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# Mānuka honey-derived methylglyoxal enhances microbial sensing by mucosal-associated invariant T cells†

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Methylglyoxal (MGO) is the main antimicrobial determinant associated with using Mānuka Honey as a topical dressing. While direct mechanisms of Mānuka honey MGO's antimicrobial activity have been demonstrated, such as disruption of bacterial fimbria and flagella, no interaction of Mānuka honey-derived MGO with antimicrobial effector cells of the immune system, such as mucosal-associated invariant T cells (MAIT cells), has yet been reported. MAIT cells are an abundant subset of human T cells, critical for regulating a diverse range of immune functions, including antimicrobial defense mechanisms but also mucosal barrier integrity. MAIT cells become activated by recognition of an important microbial metabolite, 5-amino-6-D-ribitylaminouracil (5-A-RU), which is produced by a wide range of microbial pathogens and commensals. Recognition is afforded when 5-A-RU condenses with mammalian-cell derived MGO to form the potent MAIT cell activator, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). Formation of 5-OP-RU and its subsequent presentation to MAIT cells by major histocompatibility (MHC)-related molecule 1 (MR1) facilitates host–pathogen and host–commensal interactions. While MGO is a metabolite naturally present in mammalian cells, it is unclear whether exogenous dietary MGO sources, such as those obtained from Mānuka honey intake, can contribute to 5-OP-RU formation and enhance MAIT cell activation. In this work, we report that endogenous MGO is the rate-limiting substrate for converting microbial 5-A-RU to 5-OP-RU and that Mānuka honey-derived MGO significantly enhances MAIT cell activation *in vitro*. Our findings posit a novel mechanism by which intake of a food item, such as Mānuka honey, can potentially support immune homeostasis by enhancing MAIT cell-specific microbial sensing.

## 1. Introduction

Honey, a complex natural product generated by bees from the flower nectar of melliferous plants, has long been used as a wound dressing and is notable for its antimicrobial properties.<sup>1,2</sup>

Monofloral honey types derived from different flowering plants exhibit substantial differences in antimicrobial activities, when tested against various clinically-relevant microbial isolates.<sup>1,2</sup> This observation is often linked to differences in the chemical composition of active compounds found in different monofloral honey types.<sup>1</sup>

One monofloral honey type that exhibits a particularly broad spectrum of antimicrobial activity is Mānuka honey,<sup>1–3</sup> derived from the flowers of the Mānuka bush (*Leptospermum species*).<sup>4</sup> Unlike many honeys that contain hydrogen peroxide as a major antimicrobial component, the main antimicrobial determinant of Mānuka honey is methylglyoxal (MGO).<sup>5,6</sup> This is an  $\alpha$ -dicarbonyl compound, formed from the spontaneous dehydration of dihydroxyacetone, which naturally occurs in the nectar of flowers of some species belonging to the *Leptospermum* genus, including *Leptospermum scoparium*, *Leptospermum polygalifolium*, and some related *Leptospermum species* native to New Zealand and Australia.<sup>7–9</sup> While MGO is especially abundant in Mānuka honey, its occurrence in other honey types are limited, and if present, are typically found in lower amounts than that in Mānuka honey.<sup>1,10</sup>

While the direct mechanisms of antimicrobial activity of honey MGO have been reported, such as the ability to limit bacterial adherence and motility by disrupting the structure of fimbriae and flagella,<sup>11</sup> to date, no studies have reported the ability of Mānuka honey MGO to enhance microbial sensing by cells of the immune system.

Mucosal-associated invariant T cells (MAIT cells) are a recently-described, abundant subset of T cells in humans, typically enriched in mucosal surfaces, such as the oral and gastrointestinal tracts.<sup>12</sup> They are also abundant in peripheral blood and the liver. MAIT cells can protect the body from bacterial pathogens by the recognition of small molecule vitamin B2-

