised *E. coli* Δ*ribD* (Figure 4.4B, C, D, E). Furthermore, no change in MAIT cell activation with F-1,6-BP pre-treatment was seen with 5-OP-RU treatment (Figure 4.4B, C, D, E).

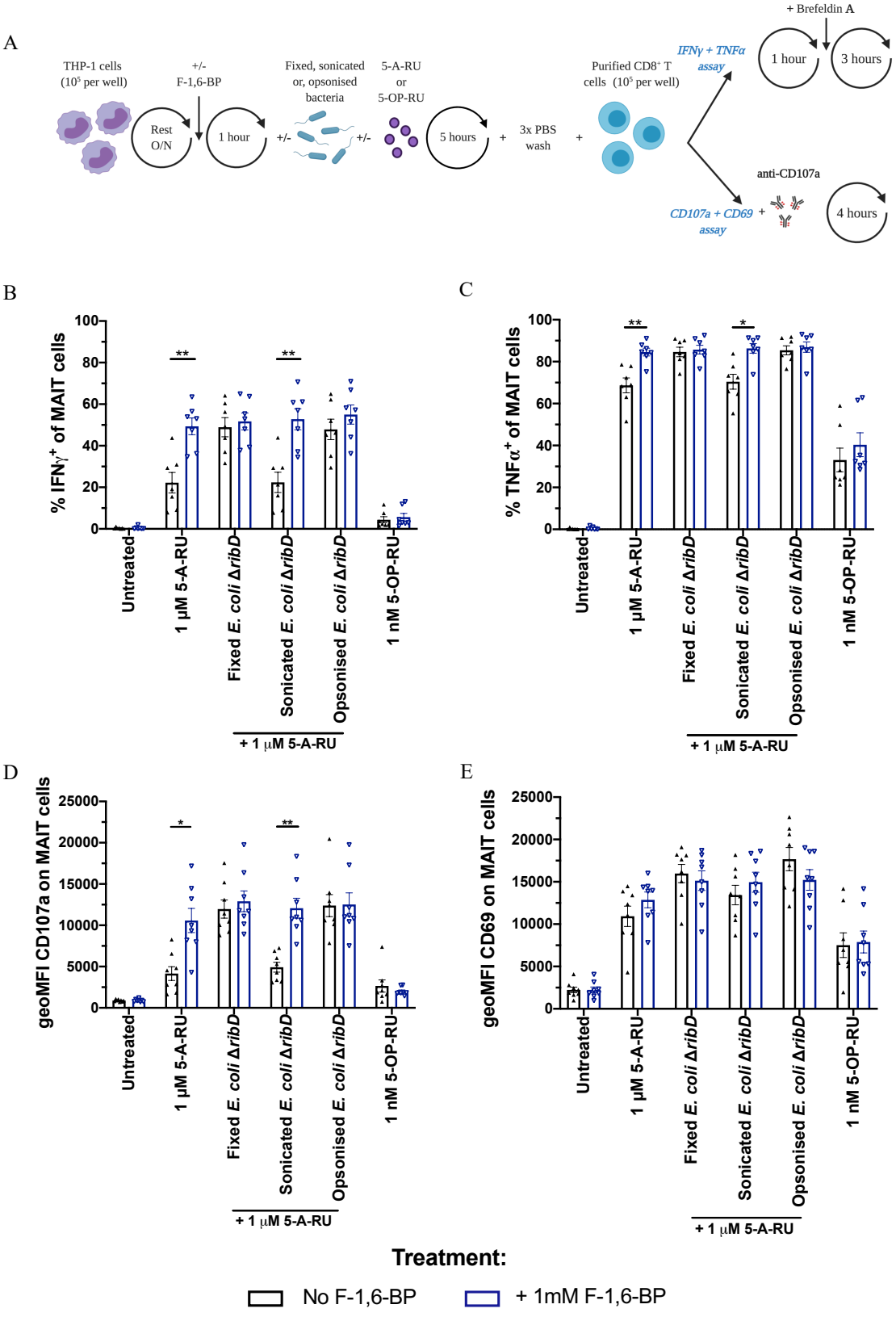



***Figure 4.4 Enhancement of glycolysis in THP-1 cells augments MAIT cell activation by 5-A-RU in cultures lacking whole bacteria.*** *(A) Schematic depicting MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic*

*created with BioRender.com. (B - E) THP-1 cells were pre-treated with F-1,6-BP for 1 hour before treatment with 1 μM 5-A-RU ± 10 BPC fixed, opsonised, or equivalent sonicated E. coli ΔribD or 10 nM 5-OP-RU alone for 5 hours. After washing, isolated CD8$^+$ T cells were added and incubated for a further 4 hours, with addition of brefeldin A (3 μg/mL) for the final 3 hours or anti-CD107a for the final 4 hours. MAIT cell IFNγ (B), TNFα (C) production and CD107a (D) and CD69 (E) surface expression was assessed. Data points represent individual blood donors tested in three independent experiments (n = 8). Treatments were analysed by an arcsine transformation with repeated measures two-way ANOVA with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test (B, C) or repeated measures two-way ANOVA with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test (D, E). *, p<0.05; **, p<0.01.*

The effect F-1,6-BP pre-treatment of THP-1 cells treated with bacteria containing an intact riboflavin synthesis pathway was then assessed. THP-1 cells were pre-treated with F-1,6-BP for one hour before treatment with *E. coli* HB101 for 5 hours (Figure 4.5A). CD8$^+$ T cells were subsequently co-cultured with THP-1 cells for 4 hours. MAIT cell activation was assessed by staining for characteristic proinflammatory cytokines, IFNγ and TNFα.

No change was observed in MAIT cell IFNγ and TNFα production with F-1,6-BP pre-treatment of THP-1 cells in samples treated with fixed *E. coli* HB101 (Figure 4.5B, C). This is consistent with the results observed when THP-1 cells were treated with fixed *E. coli* Δ*ribD* and 1 μM 5-A-RU (Figure 4.4B, C).

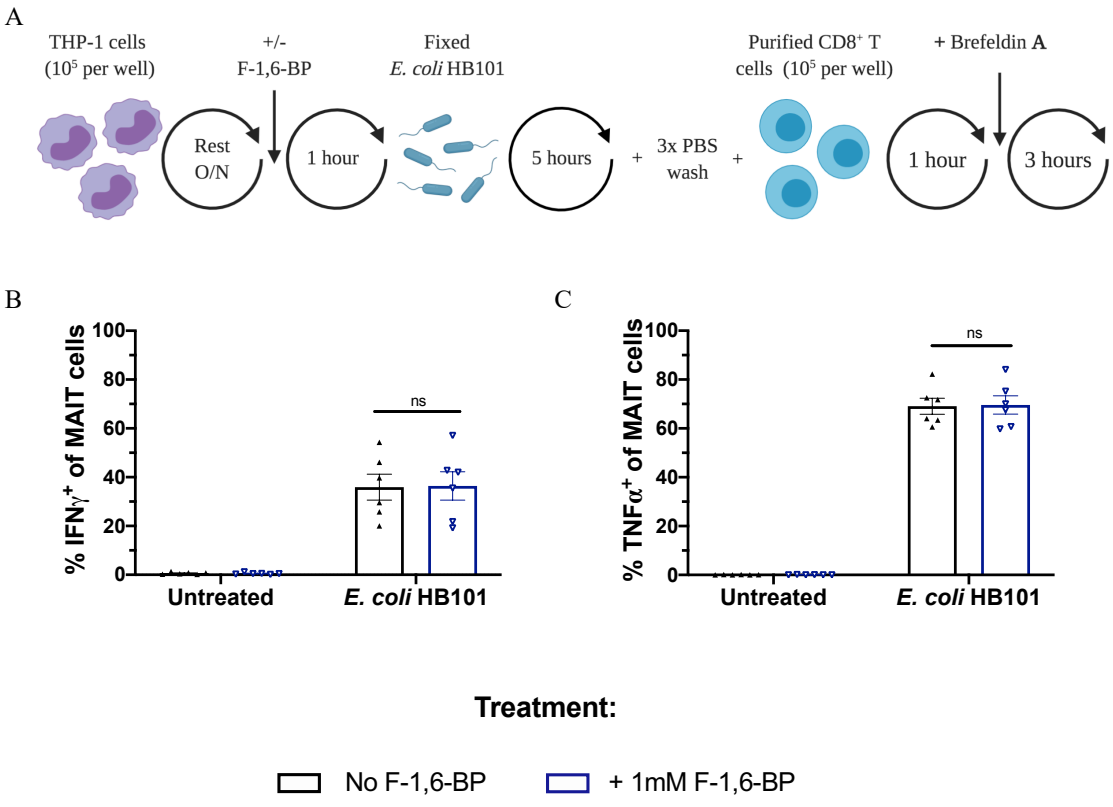



***Figure 4.5: Enhancement of THP-1 glycolysis does not alter MAIT cell activation by E. coli HB101.*** *(A) Schematic depicting MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B - C) THP-1 cells were pre-treated with F-1,6-BP for 1 hour before treatment with 10 BPC fixed E. coli HB101 for 5 hours. Isolated CD8⁺ T cells were added and incubated for a further 4 hours, with addition of brefeldin A (3 µg/mL) for the final 3 hours. MAIT cell IFNγ (B) and TNFα (C) production was assessed. Data points represent individual blood donors tested in two independent experiments (n = 6). Treatments were analysed by repeated measures two-way ANOVA with Bonferroni's multiple comparisons test (B, C). ns, non-significant.*

### 4.3.2 Inhibition of THP-1 cell glycolysis does not alter MAIT cell activation

To further explore the role of glycolysis in THP-1 cells on MAIT cell function, glycolysis was inhibited in THP-1 cells. I hypothesised that upon inhibition of glycolysis, MAIT cell activation would be impaired in samples treated with 5-A-RU and whole bacteria, with activation returning to a similar level as seen in samples treated with sonicated bacteria. To test this, 2-deoxy-D-glucose (2-DG), a glucose analogue lacking a reactive hydroxyl group, was

used to inhibit glycolysis.[83] THP-1 cells were rested in glucose free media (GF-R10) for 2 hours before the addition of either D-glucose or 2-DG for 1 hour (Figure 4.6A). THP-1 cells were then treated with 5-A-RU and fixed, sonicated, or opsonised *E. coli* Δ*ribD*, or 5-OP-RU or fixed *E. coli* HB101. After 5 hours, THP-1 cells were washed three times in PBS before being resuspended in glucose containing-R10. $CD8^+$ T cells were then added and co-cultured for 4 hours. MAIT cell activation was determined by measurement of production of proinflammatory cytokines, IFNγ and TNFα. (Figure 4.6A).

No change in MAIT cell activation was observed upon 2-DG treatment of THP-1 cells, irrespective of bacterial treatment (Figure 4.6B, C). A trend of decreased IFNγ and TNFα production by MAIT cells was observed with 2-DG treated THP-1 cells and treatment with fixed *E. coli* HB101, however, this change was not statistically significant (Figure 4.6B, C).