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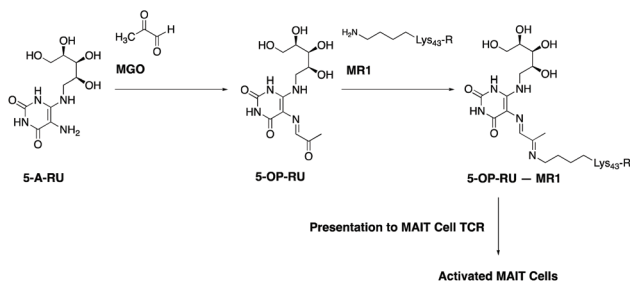
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**Fig. 1** Formation of 5-OP-RU, a potent activator of mucosal-associated invariant T cells (MAIT cells), from the condensation of microbial 5-A-RU with methylglyoxal. Once formed, 5-OP-RU can be subsequently presented to MAIT cells by major histocompatibility (MHC)-related molecule 1 (MR1). This activates MAIT cells and facilitates host–pathogen and host–commensal interactions. (Abbreviations: 5-A-RU, 5-amino-6-D-ribitylamino-uracil; MGO, methylglyoxal; 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylamino-uracil; MR1, major histocompatibility complex-related protein-1; 5-OP-RU – MR1, 5-OPRU covalently-bonded to MR1; TCR, T-cell receptor).

based metabolite intermediates produced through the riboflavin biosynthesis pathway, which is present in a wide range of bacteria, mycobacteria and yeasts.<sup>13–17</sup> A most notable early intermediate in this pathway is 5-amino-6-D-ribitylamino-uracil (5-A-RU). Recognition of 5-A-RU by MAIT cells is facilitated by formation of the potent MAIT cell activator, 5-(2-oxopropylideneamino)-6-D-ribitylamino-uracil (5-OP-RU),<sup>18,19</sup> through the non-enzymatic condensation of microbial 5-A-RU with endogenous MGO (Fig. 1). The latter metabolite (MGO) is naturally formed in mammalian cells by virtue of a combination of glycolysis, ketone body and threonine metabolisms.<sup>20</sup> Once formed, 5-OP-RU can form a Schiff base with Lys43 of major histocompatibility complex (MHC)-related protein-1 (MR1),<sup>15,18</sup> which is subsequently presented to the ‘semi-invariant’  $\alpha\beta$  T cell receptor (TCR) expressed on MAIT cells (Fig. 1). This antigen presentation activates MAIT cells, leading to the production of a variety of cytokines, chemokines and responses that in turn protect the body against microbial infection.<sup>21,22</sup>

While MGO is an endogenous metabolite naturally present in mammalian cells, it is unclear whether exogenous dietary MGO sources, such as those obtained from Mānuka honey intake, can contribute to 5-OP-RU formation and enhance MAIT cell activation. In this communication, we report that endogenous MGO is the rate-limiting substrate for the conversion of microbial 5-A-RU to 5-OP-RU and that Mānuka honey-derived MGO significantly enhances MAIT cell activation *in vitro* in the presence of 5-A-RU. Our finding posits a novel mechanism by which Mānuka honey-derived MGO can contribute to the body’s immune responses against microbial infection.

## 2. Materials and methods

### 2.1. Synthesis of 5-amino-6-D-ribitylamino-uracil (5-A-RU)

5-A-RU was produced by synthetic methods based on a published procedure.<sup>23</sup> In short, D-ribitylamine and 6-chlorouracil

were heated to 170 °C in a sealed vessel with triethylamine for 45 min before purification on an ion-exchange resin (AG-1X8), which gave 6-(D-ribitylamino)uracil. 5-Nitrosylation was achieved by treating 6-(D-ribitylamino)uracil with NaOH and NaNO<sub>2</sub> at 0 °C followed by the addition of aqueous acetic acid. Ion-exchange chromatography (AG-1X8) gave 5-nitroso-6-(D-ribitylamino)uracil which, upon reduction with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, afforded 5-A-RU which was used without further purification.

### 2.2. Methylglyoxal analysis in Mānuka honey

Mānuka Health Mānuka honey MGO<sup>TM</sup> 30+ and MGO<sup>TM</sup> 400+ produced by European honeybees (*Apis mellifera*) from the nectar of the native New Zealand Mānuka bush, *Leptospermum scoparium* were supplied by Mānuka Health New Zealand (grade MGO<sup>TM</sup> 30+, batch FMH3710 with 72.6 mg kg<sup>-1</sup> methylglyoxal, and grade MGO<sup>TM</sup> 400, batch FMH3563 with 451.4 mg kg<sup>-1</sup> methylglyoxal). This honey was tested for purity and quality and has a certified MGO content.<sup>24</sup>

### 2.3. Characterisation of 5-(2-oxopropylideneamino)-6-D-ribitylamino-uracil (5-OP-RU) by HPLC-MS and <sup>1</sup>NMR analyses

Formation of 5-OP-RU was obtained by reacting a solution of 5-A-RU (5 mg mL<sup>-1</sup> in DMSO) with methylglyoxal (2 equivalents) and monitoring the reaction by HPLC-MS (ESI Scheme 1†). After 30 min post mixing, analysis showed almost complete consumption of 5-A-RU and the formation of 5-OP-RU alongside a small amount of the less-active degradative species,<sup>25</sup> 7-methyl-8-D-ribityllumazine (RL-7-Me) (ESI Fig. 1†).

### 2.4. Isolation and *in vitro* stimulation of human peripheral blood mononuclear cells (PBMC)

Anticoagulated blood was obtained from healthy volunteers. The study was approved by the Victoria University of Wellington Human Ethics Committee (ref: 19966) and conducted in adherence to standard biosecurity and institutional safety procedures. Informed consent was obtained from all subjects. Peripheral blood mononuclear cells (PBMC) were isolated from blood by means of density gradient centrifugation using Leucosep tubes (Sigma, St Louis, MO). PBMC were resuspended in 10% (v/v) DMSO in heat-inactivated (56 °C, 30 min) fetal bovine serum (ThermoFisher Scientific, Rockford, IL) and stored in liquid nitrogen until use.

PBMC were resuspended in an RPMI medium (ThermoFisher Scientific, Rockford, IL), supplemented with 10% heat-inactivated fetal bovine serum and plated in a 96-well round-bottom plate at a concentration of  $2.5 \times 10^6$  cell per mL. Cells were treated with 5-A-RU<sup>26</sup> in the presence or absence of equimolar amounts (0.5–1000 nM) of synthetic methylglyoxal (Sigma, St Louis, MO) or PBS dilutions of commercial Mānuka honey products MGO<sup>TM</sup> 30+ (0.46 μg honey per mL–1 mg honey per mL) or MGO<sup>TM</sup> 400+ (0.16 mg honey per mL). The admixtures of 5-A-RU and either synthetic or honey-derived MGO were prepared immediately prior to use. The PBMC cultures were treated at 37 °C overnight before flow cytometric analysis.



## 2.5. Flow cytometry

Samples were incubated with Zombie Aqua™ dye (BioLegend, San Diego, CA) for 15 min in the dark at room temperature, to distinguish live cells from the dead, and were subsequently washed with FACS buffer. Privigen normal immunoglobulin (CSL Behring, Bern, Switzerland) was used as an Fc-block in staining and wash buffers. Antibody staining was carried out for 20 min in the dark on ice. Antibodies were sourced from BioLegend unless otherwise mentioned. The following antibodies were used throughout: anti-CD3 (UCHT1), anti-CD161 (HP-3G10), anti-TCR V $\alpha$ 7.2 (3C10), anti-CD137 (4B4-1).

Stained cells were measured using an LSR-II flow cytometer (BD, Heidelberg, Germany). Data were analysed with FlowJo 10 for Mac (Tree Star, Ashland, Ore).