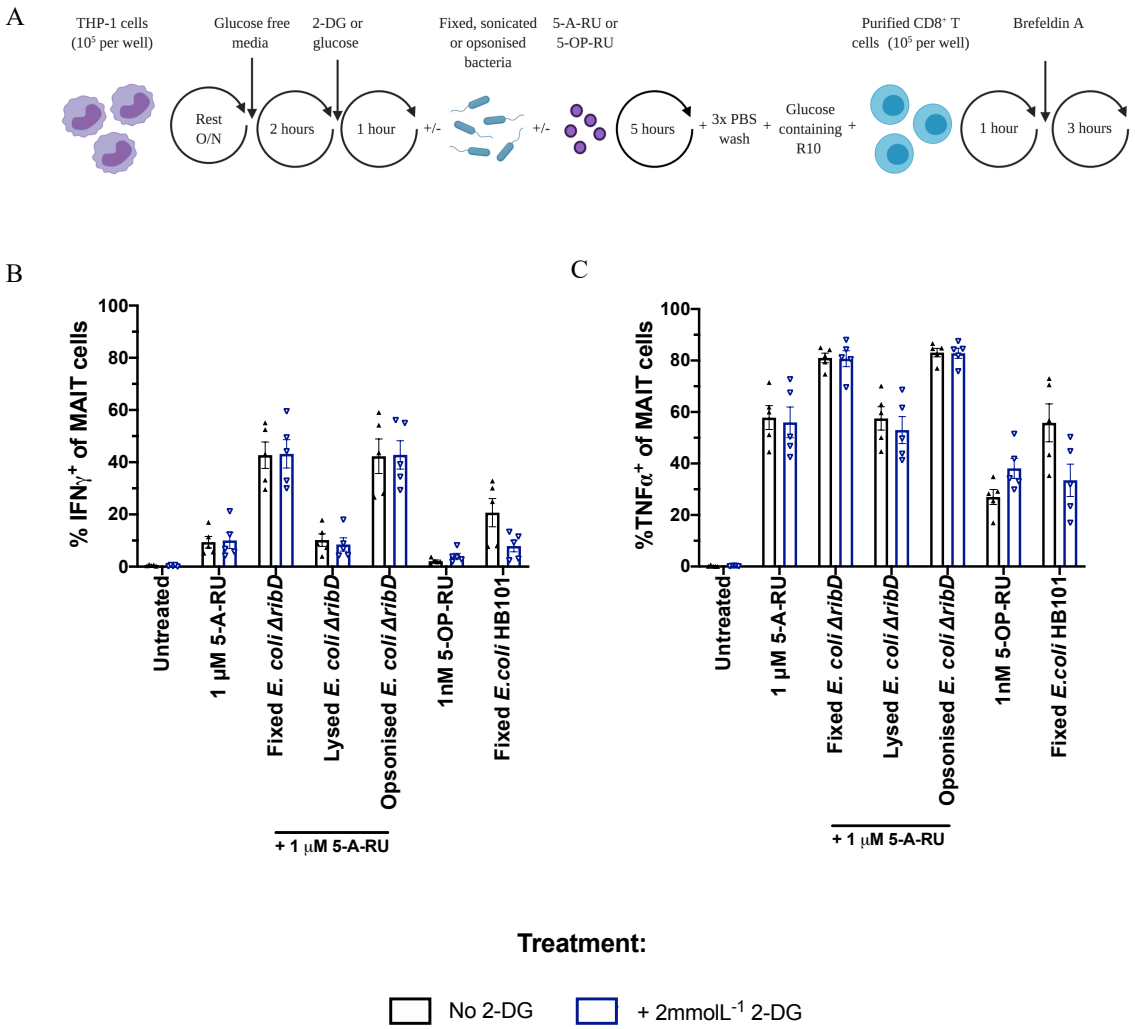



***Figure 4.6: Inhibition of glycolysis in THP-1 cells does not alter MAIT cell activation.*** *(A) Schematic depicting MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B and C) THP-1 cells were incubated in GF-R10 for 2 hours before pre-treatment with 2 mmolL⁻¹ 2-DG or 11 mM D-glucose. THP-1 cells were then treated with 1 µM 5-A-RU ± 10 BPC fixed, opsonised, or equivalent sonicated E. coli ΔribD, or 10 nM 5-OP-RU, or fixed E. coli HB101. Isolated CD8⁺ T cells were added and incubated for a further 4 hours, with addition of brefeldin A (3 µg/mL) for the final 3 hours. MAIT cell IFNγ (B) and TNFα (C) production was assessed. Data points represent individual blood donors tested in two independent experiments (n = 5). Treatments were analysed by repeated measures two-way ANOVA with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test (B, C).*

### 4.3.3 2-DG treatment of THP-1 cells does not affect MAIT cell activation, regardless of concentration

To determine whether higher concentrations of 2-DG would inhibit MAIT cell activation, a 2-DG titration was performed. To achieve this, THP-1 cells were rested in GF-R10 for 2 hours

before addition of D-glucose or a range of 2-DG concentrations (Figure 4.7A). After 1 hour of pre-treatment, cells were treated with 5-A-RU and fixed *E. coli* Δ*ribD*. THP-1 cells were incubated for 5 hours then washed three times in PBS before addition of $CD8^+$ T cells in glucose containing R10. $CD8^+$ T cells were co-cultured for 4 hours. MAIT cell activation was determined by measurement of production of proinflammatory cytokines, IFNγ and TNFα activation (Figure 4.7A).

2-DG treatment did not impact MAIT cell activation, irrespective of 2-DG concentration. No significant change was observed in production of either IFNγ or TNFα by MAIT cells (Figure 4.7B, C, E, F). A slight decrease in IFNγ, but not TNFα, production was observed at 100 and 150 $mmolL^{-1}$ 2-DG (Figure 4.7E, F). Furthermore, 2-DG treatment did not alter THP-1 cell viability (Figure 4.7D, G). These results indicated that increasing concentrations of 2-DG treatments does not alter MAIT cell activation.

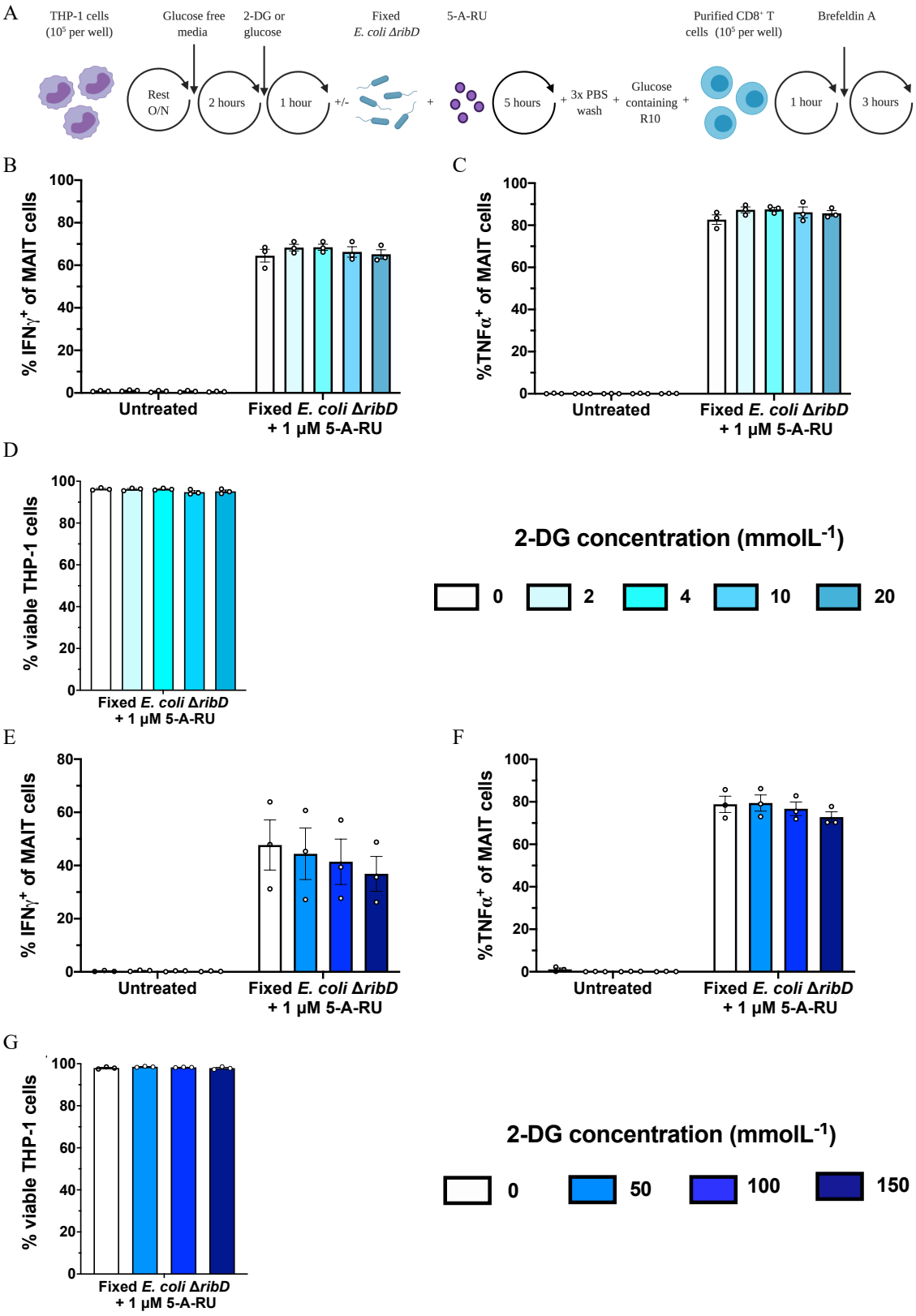



***Figure 4.7: Pre-treatment of THP-1 cells with increased concentrations of 2-DG does not alter MAIT cell activation.*** *(A) Schematic depicting MAIT cell activation assay using a 1:1 co-culture of THP-1 cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B - G) THP-1 cells were incubated in GF-R10 for 2 hours before pre-*

*treatment with 11 mM D-glucose (labelled 0 mmolL$^{-1}$ 2-DG) or 2, 4, 10, 20, 50, 100, or 150 mmolL$^{-1}$ 2-DG. THP-1 cells were then treated with 10 BPC fixed E. coli ΔribD + 1 μM 5-A-RU. Cells were washed and isolated CD8$^+$ T cells were added and incubated for a further 4 hours, with addition of brefeldin A (3 μg/mL) for the final 3 hours. MAIT cell IFNγ (B and E) and TNFα (C and F) production was assessed. Viability of THP-1 cells was assessed by live/dead staining (D and G) Data points represent individual blood donors tested in one independent experiment (n = 3). Due to the COVID-19, testing was limited to three donors, therefore statistical comparisons were not made.*

### 4.3.4 THP-1 PI3K/mTORC inhibition in THP-1 cells does not impede MAIT cell activation

The cell signalling proteins PI3K and mTORC have been previously implicated in the glycolytic switch that occurs after bacterial stimulation.[76, 84] Therefore, as an alternative mechanism to inhibit glycolysis, PI3K and mTORC1 and mTORC2 were inhibited in THP-1 cells. THP-1 cells were pre-treated with the PI3K inhibitor LY294002 (LY), the mTORC1 inhibitor rapamycin (Rapa), or the dual mTORC1 and mTORC2 inhibitor KU-0063794 (KU). After a 1 hour incubation, THP-1 cells were then treated with 5-A-RU with fixed, sonicated, or opsonised *E. coli* Δ*ribD,* or 5-OP-RU, or fixed *E. coli* HB101. After 5 hours THP-1 cells were washed 3 times in PBS before resuspension in R10. CD8$^+$ T cells were then added and co-cultured for 4 hours. MAIT cell production of IFNγ and TNFα was measured.

No change in MAIT cell activation was observed upon inhibition of PI3K or mTORC1 and mTORC2 in THP-1 cells (Figure 4.8B, C). No change in THP-1 cell viability was observed, irrespective of drug treatments (Figure 4.8D).

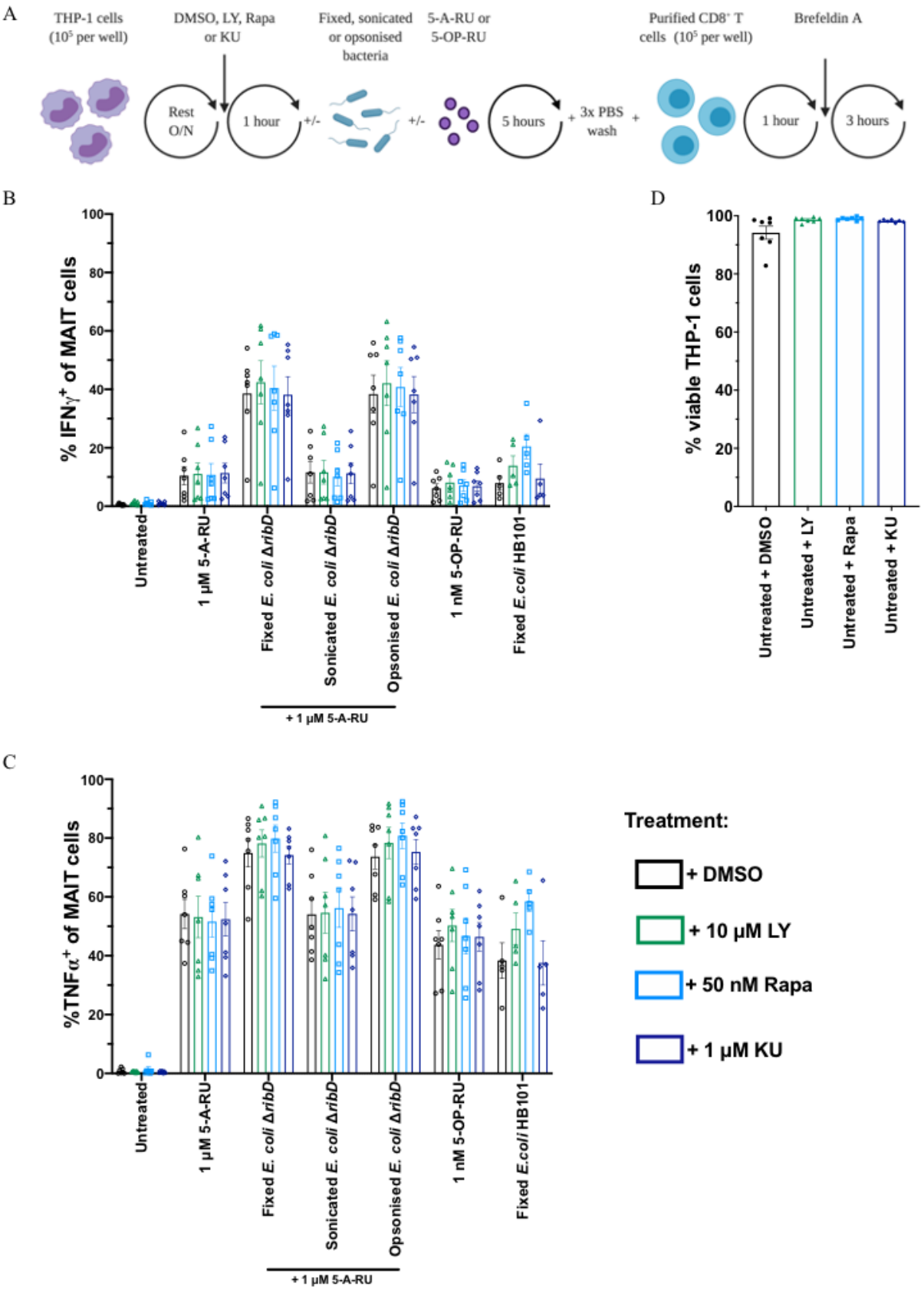



***Figure 4.8: Inhibition of THP-1 PI3K and mTORC1 and mTORC2 does not alter MAIT cell activation.*** *(A) Schematic depicting MAIT cell activation assay using a 1:1 co-culture of THP-1*

*cells and purified human CD8⁺ T cells. Schematic created with BioRender.com. (B - D) THP-1 cells were pre-treated with either DMSO, 10 μM LY294002 (LY), 50 nM rapamycin (Rapa), or 1 μM KU0063794 (KU). THP-1 cells were then treated with 1 μM 5-A-RU ± 10 BPC fixed, opsonised, or equivalent sonicated E. coli ΔribD, or 1 nM 5-OP-RU or fixed E. coli HB101. Isolated CD8⁺ T cells were added for a further 4 hours, with addition of brefeldin A (3 μg/mL) for the final 3 hours. MAIT cell IFNγ (B) and TNFα (C) production was assessed. Viability of THP-1 cells (D) was assessed by live/dead staining. Data points represent individual blood donors tested in four independent experiments (n = 7). Two donors were excluded for E. coli HB101 treatments only, due to non-responsiveness to a new bacterial stock; time limitations meant repeats could not be attempted (n=5). Treatments were analysed by an arcsine transformation with repeated measures two-way ANOVA with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test (B, C) or repeated measures one-way ANOVA with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test (D).*

### 4.4 THP-1 cells do not alter glucose uptake upon *E. coli* treatment

After determining that enhancement of glycolysis in THP-1 cells with F-1,6-BP alters MAIT cell activation in response to 5-A-RU, I next sought to determine whether THP-1 cells showed an increase of glucose uptake upon bacterial treatment. 2-NBDG, a fluorescent glucose analogue, was used to assess glucose uptake. To ensure there was no competition for glucose transporters, THP-1 cells were resuspended in GF-R10, allowing 2-NBDG uptake into THP-1 cells (Figure 4.9A). Every ten minutes over an hour, 2-NBDG was added to THP-1 cells either with or without fixed *E. coli* Δ*ribD*. Cells were then fixed and the geoMFI of 2-NBDG calculated and normalised to samples treated in the same manner without 2-NBDG treatment (Figure 4.9A).

A gradual increase in 2-NBDG uptake, measured as geoMFI of 2-NBDG, occurred for both untreated THP-1 cells and for cells treated with fixed *E. coli* Δ*ribD* (Figure 4.9B). No statistically significant difference was observed in THP-1 cells treated with fixed *E. coli* Δ*ribD* compared to untreated THP-1 cells across all timepoints (Figure 4.9B, C). Changes to multiple parameters were tested for protocol optimisation, including incubation time, live/dead staining, fixation, or pre-treatment of THP-1 cells with GF-R10. These changes did not alter the results observed (data not shown).

Expression of the glucose uptake channel, GLUT-1, was also measured. At hourly intervals THP-1 cells were left untreated or treated with fixed *E. coli* Δ*ribD*, over a 4 hour time course (Figure 4.9D). THP-1 cells were then stained for GLUT-1 on the cell surface (Figure 4.9D). The cells were fixed and the geoMFI of GLUT-1 calculated and normalised to samples treated in the same manner without GLUT-1 staining.

No change was seen in GLUT-1 expression over the time course irrespective of treatment (Figure 4.9E). Furthermore there was no statistically significant change in GLUT-1 expression in THP-1 cells treated with fixed *E. coli* Δ*ribD,* compared to untreated THP-1 cells (Figure 4.9E, F). These results indicated that upon stimulation, THP-1 cells do not appear to display a classical switch to glycolysis.

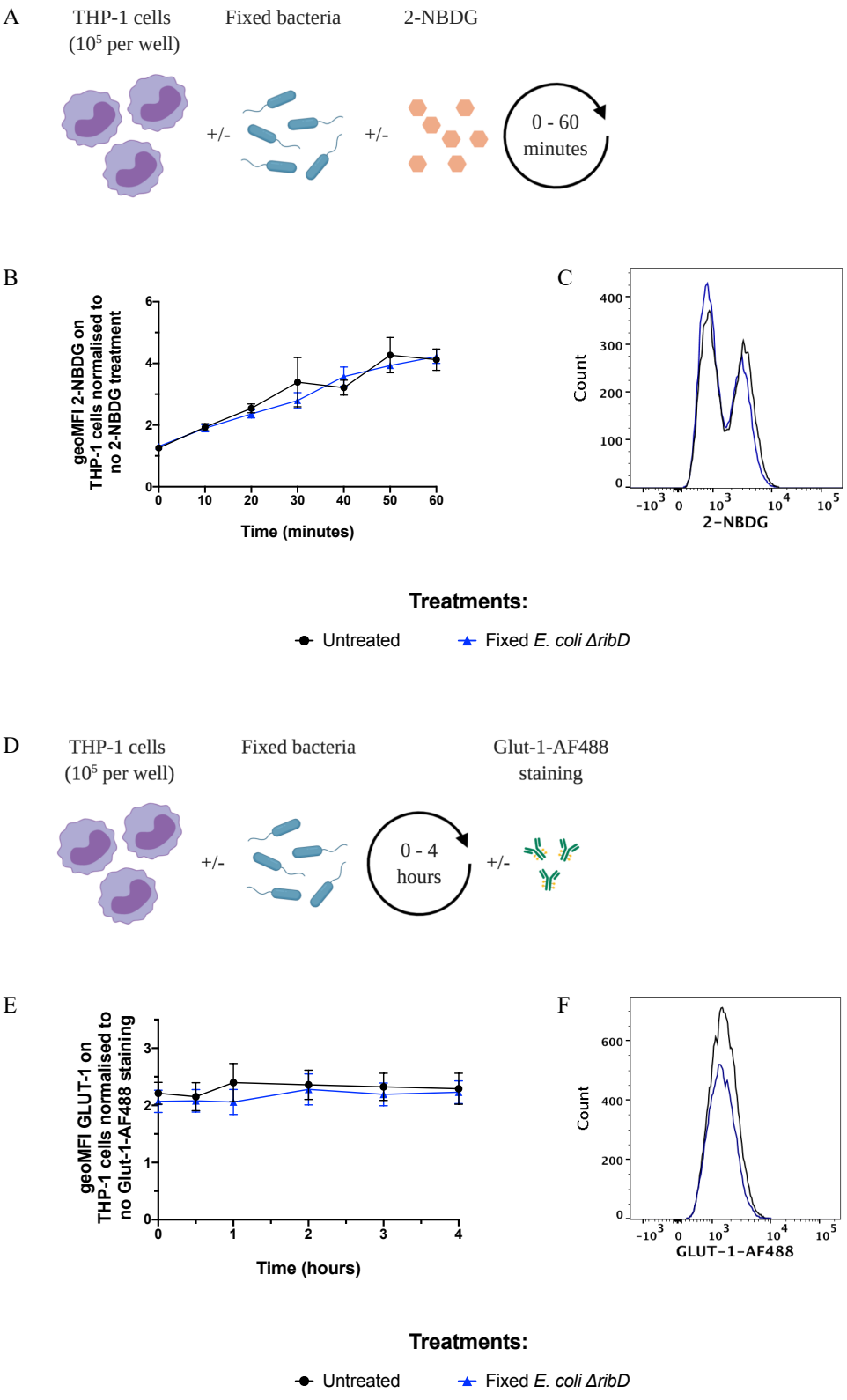



***Figure 4.9: THP-1 cells do not alter glucose uptake upon bacterial treatment.*** *(A) Schematic to demonstrate 2-NBDG uptake assay using THP-1 cells. Schematic created with BioRender.com. (B and C) THP-1 cells were cultured in glucose free (GF) R10 and treated with ±—50 µM 2-NBDG ± 10 BPC fixed E. coli ΔribD for 0 - 60 minutes. (D) Schematic*

*depicting GLUT-1 expression assay. Schematic created with BioRender.com. (E and F) THP-1 cells were cultured in R10 treated with ± 10 BPC fixed E. coli ΔribD for 0 - 4 hours, cells were then stained ± GLUT-1-AF488. The geoMFI of (B) 2-NBDG uptake and (E) GLUT-1 expression by THP-1 cells was assessed by flow cytometry. Results were normalised to samples of the same treatment lacking addition of 2-NBDG (B) or GLUT-1-AF488 (E). Exemplar histograms of (C) 2-NBDG uptake and (F) GLUT-1 expression. All experiments were repeated three times in triplicate. Treatments were compared by two-way ANOVA with Geisser-Greenhouse correction and Sidak's multiple comparison test.*

# 5 Discussion

Understanding the regulation of MAIT cell activation is of vital importance to further elucidate their role within the antibacterial immune response. A large proportion of bacteria present at mucosal surfaces produce riboflavin, and the riboflavin derived MAIT cell ligand which is capable of crossing mucosal surfaces.[85] It is hypothesised that tight regulation of MAIT cells is crucial to prevent hyperactivation and resulting immunopathology, while still allowing for robust responses to infection.[64] In this study both phagocytosis and glycolysis within the APC were implicated in MAIT cell regulation. MAIT cells were activated to a greater extent by antigen presenting THP-1 cells treated with fixed *E. coli* Δ*ribD* and 5-A-RU, compared to treatment with sonicated *E. coli* Δ*ribD* and 5-A-RU or 5-A-RU alone. This difference was not observed with *E. coli* Δ*ribD* and 5-OP-RU treated THP-1 cells. Enhancement of THP-1 cell glycolysis augmented MAIT cell activation to 5-A-RU in the absence of whole bacteria to levels that are similar to those seen with 5-A-RU in the presence of whole bacteria. However, inhibition of THP-1 cell glycolysis did not impact MAIT cell activation. Inhibition of the PI3K/AKT/mTOR signalling pathway, responsible for glycolysis regulation, did not alter MAIT cell activation. Finally, THP-1 glucose uptake and glucose transporter expression was not altered upon bacterial stimulation. Together, these results suggest that phagocytosis and glycolysis within the APC may regulate MAIT cell activation. Further research is required to fully understand the interconnected nature of the cellular metabolic pathways impacting this process.

## 5.1 Phagocytosis is required to achieve robust 5-A-RU mediated MAIT cell activation

Phagocytosis of riboflavin-producing whole bacteria into an acidified endosomal compartment has previously been implicated in MAIT cell activation.[64] Conversely, MR1 ligand alone,

obtained from riboflavin-producing bacterial cell lysate or bacterial culture supernatant, fails to strongly activate MAIT cells.[64] Interestingly, phagocytosis of the non-riboflavin producing bacteria, *Enterococcus faecalis*, did not enhance the ability of bacterial culture supernatant to activate MAIT cells.[64] It is not yet known why this occurred, though it was hypothesised that insufficient MR1 ligand was phagocytosed.[64] The inability to standardise ligand concentration or to isolate it from other bacterial components made it difficult to draw meaningful conclusions with regard to the role of phagocytosis in the regulation of MAIT cell activation.

The production of synthetic 5-A-RU has allowed investigation into how the ligand alone and the additive effect of bacterial derived signals that occur upon addition of whole bacteria impact MAIT cell activation. Using 5-A-RU, previous work in the Ussher Laboratory indicated that MR1 is upregulated on the APC cell surface in response to whole *E. coli* Δ*ribD* and 5-A-RU.[81] This effect was lost upon inhibition of phagocytosis or sonication of *E. coli* Δ*ribD*.[81] Furthermore, MAIT cell activation by 5-A-RU was enhanced with the addition of *E. coli* Δ*ribD* treatment in PBMCs, as well as in THP-1 and $CD8^+$ T cell co-cultures.[63]

Consistent with previous results, this study found an MR1 dependent synergistic effect on MAIT cell activation of co-treatment with 5-A-RU and fixed *E. coil* Δ*ribD* (Figure 4.1B, E). This effect was lost upon THP-1 treatment with sonicated *E. coli* Δ*ribD*, where MAIT cell activation reduced to levels similar to those seen upon 5-A-RU treatment alone (Figure 4.1B, C, E, F). The effect of sonication was seen in treatments with both *E. coli* Δ*ribD*, and the riboflavin-producing *E. coli* HB101 strain (Figure 4.1B, E).

There were multiple discrepancies between the results obtained with the precursor ligand, 5-A-RU, and those obtained with the final ligand, 5-OP-RU. Firstly, there was a significant difference in the concentration required to sufficiently activate MAIT cells. 5-OP-RU was added at concentrations a hundred-fold lower than that of 5-A-RU to achieve similar levels of

MAIT cell activation (Figure 4.1B, E). Secondly, the synergistic effect of whole bacteria addition was greatly reduced by 5-OP-RU treatment (Figure 4.1B, E). This may have been in part due to differences in MR1 binding between 5-A-RU and 5-OP-RU. As the intermediate ligand, 5-A-RU requires condensation with MG to form the activating MAIT cell ligand, 5-OP-RU, before binding to MR1.[4] 5-OP-RU binding to MR1 has been postulated to occur in multiple different locations within the cell. 5-OP-RU may displace endogenous ligand bound to MR1 to bind directly at the cell surface, or within endosomes.[29, 34] The ability of 5-OP-RU to bind directly and independently of phagocytosis may allow for a mechanism of robust 5-OP-RU mediated MAIT cell activation, regardless of bacterial treatment. In support of this, the levels of MAIT cell activation were unchanged when ER to Golgi trafficking was inhibited in 5-OP-RU treated PBMC samples, suggesting that 5-OP-RU binding can occur outside of the ER-Golgi system.[63] To further investigate this theory folate free media, which would sequester MR1 to the ER, could be used to prevent ligand binding in the endosome.[27, 29] This would determine whether 5-OP-RU binding in our system occurs in endosomes or on the cell surface.

While small in magnitude, a synergistic effect was still observed with addition of whole bacteria to 5-OP-RU treated samples (Figure 4.1B, E). In part, this enhancement of MAIT cell activation may be due to a role for other bacteria-derived signals in activating robust immune responses. Stimulation of TLR signalling pathways by bacteria enhances production of a range of co-stimulatory molecules, including IL-6, IL-12 and IFNγ.[86] Stimulation of APCs with TLR agonists prior to incubation with whole bacteria has been shown to enhance early activation of MAIT cells.[64] This may explain the small increase in MAIT cell activation upon treatment with 5-OP-RU and whole bacteria, but does not fully explain the large enhancement observed with the addition of whole bacteria to 5-A-RU treatment of THP-1 cells.

The key difference between 5-A-RU and 5-OP-RU-mediated MAIT cell activation is the requirement for 5-A-RU to react with MG in order to produce the final ligand.[4] Previous unpublished work in the Ussher Laboratory using PBMC cultures indicated that glycolysis may be crucial in MAIT cell regulation, potentially through the production of the glycolytic by-product, MG.[63] Indeed, the addition of MG derived from Mānuka honey has been shown to enhance 5-A-RU mediated MAIT cell activation.[87] Multiple papers have indicated that bacterial signalling is capable of enhancing glycolysis and leads to the production of effector functions within APCs.[74, 75] Furthermore, both cell surface and endosomal TLR signalling has been shown to induce a switch from oxidative phosphorylation to glycolysis as a mechanism of activation.[74, 76] Despite the current research suggesting a link between phagocytosis and glycolysis, limited research has occurred to demonstrate if there is a causal effect of phagocytosis on glycolysis enhancement within APCs. I observed an increase in 5-A-RU-mediated MAIT cell activation with APC phagocytosis of intact bacteria (Figure 4.1B, E). Therefore, phagocytosis of intact bacteria by APCs may cause an upregulation of glycolysis resulting in increased MG production and availability to react with 5-A-RU, thereby producing more 5-OP-RU, to robustly activate MAIT cells in a TCR dependent manner.

### 5.2 The ability of APCs to activate MAIT cells increases with time

MR1-mediated MAIT cell activation was dependent on APC activation (Figure 4.2, 4.3).[64] There has been limited research into the optimal pre-treatment time of THP-1 cells to allow for an effective MAIT cell response. Previous work in the Ussher Laboratory used a THP-1 treatment time of 6 hours, followed by 4 hours of MAIT cell co-culture, based on data showing peak MR1 surface expression at this time.[81] However, the TCR-dependent response relies on multiple factors, including ligand loading, presentation of MR1 on the cell surface, and the production of co-stimulatory molecules or cytokines.[88] As a consequence, maximal MR1

expression alone does not necessarily correlate to optimal MAIT cell activation. LPS stimulation of DCs was found to cause a shift to glycolysis within 50 minutes of treatment.[74] Furthermore, peak MR1 expression in the C1R cell line was found to occur four hours after 5-OP-RU treatment.[29] Therefore this study sought to optimise the co-culture model in order to obtain the peak MAIT cell activation.

MAIT cells were readily activated at all time points tested, with optimal activation observed at a nine hour total incubation time across all treatments (Figure 4.2B, 4.2C, 4.3B, 4.3C). Innate signalling has been identified as a necessary component for MR1-mediated MAIT cell activation. Therefore, it was speculated that washing of THP-1 cells before addition of $CD8^+$ T cells might alter the magnitude of MAIT cell activation. Counter to this hypothesis, MAIT cell activation was not demonstrably altered between protocols with or without washing (Figure 4.2B, 4.2C, 4.3B, 4.3C). Potentially this was due to MAIT cell activation at early timepoints being largely mediated by MR1 and modulated by the expression of co-stimulatory molecules on the APC surface.[24]

## 5.3 Modulation of THP-1 cell glycolysis has varied impact on MAIT cell activation

### 5.3.1 Enhancement of glycolysis augments MAIT cell activation in the absence of whole fixed bacteria

Enhancement of THP-1 glycolysis augmented MAIT cell activation in samples lacking whole bacteria. A significant increase in MAIT cell activation occurred when THP-1 cells were treated with F-1,6-BP and 5-A-RU alone or sonicated *E. coli* Δ*ribD* and 5-A-RU, compared with the same treatments lacking F-1,6-BP (Figure 4.4B, C, D). Augmentation of glycolysis in samples lacking whole bacterial treatments enhanced MAIT cell activation to comparable levels to those seen in samples treated with whole bacteria. Interestingly, MAIT cell activation was not further enhanced with augmented glycolysis in samples treated with whole bacteria

(Figure 4.4, 4.5). Conversely, unpublished work from the Ussher Laboratory observed increased MAIT cell activation in response to ligand and whole bacteria treatment in PBMC cultures upon enhancement of glycolysis with F-1,6-BP.[63] This is likely due to effects of F-1,6-BP on cell types other than APCs. MAIT cells are reliant on a switch to glycolysis upon stimulation to produce characteristic effector functions including granzyme B and IFNγ.[62, 89] Therefore, in PBMC studies, glycolysis enhancement in MAIT cells may have directly upregulated effector functions, confounding the true effect of enhancing APC glycolysis. The experimental system used in the current study specifically targeted the APC, by washing away any modulators before the addition of MAIT cells. Therefore, the observed effects can be attributed solely to APC function.

As MG is a metabolic by-product produced during glycolysis, enhancement of glycolysis by phagocytosis of bacteria may increase MG availability for condensation with 5-A-RU to enable efficient formation of 5-OP-RU. Stimulation of endosomal TLRs, TLR3, TLR7/8, and TLR9, by bacterial TLR agonists induces a switch to glycolytic metabolism.[74] Furthermore, inhibition of phagocytosis by cytochalasin D has been shown to reduce MAIT cell activation in response to riboflavin-producing bacteria.[63, 64] This further supports the data obtained which demonstrated that MAIT cell activation was not enhanced upon THP-1 treatment with F-1,6-BP and intact bacteria or 5-OP-RU (Figure 4.4B, C, D, E). Potentially phagocytosis had already induced an upregulation in glycolysis which cannot be further enhanced. Activation of MAIT cells can still occur upon cytochalasin D treatment, potentially due to uptake of 5-A-RU into the APC by alternative mechanisms.[63] Phagocytosis may also enable efficient delivery of soluble ligand into the APC and therefore enhance MAIT cell activation. Overall, these results supported the hypothesis that phagocytosis of whole bacteria causes an upregulation of glycolysis. Potentially enhancement of APCs glycolytic capacity when treated with whole bacteria allowed for sufficient MG production which was unable to be enhanced further.

Enhanced MAIT cell activation upon treatment with opsonised bacteria has been observed in the published literature.[44, 90] The process of opsonisation involves coating bacteria in antibodies, enabling enhanced phagocytosis by APCs. Contrary to published data, no difference was observed with bacterial opsonisation in our system (Figure 4.4, 4.6, 4.8).[44, 90] Studies by Bánki et al and Boulouis et al observed enhanced MAIT cell activation when co-cultured for 20 and 24 hours, respectively, with THP-1 cells treated with riboflavin synthesising bacteria.[44, 90] As activation of MAIT cells at later time points is dependent on both TCR- and cytokine-mediated activation, the observed increase in MAIT cell activation reported may be due to enhanced co-stimulatory molecules produced by THP-1 cells, such as TNFα.[24, 37, 44] Bánki et al indicated that THP-1 cells reached a maximum rate of bacterial uptake at 2 BPC; potentially the higher concentration of bacteria used in this study (10 BPC) may have mitigated any gain in activation observed with opsonisation.[44]

The effects of glycolysis on expression of CD69, an activation marker on multiple lymphocytes, was also assessed. MAIT cell CD69 expression has previously been shown to be independent of glycolysis in MAIT cells.[89] In this study, MAIT cell CD69 expression was also not altered by enhancement of THP-1 cell glycolysis (Figure 4.4E). NK cells have been found to upregulate CD69 in an IFNγ dependent mechanism.[91] However, this present study involved washing away the media containing any cytokines produced by the APCs before the addition of $CD8^+$ T cells (Figure 4.4A). Therefore, the amount of IFNγ present in the media produced by THP-1 cells is likely to have been reduced to a level where no effect could be observed.[92] It is unlikely that a substantial amount of IFNγ would have been produced after washing due to the addition of brefeldin A after one hour, which sequesters cytokines within the APC. Potentially enhancement of CD69 expression was not observed upon glycolytic enhancement as expression is largely dependent on co-stimulatory molecule production, as opposed to a TCR

dependent mechanism which is hypothesised to be enhanced upon enhancement of THP-1 cell glycolysis.

### 5.3.2 Inhibition of glycolysis in THP-1 cells does not alter MAIT cell activation

In contrast, inhibition of THP-1 glycolysis by the glucose analogue 2-DG did not alter MAIT cell activation, irrespective of bacterial treatment (Figure 4.6B, C). This was true even at concentrations of 2-DG that were significantly higher than the glucose concentration found in glucose-containing R10 (11 μM) (Figure 4.7B, C, E, F). As such, drug dosage was ruled out as a contributing factor to the observed results.

The lack of MAIT cell inhibition may reflect differences in cell biology and subsequent effectiveness of 2-DG treatment between primary monocytes and THP-1 cells. 2-DG has been shown to inhibit glycolysis and cell activation in primary monocytes.[93] Conversely, THP-1 cells are reasonably resistant to 2-DG.[94, 95, 96] Cell viability, glucose uptake, and the production of glycolysis by-products by THP-1 cells remain relatively unchanged upon treatment.[94, 95, 96] It is of note that a reduction in THP-1 cell metabolic activity is observed at high concentrations of 2-DG, indicating that some level of THP-1 cell glycolysis inhibition may have occurred (Figure 4.7E, F).[95] However, this was insufficient to inhibit MAIT cell activation.

Modification to MAIT cell activation upon 2-DG treatment may not have been observed due to alternate MG production pathways (Figure 5.1). THP-1 cells are partially reliant on fatty acid metabolism, where MG is produced during a two-step enzymatic reaction of acetone.[94, 96, 97, 98] Glucose deprivation has been shown to enhance fatty acid metabolism in monocytes.[99] Therefore, as the THP-1 cells were cultured GF-R10, it is possible that this pathway was enhanced in the THP-1 cells. Additionally pyruvate, present in the GF-R10 media, can be converted to MG in a series of steps.[100] Because of these alternate pathways, MG production

may have been unaffected by partial impairment of THP-1 glycolysis upon 2-DG treatment (Figure 5.1).

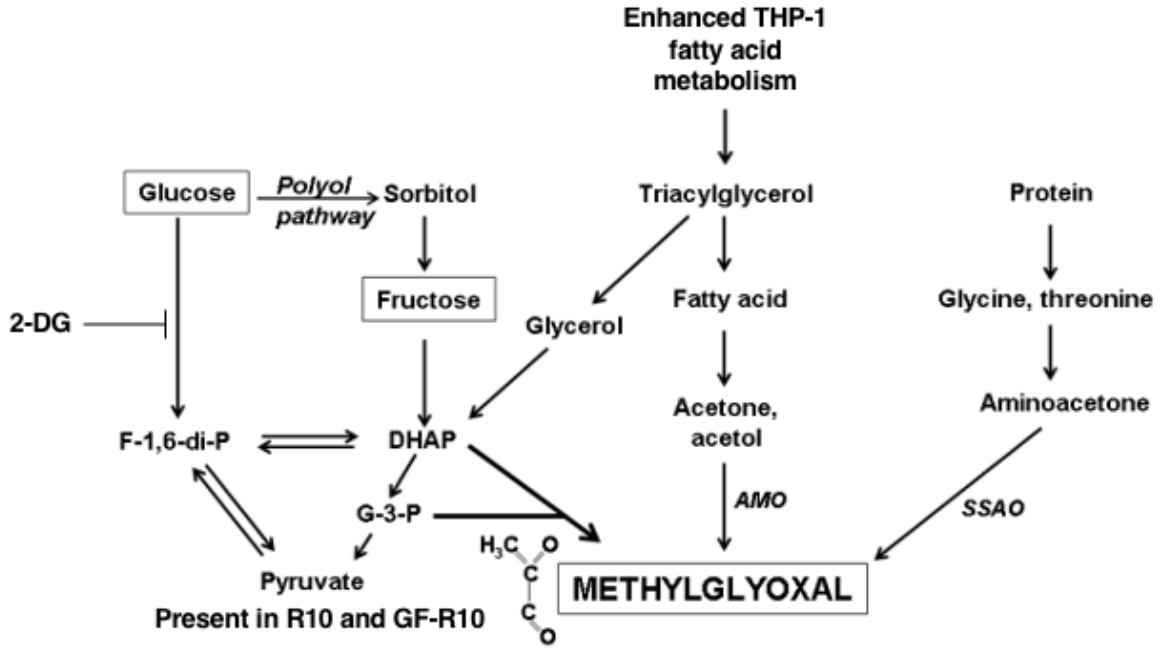



***Figure 5.1: Metabolic pathways leading to MG production.*** *THP-1 cells exhibit partial reliance on fatty acid metabolism, leading to enhanced MG production. Adapted from Desai et al, 2010.*

The role of MG production by APCs in MAIT cell activation could be assessed in future experiments by MG detoxification. This process reduces the amount of MG available to react with 5-A-RU in APCs. MG is detoxified in an enzymatic reaction with glutathione (GSH), catalysed by glyoxalase (glo) enzymes.[101] Supplementation of THP-1 cells with GSH may enhance MG breakdown, thereby reducing MG build-up in cells. This may provide a more viable inhibition protocol, as inhibition of all of the individual pathways responsible for MG production would likely lead to cell death or other non-desirable off target effects.

### 5.3.3 PI3K/mTOR inhibition does not impede MAIT cell activation

PI3K and mTOR have been implicated in the regulation of glycolysis in immune cells.[76, 84, 93] Therefore, it was hypothesised that inhibition of these cell signalling proteins would reduce MG production and subsequent MAIT cell activation. However, inhibition of THP-1 cell PI3K

and mTORC1 and -2 inhibition did not impede MAIT cell activation (Figure 4.8B, C). This data provides additional evidence that other MG production pathways in THP-1 cells are involved in MAIT cell activation. PI3K serves as a pivotal enzyme that, when activated, creates a cascade of downstream effects, including the activation of mTORC1 and subsequent upregulation of glycolysis.[76, 84] LPS stimulation of primary monocytes causes an mTORC1 dependent increase in GLUT-1 expression and glycolysis.[93] Inhibition of mTORC1 by rapamycin significantly reduces the activation and glycolytic profile of primary monocytes.[93] However, there is a degree of discord within the literature, with Evarts et al reporting that inhibition of PI3K and mTOR was insufficient to reduce the surge in glycolysis after DC activation.[74] An alternative mechanism was proposed, suggesting that kinases independent from PI3K activated AKT in order to enhance glycolysis.[74] Cell signalling pathways are strongly related, a range of cell signalling proteins, including PI3K and mTOR, and external signals may be needed to produce a robust switch to glycolysis. Additionally, the wide range of interconnected metabolic pathways may also contribute to MG production.

Due to the interconnected nature of cell signalling, inhibition of cell signalling proteins can cause a range of off-target effects. It is possible that the observed MAIT cell activation was due to secondary effects of PI3K and mTOR inhibition within the THP-1 cells. Indeed, treatment of DCs with either LPS or whole *S. aureus* paired with mTORC1 inhibition results in enhanced production of multiple proinflammatory cytokines via the enhancement of NF-κB signalling.[102, 103] Inhibition of mTORC1 in monocytes increases the production of IL-12 in a GSK3-β and NF-κB dependent manner.[104] There is limited research into the effects of mTORC1 inhibition in THP-1 cells. Sun et al reported that rapamycin inhibition reduced proinflammatory cytokine production by THP-1 cells.[105] However, only the TLR2 agonist was used to assess activation, and IL-12 production was not investigated.[105] Further, any proinflammatory cytokines produced in our system would have been washed away, so cytokine

mediated effects were not expected to have a large impact on the results observed. The specific TLR stimulated may also alter the mechanism of activation and effector functions. For example, treatment of THP-1 cells with specifically the TLR1, 2, or 6 agonists, and not other agonists, enhanced MAIT cell activation to subsequent treatment with *E. coli*.[64] This data suggests that different signalling pathways may lead to differing APC effector functions. Differential TLR stimulation may have different effects on cell metabolism. In $CD14^+$ monocytes TLR4 stimulation with LPS enhanced glycolysis and downregulated oxidative phosphorylation while TLR2 stimulation upregulated both glycolysis and oxidative phosphorylation.[106]

THP-1 cells express high levels of glo1, one of the two enzymes (glo1 and glo2) which break down MG into lactate.[100, 101, 107] Expression of glo1 is dependent on PI3K, a pathway that is hyperactivated in multiple cancer cell lines.[108] Talesa et al demonstrated in prostate cancer cell lines that inhibition of both PI3K and mTOR reduced the expression of glo1 and glo2.[108] Therefore, inhibition of PI3K in THP-1 cells may result in increased levels of MG due to lower expression of glo1 and the subsequent lack of MG breakdown. The decreased amount of MG breakdown may counter any reduction in MG production caused by glycolysis inhibition, still allowing for sufficient MG production, and therefore MAIT cell activation.

Another possible explanation for the lack of an effect on MAIT cell activation upon THP-1 PI3K and mTOR1 inhibition lies in the interconnected nature of cell signalling pathways and THP-1 cell biology (Figure 5.2). Enhancement of fatty acid metabolism, coupled with reduced MG break down may lead to an accumulation of MG within THP-1 cells.[96] This would offset the lack of glycolytic MG production, allowing for effective MAIT cell activation. Additionally, inhibition of mTOR in APCs may stimulate the production of proinflammatory cytokines, enhancing MAIT cell activation.[102, 103, 104] It is unclear how large a role this would have as MAIT cell activation is largely TCR-dependent at earlier timepoints. Inhibition of these cell

signalling molecules also impacts on a wide range of cellular functions such as autophagy and on the production of reactive oxygen species, which may have further impacted the observed results (Figure 5.2).[109]

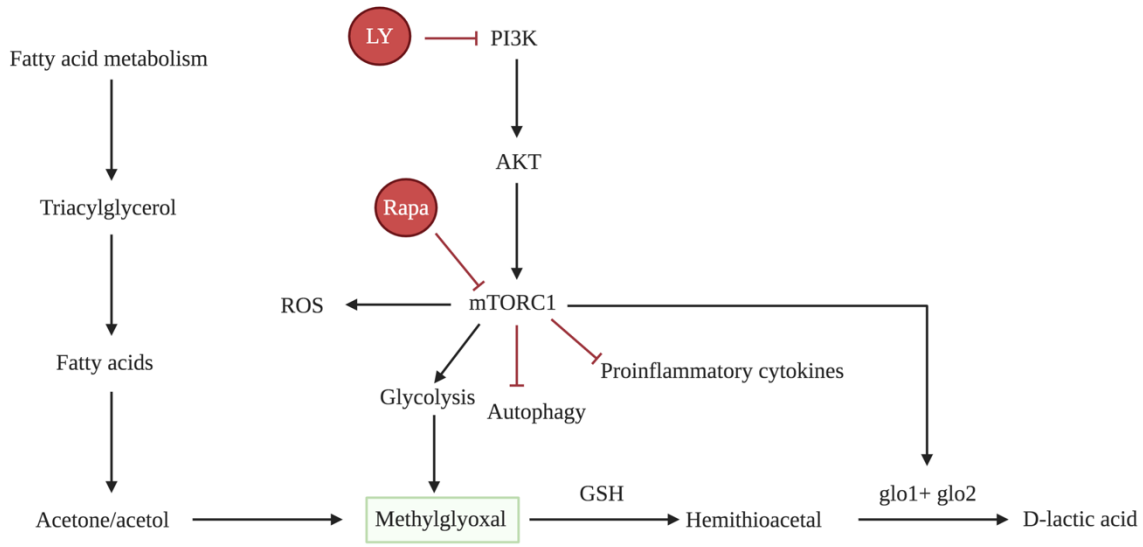



***Figure 5.2: Role of interconnected cellular metabolic and signalling pathways in MG production.*** *The integration of multiple pathways is required for MG production, which can be modulated by inhibition of PI3K with LY294002 (LY) and mTORC1 inhibition with Rapamycin (Rapa) treatments. Created with BioRender.com.*

## 5.4 THP-1 cells do not show an increase in glucose uptake upon bacterial stimulation

To assess whether glycolysis is upregulated in response to whole bacteria, both uptake of glucose and expression of the glucose transporter, GLUT-1, were assessed. No significant differences occurred between bacterially treated and untreated THP-1 cells in assessment of both uptake of the florescent glucose analogue, 2-NBDG, and GLUT-1 expression (Figure 4.9B, D). These results were surprising and contradictory to published literature. Multiple APCs, including DCs, monocytes, and macrophages have been shown to increase glucose uptake, usage, and GLUT-1 expression following LPS stimulation.[74, 93, 110, 111, 112] There is a degree of discord in the published literature surrounding glucose uptake by the THP-1 cell line.[113, 114] THP-1 cells express mRNA for multiple GLUT transporters, including GLUT-1.[112] Singh et al saw increased GLUT-1 surface expression and 2-NBDG uptake upon a three hour

incubation of THP-1 cells with an *M. tuberculosis* antigen.[113] Conversely, Ubanako et al saw enhanced lactate production and glycolytic polarisation of THP-1 cells following 48 hours of stimulation with LPS.[114] However, this change was not observed at a 24 hour timepoint.[114] A transcriptomic approach was also used to observe upregulation of multiple glycolytic enzymes.[114] As THP-1 cells are already expressing substantial amounts of GLUT transporters, potentially glucose uptake is already occurring at the maximum rate, and therefore is not enhanced further upon bacterial stimulation.[112, 113] Instead, glycolytic enzymes may be limiting glycolysis in THP-1 cells. It is also of note that there is limited research in respect to THP-1 cells glucose uptake following whole bacteria treatment. To best address this discord 2-NBDG uptake and GLUT-1 expression could be analysed using primary monocytes. This would mitigate the effects of using a cancer cell line, such as THP-1 cells, which show enhanced glucose uptake, 2-DG resistance, and over expression of multiple enzymes including glo1.[94, 107, 112]

### 5.5 Investigation into the role of phagocytosis in glycolysis upregulation

A key question that remains is whether phagocytosis directly leads to increased MG production in order to form the final MAIT cell activating ligand. It is unknown what form of ligand, 5-A-RU or 5-OP-RU, is present in intact bacteria. If 5-A-RU is the predominant ligand found in intact bacteria then upregulation of MG production pathways, including glycolysis, would be crucial in ensuring the formation of 5-OP-RU. These mechanisms of MG product would become less essential if 5-OP-RU is present in large quantities in intact bacteria. Although stimulation of endosomal TLRs, which come into contact with bacteria upon phagocytosis can cause an upregulation of glycolysis, a causative link between phagocytosis and glycolysis upregulation remains to be determined.[74]

Multiple techniques could be used to identify both a metabolic switch and MG upregulation upon phagocytosis. RNA sequencing of primary monocytes treated with either fixed or sonicated *E. coli* Δ*ribD* could be performed. Expression of key glycolytic enzymes and glucose transporters could then be assessed to deduce if phagocytosis causes an upregulation of the glycolytic machinery, and subsequently, MG production. A metabolomic approach could also be used to assess the cellular metabolism occurring after bacterial stimulation. A Seahorse XF analyser could be used to determine if a switch to glycolysis is occurring upon bacterial stimulation by measuring media acidification that occurs due to lactate production. This technique has been used to identify a glycolytic switch upon activation of a range of immune cells including monocytes, DCs and MAIT cells.[74, 89, 93] A metabolomic approach should indicate whether a clear switch from oxidative phosphorylation to glycolytic metabolism is occurring.

### 5.6 Proposed model for MAIT cell activation

Based on the published literature, previous unpublished data, and the data generated here, I propose the following mechanism of MAIT cell activation (Figure 5.3). Whole bacteria and bacterial ligands activate extracellular TLRs (1). External TLR stimulation causes a change in gene expression (2), leading to the expression of cytokines and co-stimulatory molecules (3). Phagocytosis of intact bacteria possibly enhances 5-A-RU delivery into APC endosomes (4). TLRs present in the endosome are activated by phagocytosed bacteria (5). Stimulation of both TLRs on the cell surface and in the endosome and PRRs cause an upregulation of the glycolytic pathway (6). Upregulation of glycolysis leads to augmented MG production (7). Fatty acid metabolism provides an alternate pathway for MG production (8). Bacteria are lysed in the phagolysosome, releasing 5-A-RU and 5-OP-RU formed in the bacteria (not shown) (9). The non-enzymatic condensation reaction of 5-A-RU and MG occurs in the phagolysosome, to

form 5-OP-RU (10). 5-OP-RU binds to MR1 and is trafficked to the cell surface (11). MAIT cells are activated in response to MR1-TCR interactions, secretory cytokines and co-stimulatory molecules produced by the APC (12, 13). The integration of the two signalling pathways causes changes in MAIT cell gene expression (14), leading to a robust production of pro-inflammatory cytokines and cytotoxic molecules by MAIT cells (15).

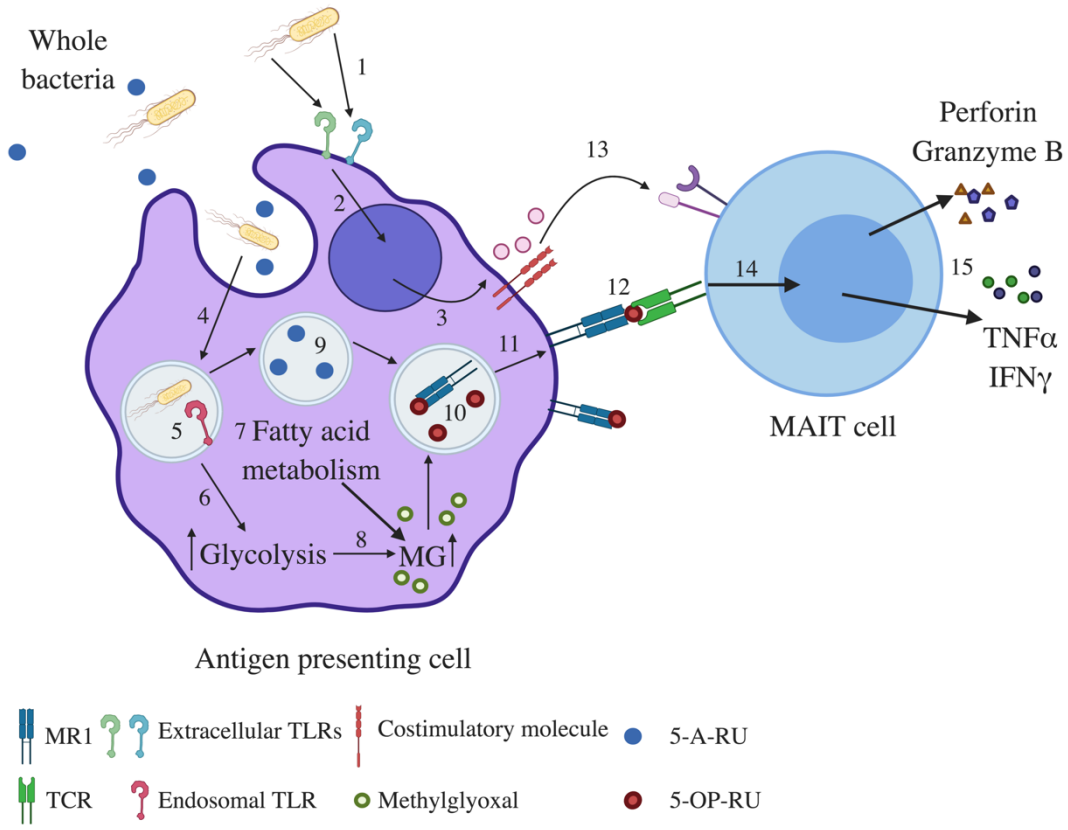



***Figure 5.3: Proposed model of regulation of MAIT cell activation by phagocytosis of whole bacteria and subsequent glycolysis upregulation.*** *Refer to section 1.5 for detailed description. Created with BioRender.com.*

## 5.7 Conclusions and future directions

Overall this study has partially elucidated the role of both phagocytosis and glycolysis within the APC for MAIT cell activation. Phagocytosis of whole bacteria is required for the robust activation of MAIT cells by 5-A-RU, but not 5-OP-RU. This suggests a role of phagocytosis in enhancement of MG production. Augmentation of glycolysis in THP-1 cells enhanced MAIT cell activation by 5-A-RU in the absence of intact bacteria to levels seen with intact

bacterial treatment. Activation by treatment with whole bacteria could not be further enhanced with F-1,6-BP treatment, suggesting that glycolysis is enhanced with phagocytosis of whole bacteria and cannot be further enhanced. However, inhibition of glycolysis did not reduce MAIT cell activation, likely due to the production of MG by other metabolic pathways. THP-1 cells do not exhibit enhanced glucose uptake upon bacterial stimulation, highlighting limitations of using the THP-1 cell line.

There is still a large amount of work to be done in order to dissect the full mechanism of regulation occurring in APCs. Usage of bioinformatic techniques, such as RNA sequencing and Seahorse acidification and oxygenation measurements could be employed in order to confirm the switch to glycolytic metabolism following bacterial stimulation. To overcome the limitations of using the THP-1 cell line, isolated primary monocytes could be treated with a range of bacterial preparations and TLR agonists to identify which signals are responsible for the upregulation of glycolysis.

MAIT cells are widely regarded as playing a pivotal role in the antibacterial immune response. Despite this, the mechanism for MAIT cell regulation and activation remains largely unresearched. This research implicates both phagocytosis and glycolysis upregulation in APCs in the regulation of MAIT cell activation. These findings provide a strong baseline of research which can be built upon in order to fully dissect and understand the regulatory mechanisms occurring in MAIT cell activation.

# 6 References


1. Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M. & Ugolini, S. Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49 (2011).

2. Lanier, L.L. Shades of grey — the blurring view of innate and adaptive immunity. *Nat Rev Immunol* **13**, 73-74 (2013).

3. Zeng, X., Wei, Y.L., Huang, J., Newell, E.W., Yu, H., Kidd, B.A., Kuhns, M.S., Waters, R.W., Davis, Mark M., Weaver, C.T. & Chien, Y.H. γδ T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity* **37**, 524-534 (2012).

4. Corbett, A.J., Eckle, S.B.G., Birkinshaw, R.W., Liu, L., Patel, O., Mahony, J., Chen, Z., Reantragoon, R., Meehan, B., Cao, H., Williamson, N.A., Strugnell, R.A., Van Sinderen, D., Mak, J.Y.W., Fairlie, D.P., Kjer-Nielsen, L., Rossjohn, J. & McCluskey, J. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361-365 (2014).

5. Reantragoon, R., Corbett, A.J., Sakala, I.G., Gherardin, N.A., Furness, J.B., Chen, Z., Eckle, S.B.G., Uldrich, A.P., Birkinshaw, R.W., Patel, O., Kostenko, L., Meehan, B., Kedzierska, K., Liu, L., Fairlie, D.P., Hansen, T.H., Godfrey, D.I., Rossjohn, J., McCluskey, J. & Kjer-Nielsen, L. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med* **210**, 2305-2320 (2013).

6. Tilloy, F., Treiner, E., Park, S.-H., Garcia, C., Lemonnier, F., de la Salle, H., Bendelac, A., Bonneville, M. & Lantz, O. An invariant T cell receptor α chain defines a novel TAP-independent major histocompatibility complex class Ib–restricted α/β T cell subpopulation in mammals. *J Exp Med* **189**, 1907-1921 (1999).

7. Porcelli, S., Yockey, C.E., Brenner, M.B. & Balk, S.P. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* **178**, 1-16 (1993).

8. Reantragoon, R., Kjer-Nielsen, L., Patel, O., Chen, Z., Illing, P.T., Bhati, M., Kostenko, L., Bharadwaj, M., Meehan, B., Hansen, T.H., Godfrey, D.I., Rossjohn, J. & McCluskey, J. Structural insight into MR1-mediated recognition of the mucosal associated invariant T cell receptor. *J Exp Med* **209**, 761-774 (2012).

9. Takahashi, T., Dejbakhsh-Jones, S. & Strober, S. Expression of CD161 (NKR-P1A) defines subsets of human CD4 and CD8 T cells with different functional activities. *J Immunol* **176**, 211-216 (2006).

10. Martin, E., Treiner, E., Duban, L., Guerri, L., Laude, H., Toly, C., Premel, V., Devys, A., Moura, I.C., Tilloy, F., Cherif, S., Vera, G., Latour, S., Soudais, C. & Lantz, O. Stepwise development of MAIT cells in mouse and human. *PLoS Biol* **7**, e54 (2009).

11. Fergusson, J.R., Smith, K.E., Fleming, V.M., Rajoriya, N., Newell, E.W., Simmons, R., Marchi, E., Björkander, S., Kang, Y.H., Swadling, L., Kurioka, A., Sahgal, N., Lockstone, H., Baban, D. & Freeman, G.J. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep* **9**, 1075-1088 (2014).

12. Lanier, L.L., Chang, C. & Phillips, J.H. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J Immunol* **153**, 2417-2428 (1994).

13. Koay, H.F., Gherardin, N.A., Enders, A., Loh, L., Mackay, L.K., Almeida, C.F., Russ, B.E., Nold-Petry, C.A., Nold, M.F., Bedoui, S., Chen, Z., Corbett, A.J., Eckle, S.B.G., Meehan, B., d'Udekem, Y., Konstantinov, I.E., Lappas, M., Liu, L., Goodnow, C.C., Fairlie, D.P., Rossjohn, J., Chong, M.M., Kedzierska, K., Berzins, S.P., Belz, G.T., McCluskey, J., Uldrich, A.P., Godfrey, D.I. & Pellicci, D.G. A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat Immunol* **17**, 1300-1311 (2016).

14. Kurioka, A., Jahun, A.S., Hannaway, R.F., Walker, L.J., Fergusson, J.R., Sverremark-Ekström, E., Corbett, A.J., Ussher, J.E., Willberg, C.B. & Klenerman, P. Shared and distinct phenotypes and functions of human CD161++ Vα7.2+ T cell subsets. *Front Immunol* **8**, 1031 (2017).

15. Dias, J., Boulouis, C., Gorin, J.B., van Den Biggelaar, R.H.G.A., Lal, K.G., Gibbs, A., Loh, L., Gulam, M.Y., Sia, W.R., Bari, S., Hwang, W.Y.K., Nixon, D.F., Nguyen, S.,

Betts, M.R., Buggert, M., Eller, M.A., Broliden, K., Tjernlund, A., Sandberg, J.K. & Leeansyah, E. The CD4CD8 MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8 MAIT cell pool. *Proc Natl Acad Sci U S A* **115**, E11513-E11522 (2018).

16. Leeansyah, E., Svärd, J., Dias, J., Buggert, M., Nyström, J., Quigley, M.F., Moll, M., Sönnerborg, A., Nowak, P. & Sandberg, J.K. Arming of MAIT cell cytolytic antimicrobial activity is induced by IL-7 and defective in HIV-1 infection. *PLoS Pathog* **11**, e1005072 (2015).

17. Dusseaux, M., Martin, E., Serriari, N., Péguillet, I., Premel, V., Louis, D., Milder, M., Le Bourhis, L., Soudais, C., Treiner, E. & Lantz, O. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* **117**, 1250-1259 (2011).

18. Treiner, E., Duban, L., Bahram, S., Radosavljevic, M., Wanner, V., Tilloy, F., Affaticati, P., Gilfillan, S. & Lantz, O. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164-169 (2003).

19. Gold, M.C., Cerri, S., Smyk-Pearson, S., Cansler, M.E., Vogt, T.M., Delepine, J., Winata, E., Swarbrick, G.M., Chua, W.J., Yu, Y.Y.L., Lantz, O., Cook, M.S., Null, M.D., Jacoby, D.B., Harriff, M.J., Lewinsohn, D.A., Hansen, T.H. & Lewinsohn, D.M. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* **8**, e1000407 (2010).

20. Sobkowiak, M.J., Davanian, H., Heymann, R., Gibbs, A., Emgard, J., Dias, J., Aleman, S., Kruger-Weiner, C., Moll, M., Tjernlund, A., Leeansyah, E., Chen, M.S. & Sandberg, J.K. Tissue-resident MAIT cell populations in human oral mucosa exhibit an activated profile and produce IL-17. *Eur J Immunol* **49**, 133-143 (2019).

21. Freeman, C.M., Curtis, J.L. & Chensue, S.W. CC chemokine receptor 5 and CXC chemokine receptor 6 expression by lung CD8 + cells correlates with chronic obstructive pulmonary disease severity. *Am J Pathol* **171**, 767-776 (2007).

22. Sato, T., Thorlacius, H., Johnston, B., Staton, T., Xiang, W., Littman, D. & Butcher, E. Role for CXCR6 in recruitment of activated CD8 super(+) lymphocytes to inflamed liver. *J Immunol* **174**, 277-283 (2005).

23. Billerbeck, E., Kang, Y.H., Walker, L., Lockstone, H., Grafmueller, S., Fleming, V., Flint, J., Willberg, C.B., Bengsch, B., Seigel, B., Ramamurthy, N., Zitzmann, N., Barnes, E.J., Thevanayagam, J., Bhagwanani, A., Leslie, A., Oo, Y.H., Kollnberger, S., Bowness, P., Drognitz, O., Adams, D.H., Blum, H.E., Thimme, R. & Klenerman, P. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A* **107**, 3006-3011 (2010).

24. Ussher, J.E., Bilton, M., Attwod, E., Shadwell, J., Richardson, R., Lara, C., Mettke, E., Kurioka, A., Hansen, T.H., Klenerman, P. & Willberg, C.B. CD 161 ++ CD 8 + T cells, including the MAIT cell subset, are specifically activated by IL ‐12+ IL ‐18 in a TCR ‐independent manner. *Eur J Immunol* **44**, 195-203 (2014).

25. Yamaguchi, H., Hirai, M., Kurosawa, Y. & Hashimoto, K. A highly conserved major histocompatibility complex class I-related gene in mammals. *Biochem Biophys Res Commun* **238**, 697-702 (1997).

26. Le Bourhis, L., Martin, E., Péguillet, I., Guihot, A., Froux, N., Coré, M., Lévy, E., Dusseaux, M., Meyssonnier, V., Premel, V., Ngo, C., Riteau, B., Duban, L., Robert, D., Huang, S., Rottman, M., Soudais, C. & Lantz, O. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol* **11**, 701–708 (2010).

27. Kjer-Nielsen, L., Patel, O., Corbett, A., J. , Le Nours, J., Meehan, B., Liu, L., Bhati, M., Chen, Z., Kostenko, L., Reantragoon , R., Williamson, N., A., Purcell, A., W. , Dudek, N., L., McConville, M., J., O'hair, R., A. J. , Khairallah, G., N., Godfrey, D., I., Fairlie, D., P., Rossjohn, J. & McCluskey, J. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717–723 (2012).

28. López-Sagaseta, J., Dulberger, C.L., McFedries, A., Cushman, M., Saghatelian, A. & Adams, E.J. MAIT recognition of a stimulatory bacterial antigen bound to MR1. *J Immunol* **191**, 5268-5277 (2013).

29. Mcwilliam, H.E.G., Eckle, S., B. G., Theodossis, A., Liu, L., Chen, Z., Wubben, J., M., Fairlie, D., P., Strugnell, R., A., Mintern, J., D., Mccluskey, J., Rossjohn, J. & Villadangos, J., A. The intracellular pathway for the presentation of vitamin B–related antigens by the antigen-presenting molecule MR1. *Nat Immunol* **17**, 531–537 (2016).

30. Aldemir, H. Novel MHC class I-related molecule MR1 affects MHC class I expression in 293T cells. *Biochem Biophys Res Commun* **366**, 328-334 (2008).

31. Miley, M.J., Truscott, S.M., Yu, Y.Y.L., Gilfillan, S., Fremont, D.H., Hansen, T.H. & Lybarger, L. Biochemical features of the MHC-related protein 1 consistent with an immunological function. *J Immunol* **170**, 6090-6098 (2003).

32. Yamaguchi, H. & Hashimoto, K. Association of MR1 protein, an MHC class I-related molecule, with β2-microglobulin. *Biochem Biophys Res Commun* **290**, 722-729 (2002).

33. Lamichhane, R. & Ussher, J.E. Expression and trafficking of MR1. *Immunology* **151**, 270-279 (2017).

34. Harriff, M.J., Karamooz, E., Burr, A., Grant, W.F., Canfield, E.T., Sorensen, M.L., Moita, L.F. & Lewinsohn, D.M. Endosomal MR1 trafficking plays a key role in presentation of Mycobacterium tuberculosis ligands to MAIT cells. *PLoS Pathog* **12**, e1005524 (2016).

35. Le Bourhis, L., Dusseaux, M., Bohineust, A., Bessoles, S., Martin, E., Premel, V., Coré, M., Sleurs, D., Serriari, N.E., Treiner, E., Hivroz, C., Sansonetti, P., Gougeon, M., Soudais, C. & Lantz, O. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog* **9**, e1003681 (2013).

36. Kurioka, A., Ussher, J.E., Cosgrove, C., Clough, C., Fergusson, J.R., Smith, K., Kang, Y.H., Walker, L.J., Hansen, T.H., Willberg, C.B. & Klenerman, P. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol* **8**, 429–440 (2014).

37. Lamichhane, R., Schneider, M., de La Harpe, S.M., Harrop, T.W.R., Hannaway, R.F., Dearden, P.K., Kirman, J.R., Tyndall, J.D.A., Vernall, A.J. & Ussher, J.E. TCR- or cytokine-activated CD8+ mucosal-associated invariant T cells are rapid polyfunctional effectors that can coordinate immune responses. *Cell Rep* **28**, 3061-3076 (2019).

38. Meierovics, A., Yankelevich, W.J.C. & Cowley, S.C. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proc Natl Acad Sci U S A* **110**, E3119-E3128 (2013).

39. Tang, X.Z., Jo, J., Tan, A.T., Sandalova, E., Chia, A., Tan, K.C., Lee, K.H., Gehring, A.J., De Libero, G. & Bertoletti, A. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol* **190**, 3142-3152 (2013).

40. Leeansyah, E., Ganesh, A., Quigley, M.F., Sonnerborg, A., Andersson, J., Hunt, P.W., Somsouk, M., Deeks, S.G., Martin, J.N., Moll, M., Shacklett, B.L. & Sandberg, J.K. Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. *Blood* **121**, 1124-1135 (2013).

41. Sattler, A., Dang-Heine, C., Reinke, P. & Babel, N. IL-15 dependent induction of IL-18 secretion as a feedback mechanism controlling human MAIT-cell effector functions. *Eur J Immunol* **45**, 2286-2298 (2015).

42. Lamichhane, R., Galvin, H., Hannaway, R.F., de La Harpe, S.M., Munro, F., Tyndall, J.D., Vernall, A.J., McCall, J.L., Husain, M. & Ussher, J.E. Type I interferons are important co-stimulatory signals during T cell receptor mediated human MAIT cell activation. *Eur J Immunol* **50**, 178-191 (2020).

43. Dias, J., Leeansyah, E. & Sandberg, J.K. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines. *Proc Natl Acad Sci U S A* **114**, E5434-E5443 (2017).

44. Bánki, Z., Krabbendam, L., Klaver, D., Leng, T., Kruis, S., Mehta, H., Müllauer, B., Orth-Höller, D., Stoiber, H., Willberg, C.B. & Klenerman, P. Antibody opsonization enhances MAIT cell responsiveness to bacteria via a TNF-dependent mechanism. *Immunol Cell Biol* **97**, 538-551 (2019).

45. Wong, E.B., Akilimali, N.A., Govender, P., Sullivan, Z.A., Cosgrove, C., Pillay, M., Lewinsohn, D.M., Bishai, W.R., Walker, B.D., Ndung'u, T., Klenerman, P. & Kasprowicz, V.O. Low Levels of Peripheral CD161++CD8+ Mucosal Associated Invariant T (MAIT) Cells Are Found in HIV and HIV/TB Co-Infection. *PLoS One* **8**, e83474 (2014).

46. Wang, H., D'Souza, C., Lim, X.Y., Kostenko, L., Pediongco, T.J., Eckle, S.B.G., Meehan, B.S., Shi, M., Wang, N., Li, S., Liu, L., Mak, J.Y.W., Fairlie, D.P., Iwakura,

Y., Gunnersen, J.M., Stent, A.W., Godfrey, D.I., Rossjohn, J., Westall, G.P., Kjer-Nielsen, L., Strugnell, R.A., McCluskey, J., Corbett, A.J., Hinks, T.S.C. & Chen, Z. MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun* **9**, 3350-3315 (2018).

47. Booth, J.S., Salerno-Goncalves, R., Blanchard, T.G., Patil, S.A., Kader, H.A., Safta, A.M., Morningstar, L.M., Czinn, S.J., Greenwald, B.D. & Sztein, M.B. Mucosal-associated invariant T cells in the human gastric mucosa and blood: role in Helicobacter pylori infection. *Front Immunol* **6**, 466 (2015).

48. Grimaldi, D., Le Bourhis, L., Sauneuf, B., Dechartres, A., Rousseau, C., Ouaaz, F., Milder, M., Louis, D., Chiche, J.D., Mira, J.P., Lantz, O. & Pène, F. Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections. *Intensive Care Med* **40**, 192-201 (2014).

49. Hannaway, R.F., Wang, X., Schneider, M., Slow, S., Cowan, J., Brockway, B., Schofield, M.R., Morgan, X.C., Murdoch, D.R. & Ussher, J.E. Mucosal-associated invariant T cells and Vδ2+ γδ T cells in community acquired pneumonia: association of abundance in sputum with clinical severity and outcome. *Clin Exp Immunol* **199**, 201-215 (2020).

50. Georgel, P., Radosavljevic, M., Macquin, C. & Bahram, S. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. *Mol Immunol* **48**, 769-775 (2011).

51. van Wilgenburg, B., Scherwitzl, I., Hutchinson, E., C., Leng, T., Kurioka, A., Kulicke, C., De Lara, C., Cole, S., Vasanawathana, S., Limpitikul, W., Malasit, P., Young, D., Denney, L., Moore, M., D, Fabris, P., Giordani, M.T., Oo, Y.H., Laidlaw, S., M, Dustin, L., B, Ho, L.P., Thompson, F., M., Ramamurthy, N., Mongkolsapaya, J., Willberg, C., B., Screaton, G., R. & Klenerman, P. MAIT cells are activated during human viral infections. *Nat Commun* **7**, 1-11 (2016).

52. Loh, L., Wang, Z., Sant, S., Koutsakos, M., Jegaskanda, S., Corbett, A.J., Liu, L., Fairlie, D.P., Crowe, J., Rossjohn, J., Xu, J., Doherty, P.C., McCluskey, J. & Kedzierska, K. Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc Natl Acad Sci U S A* **113**, 10133-10138 (2016).

53. Cosgrove, C., Ussher, J.E., Rauch, A., Gärtner, K., Kurioka, A., Hühn, M.H., Adelmann, K., Kang, Y.-H., Fergusson, J.R., Simmonds, P., Goulder, P., Hansen, T.H., Fox, J., Günthard, H.F., Khanna, N., Powrie, F., Steel, A., Gazzard, B., Phillips, R.E., Frater, J., Uhlig, H. & Klenerman, P. Early and nonreversible decrease of CD161++/MAIT cells in HIV infection. *Blood* **121**, 951-961 (2013).

54. Won, E.J., Ju, J.K., Cho, Y.N., Jin, H.M., Park, K.J., Kim, T.J., Kwon, Y.S., Kee, H.J., Kim, J.C., Kee, S.J. & Park, Y.W. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget* **7**, 76274-76290 (2016).

55. Limian, L., Yuyang, L., Wenwen, Z., Sen, H., Xiuqi, T., Pingwei, Z., Ming, L., Jingsong, N., Chenguang, L., Lei, W. & Yanfang, J. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci Rep* **6**, 20358 (2016).

56. Zabijak, L., Attencourt, C., Guignant, C., Chatelain, D., Marcelo, P., Marolleau, J.P. & Treiner, E. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol Immunother* **64**, 1601-1608 (2015).

57. Kelly, J., Minoda, Y., Meredith, T., Cameron, G., Philipp, M.S., Pellicci, D.G., Corbett, A.J., Kurts, C., Gray, D.H., Godfrey, D.I., Kannourakis, G. & Berzins, S.P. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol Cell Biol* **97**, 689-699 (2019).

58. Cho, Y.N., Kee, S.J., Kim, T.J., Jin, H.M., Kim, M.J., Jung, H.J., Park, K.J., Lee, S.J., Lee, S.S., Kwon, Y.S., Kee, H.J., Kim, N. & Park, Y.W. Mucosal-associated invariant T cell deficiency in systemic lupus erythematosus. *J Immunol* **193**, 3891-3901 (2014).

59. Cassius, C., Branchtein, M., Battistella, M., Amode, R., Lepelletier, C., Jachiet, M., de Masson, A., Frumholtz, L., Chasset, F., Amoura, Z., Mathian, A., Samri, A., Monfort, J.B., Bachmeyer, C., Bengoufa, D., Cordoliani, F., Bagot, M., Bensussan, A., Bouaziz, J.D. & Le Buanec, H. Persistent deficiency of mucosal-associated invariant T cells during dermatomyositis. *Rheumatology*, kez564 (2019).

60. Serriari, N.E., Eoche, M., Lamotte, L., Lion, J., Fumery, M., Marcelo, P., Chatelain, D., Barre, A., Nguyen-Khac, E., Lantz, O., Dupas, J.L. & Treiner, E. Innate mucosal-

associated invariant T ( MAIT ) cells are activated in inflammatory bowel diseases. *Clin Exp Immunol* **176**, 266-274 (2014).

61. Leng, T., Akther, H.D., Hackstein, C.P., Powell, K., King, T., Friedrich, M., Christoforidou, Z., McCuaig, S., Neyazi, M., Arancibia-Cárcamo, C.V., Hagel, J., Powrie, F., Peres, R.S., Millar, V., Ebner, D., Lamichhane, R., Ussher, J., Hinks, T.S.C. & Marchi, E. TCR and inflammatory signals tune human MAIT cells to exert specific tissue repair and effector functions. *Cell Rep* **28**, 3077-3091 (2019).

62. O'Brien, A., Loftus, R.M., Pisarska, M.M., Tobin, L.M., Bergin, R., Wood, N.A.W., Foley, C., Mat, A., Tinley, F.C., Bannan, C., Sommerville, G., Veerapen, N., Besra, G.S., Sinclair, L.V., Moynagh, P.N., Lynch, L., Finlay, D.K., O'Shea, D. & Hogan, A.E. Obesity reduces mTORC1 activity in mucosal-associated invariant T cells, driving defective metabolic and functional responses. *J Immunol* **202**, 3404-3411 (2019).

63. Dewar, P. Bacterial regulation of early activation of mucosal-associated invariant T (MAIT) cells. Bachelor of Biomedical Sciences with Honours thesis, University of Otago, Otago, Dunedin, New Zealand, 2019.

64. Ussher, J.E., Wilgenburg, B., Hannaway, R.F., Ruustal, K., Phalora, P., Kurioka, A., Hansen, T.H., Willberg, C.B., Phillips, R.E. & Klenerman, P. TLR signaling in human antigen-presenting cells regulates MR1-dependent activation of MAIT cells. *Eur J Immunol* **46**, 1600-1614 (2016).

65. Newsholme, P., Curi, R., Gordon, S. & Newsholme, E.A. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. *Biochem J* **239**, 121-125 (1986).

66. Hamilton, J.A., Vairo, G. & Lingelbach, S.R. CSF-1 stimulates glucose uptake in murine bone marrow-derived macrophages. *Biochem Biophys Res Commun* **138**, 445-454 (1986).

67. Chang, C.H., Curtis, J.D., Maggi, L.B., Faubert, B., Villarino, A.V., O'sullivan, D., Huang, S.C.C., van der Windt, G.J.W., Blagih, J., Qiu, J. & Weber, J.D. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* **153**, 1239-1251 (2013).

68. Zinser, M.E., Highton, A.J., Kurioka, A., Kronsteiner, B., Hagel, J., Leng, T., Marchi, E., Phetsouphanh, C., Willberg, C.B., Dunachie, S.J. & Klenerman, P. Human MAIT cells show metabolic quiescence with rapid glucose-dependent upregulation of granzyme B upon stimulation. *Immunol Cell Biol* **96**, 666-674 (2018).

69. Peng, M., Yin, N., Chhangawala, S., Xu, K., Leslie, C.S. & Li, M.O. Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. *Science* **354**, 481-484 (2016).

70. Moon, J.S., Hisata, S., Park, M.A., Denicola, G.M., Ryter, S.W., Nakahira, K. & Choi, A.M.K. mTORC1-induced HK1-dependent glycolysis regulates NLRP3 inflammasome activation. *Cell Rep* **12**, 102-115 (2015).

71. Freemerman, A.J., Johnson, A.R., Sacks, G.N., Milner, J.J., Kirk, E.L., Troester, M.A., Macintyre, A.N., Goraksha-Hicks, P., Rathmell, J.C. & Makowski, L. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *J Biol Chem* **289**, 7884-7896 (2014).

72. Haschemi, A., Kosma, P., Gille, L., Evans, Charles R., Burant, Charles F., Starkl, P., Knapp, B., Haas, R., Schmid, Johannes A., Jandl, C., Amir, S., Lubec, G., Park, J., Esterbauer, H., Bilban, M., Brizuela, L. & Pospisilik, J.A. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab* **15**, 813-826 (2012).

73. Bajwa, G., Deberardinis, R.J., Shao, B., Hall, B., Farrar, J.D. & Gill, M.A. Cutting edge: critical role of glycolysis in human plasmacytoid dendritic cell antiviral responses. *J Immunol* **196**, 2004-2009 (2016).

74. Everts, B., Amiel, E., Huang, S.C.-C., Smith, A.M., Chang, C.-H., Lam, W., Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J.W., Artyomov, M.N., Jones, R.G., Pearce, E.L. & Pearce, E.J. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nat Immunol* **15**, 323–332 (2014).

75. Rodríguez-Prados, J.C., Través, P.G., Cuenca, J., Rico, D., Aragonés, J., Martín-Sanz, P., Cascante, M. & Boscá, L. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *J Immunol* **185**, 605-614 (2010).

76. Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G. & Pearce, E.J. Toll-like receptor–induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* **115**, 4742-4749 (2010).

77. Phillips, S.A. & Thornalley, P.J. The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal. *Eur J Biochem* **212**, 101-105 (1993).

78. Richard, J.P. Acid-base catalysis of the elimination and isomerization reactions of triose phosphates. *J Am Chem Soc* **106**, 4926-4936 (1984).

79. Thornalley, P.J., Langborg, A. & Minhas, H.S. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* **344**, 109-116 (1999).

80. Lamichhane, R., Schneider, M., de la Harpe, S.M., Harrop, T.W.R., Hannaway, R.F., Dearden, P., Kirman, J.R., Tyndall, J.D.A., Vernall, A.J. & Ussher, J.E. Transcriptional analysis defines TCR and cytokine-stimulated MAIT cells as rapid polyfunctional effector T cells that can coordinate the immune response. *bioRxiv*, 600189 (2019).

81. Williams, T.M. The role of Toll-like receptor signalling in MR1 surface expression and mucosal associated invariant T (MAIT) cell activation Bachelor of Science with Honours thesis, University of Otago, Dunedin, New Zealand, 2017.

82. Liu, J., Desai, K., Wang, R. & Wu, L. Up-regulation of aldolase A and methylglyoxal production in adipocytes. *Br J Pharmacol* **168**, 1639-1646 (2013).

83. Wick, A.N., Drury, D.R., Nakada, H.I. & Wolfe, J.B. Localization of the primary metabolic block produced by 2-deoxyglucose. *J Biol Chem* **224**, 963 (1957).

84. Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H. & Thompson, C.B. The CD28 signaling pathway regulates glucose metabolism. *Immunity* **16**, 769-777 (2002).

85. Legoux, F., Bellet, D., Daviaud, C., El Morr, Y., Darbois, A., Niort, K., Procopio, E., Salou, M., Gilet, J., Ryffel, B., Balvay, A., Foussier, A., Sarkis, M., El Marjou, A., Schmidt, F., Rabot, S. & Lantz, O. Microbial metabolites control the thymic development of mucosal-associated invariant T cells. *Science* **366**, 494-499 (2019).

86. Barr, T.A., Brown, S., Ryan, G., Zhao, J. & Gray, D. TLR-mediated stimulation of APC: distinct cytokine responses of B cells and dendritic cells. *Eur J Immunol* **37**, 3040-3053 (2007).

87. Tang, J.S., Compton, B.J., Marshall, A., Anderson, R., Li, Y., van der Woude, H., Hermans, I.F., Painter, G.F. & Gasser, O. Mānuka honey-derived methylglyoxal enhances microbial sensing by mucosal-associated invariant T cells. *Food Funct* **11**, 5782-5787 (2020).

88. Turtle, C.J., Delrow, J., Joslyn, R.C., Swanson, H.M., Basom, R., Tabellini, L., Delaney, C., Heimfeld, S., Hansen, J.A. & Riddell, S.R. Innate signals overcome acquired TCR signaling pathway regulation and govern the fate of human CD161hi CD8α+ semi-invariant T cells. *Blood* **118**, 2752-2762 (2011).

89. Zinser, M.E., Highton, A.J., Kurioka, A., Kronsteiner, B., Hagel, J., Leng, T., Marchi, E., Phetsouphanh, C., Willberg, C.B., Dunachie, S.J. & Klenerman, P. Human MAIT cells show metabolic quiescence with rapid glucose - dependent upregulation of granzyme B upon stimulation. *Immunol Cell Biol* **96**, 666-674 (2018).

90. Boulouis, C., Gorin, J.-B., Dias, J., Bergman, P., Leeansyah, E. & Sandberg, J.K. Opsonization-enhanced antigen presentation by MR1 activates rapid polyfunctional MAIT cell responses acting as an effector arm of humoral antibacterial immunity. *J Immunol* **205**, 67-77 (2020).

91. Benlahrech, A., Donaghy, H., Rozis, G., Goodier, M., Klavinskis, L., Gotch, F. & Patterson, S. Human NK cell up-regulation of CD69, HLA-DR, interferon γ secretion and cytotoxic activity by plasmacytoid dendritic cells is regulated through overlapping but different pathways. *Sensors* **9**, 386-403 (2009).

92. Kraaij, M.D., Vereyken, E.J.F., Leenen, P.J.M., van den Bosch, T.P.P., Rezaee, F., Betjes, M.G.H., Baan, C.C. & Rowshani, A.T. Human monocytes produce interferon-gamma upon stimulation with LPS. *Cytokine* **67**, 7-12 (2014).

93. Lee, M.K.S., Al-Sharea, A., Shihata, W.A., Bertuzzo Veiga, C., Cooney, O.D., Fleetwood, A.J., Flynn, M.C., Claeson, E., Palmer, C.S., Lancaster, G.I., Henstridge, D.C., Hamilton, J.A. & Murphy, A.J. Glycolysis is required for LPS-induced activation and adhesion of human CD14+CD16− monocytes. *Front Immunol* **10**, 2054 (2019).

94. Miwa, H., Shikami, M., Goto, M., Mizuno, S., Takahashi, M., Tsunekawa-Imai, N., Ishikawa, T., Mizutani, M., Horio, T., Gotou, M., Yamamoto, H., Wakabayashi, M., Watarai, M., Hanamura, I., Imamura, A., Mihara, H. & Nitta, M. Leukemia cells demonstrate a different metabolic perturbation provoked by 2-deoxyglucose. *Oncol Rep* **29**, 2053-2057 (2013).

95. Lapa, B., Gonçalves, A.C., Jorge, J., Alves, R., Pires, A.S., Abrantes, A.M., Coucelo, M., Abrunhosa, A., Botelho, M.F., Nascimento-Costa, J.M. & Sarmento-Ribeiro, A.B. Acute myeloid leukemia sensitivity to metabolic inhibitors: glycolysis showed to be a better therapeutic target. *Med Oncol* **37** (2020).

96. Suganuma, K., Miwa, H., Imai, N., Shikami, M., Gotou, M., Goto, M., Mizuno, S., Takahashi, M., Yamamoto, H., Hiramatsu, A., Wakabayashi, M., Watarai, M., Hanamura, I., Imamura, A., Mihara, H. & Nitta, M. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation. *Leuk Lymphoma* **51**, 2112-2119 (2010).

97. Koop, D.R. & Casazza, J.P. Identification of ethanol-inducible P-450 isozyme 3a as the acetone and acetol monooxygenase of rabbit microsomes. *J Biol Chem* **260**, 13607 (1985).

98. Casazza, J.P., Felver, M.E. & Veech, R.L. The metabolism of acetone in rat. *J Biol Chem* **259**, 231 (1984).

99. Raulien, N., Friedrich, K., Strobel, S., Rubner, S., Baumann, S., von Bergen, M., Körner, A., Krueger, M., Rossol, M. & Wagner, U. Fatty acid oxidation compensates for lipopolysaccharide-induced warburg effect in glucose-deprived monocytes. *Front Immunol* **8**, 609-609 (2017).

100. Desai, K.M., Chang, T., Wang, H., Banigesh, A., Dhar, A., Liu, J., Untereiner, A. & Wu, L. Oxidative stress and aging: is methylglyoxal the hidden enemy? *Can J Physiol Pharmacol* **88**, 273-284 (2010).

101. Thornalley, P.J. Glyoxalase I – structure, function and a critical role in the enzymatic defence against glycation. *Biochem Soc Trans* **31**, 1343-1348 (2003).

102. Haidinger, M., Poglitsch, M., Geyeregger, R., Kasturi, S., Zeyda, M., Zlabinger, G.J., Pulendran, B., Hörl, W.H., Säemann, M.D. & Weichhart, T. A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation. *J Immunol* **185**, 3919-3931 (2010).

103. Ohtani, M., Nagai, S., Kondo, S., Mizuno, S., Nakamura, K., Tanabe, M., Takeuchi, T., Matsuda, S. & Koyasu, S. Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. *Blood* **112**, 635-643 (2008).

104. Wang, H., Brown, J., Gu, Z., Garcia, C.A., Liang, R., Alard, P., Beurel, E., Jope, R.S., Greenway, T. & Martin, M. Convergence of the mammalian target of rapamycin complex 1- and glycogen synthase kinase 3-β-signaling pathways regulates the innate inflammatory response. *J Immunol* **186**, 5217-5226 (2011).

105. Sun, R., Zhang, Y., Ma, S., Qi, H., Wang, M., Duan, J., Ma, S., Zhu, X., Li, G. & Wang, H. Down-regulation of mitogen-activated protein kinases and nuclear factor-κB signaling is involved in rapamycin suppression of TLR2-induced inflammatory response in monocytic THP-1 cells. *Microbiol Immunol* **59**, 614-622 (2015).

106. Lachmandas, E., Boutens, L., Ratter, J.M., Hijmans, A., Hooiveld, G.J., Joosten, L.A.B., Rodenburg, R.J., Fransen, J.A.M., Houtkooper, R.H., van Crevel, R., Netea, M.G. & Stienstra, R. Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nat Microbiol* **2**, 16246 (2016).

107. Kohro, T., Tanaka, T., Murakami, T., Wada, Y., Aburatani, H., Hamakubo, T. & Kodama, T. A comparison of differences in the gene expression profiles of phorbol 12-

myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. *J Atheroscler Thromb* **11**, 88-97 (2004).

108. Talesa, V.N., Ferri, I., Bellezza, G., Love, H.D., Sidoni, A. & Antognelli, C. Glyoxalase 2 is involved in human prostate cancer progression as part of a mechanism driven by PTEN/PI3K/AKT/mTOR signaling with involvement of PKM2 and ERα. *Prostate* **77**, 196-210 (2017).

109. Ko, J.H., Yoon, S.-O., Lee, H.J. & Oh, J.Y. Rapamycin regulates macrophage activation by inhibiting NLRP3 inflammasome-p38 MAPK-NFκB pathways in autophagy- and p62-dependent manners. *Oncotarget* **8**, 40817-40831 (2017).

110. Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H., Zheng, L., Gardet, A., Tong, Z., Jany, S.S., Corr, S.C., Haneklaus, M., Caffrey, B.E., Pierce, K., Walmsley, S., Beasley, F.C., Cummins, E., Nizet, V., Whyte, M., Taylor, C.T., Lin, H., Masters, S.L., Gottlieb, E., Kelly, V.P., Clish, C., Auron, P.E., Xavier, R.J. & O'Neill, L.A.J. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* **496**, 238-242 (2013).

111. Fukuzumi, M., Shinomiya, H., Shimizu, Y., Ohishi, K. & Utsumi, S. Endotoxin-induced enhancement of glucose influx into murine peritoneal macrophages via GLUT1. *Infect Immun* **64**, 108-112 (1996).

112. Fu, Y. Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3, and 5 in the immune response and foam cell formation. *Blood Cells Mol Dis* **32**, 182-190 (2004).

113. Singh, V., Kaur, C., Chaudhary, V.K., Rao, K.V.S. & Chatterjee, S. M. tuberculosis secretory protein ESAT-6 induces metabolic flux perturbations to drive foamy macrophage differentiation. *Sci Rep* **5**, 12906 (2015).

114. Ubanako, P., Xelwa, N. & Ntwasa, M. LPS induces inflammatory chemokines via TLR-4 signalling and enhances the Warburg Effect in THP-1 cells. *PLoS One* **14**, e0222614 (2019).