## 2.6. Statistical analysis

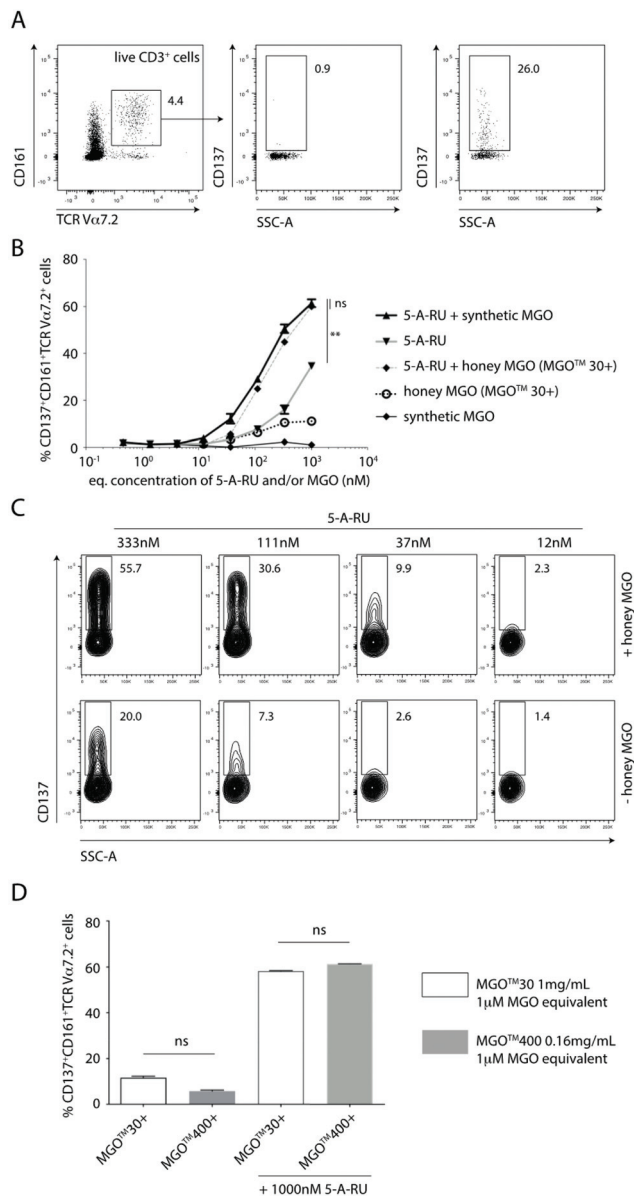
All graphs and statistical tests were carried out using GraphPad Prism 8 (GraphPad, La Jolla, CA). Statistical significance was assessed using one-way ANOVA with Tukey post-tests or non-parametric Mann-Whitney *U*-tests.

## 3. Results and discussion

To verify that 5-OP-RU is the major species produced from the reaction of 5-A-RU and MGO, HPLC-MS and <sup>1</sup>H NMR analyses were conducted on the reaction mixture. The precursor, 5-A-RU, is unstable and can undergo degradative hydrolysis to the purported compound 5,5-dihydroxy-6-(*D*-ribitylamino)pyrimidine-2,4(3*H*,5*H*)-dione (5-*D*-RP) (ESI Scheme 1†).<sup>23</sup> The formation of this compound is evidenced by the forerunning peak (*T* = 0.68 min) in the chromatogram of freshly synthesised 5-A-RU which corresponds to the chemical species with *m/z* of 294.1 (ESI Fig. 1A†).

When mixed with 2 equivalents of MGO in DMSO, 5-A-RU was converted into 5-OP-RU as a single major product (*m/z*: 331.1, *T* = 5.23 min) (ESI Fig. 1B†). Further characterisation of the reaction mixture by <sup>1</sup>H NMR spectroscopy reveals a species with spectral fingerprint (ESI Fig. 2†) matching that of the <sup>1</sup>H NMR spectrum of authentic 5-OP-RU previously reported.<sup>19</sup> A minor species that was also formed in the reaction mixture was likely to be RL-7-Me. Eluting at 1.79 min, this species has a *m/z* of 313.1 and results from a spontaneous intramolecular condensation reaction between the ribitylamine and methyl ketone groups.<sup>19,23</sup>

In this study, we used a well-established flow cytometry method to identify and quantify activated MAIT cells in human peripheral blood mononuclear cells (PBMC).<sup>26</sup> Specifically, MAIT cells were identified using a combination of antibodies specific for the three cell-surface markers CD3, TCR V $\alpha$ 7.2 and CD161. CD137 was used as an activation specific marker, as it has been shown to be upregulated upon antigen-specific activation.<sup>27</sup> Thus, non-activated *vs.* activated MAIT cell were identified as CD137<sup>-</sup>CD161<sup>+</sup>TCRV $\alpha$ 7.2<sup>+</sup> T cells *vs.* CD137<sup>+</sup>CD161<sup>+</sup>TCRV $\alpha$ 7.2<sup>+</sup> T cells (Fig. 2A).



**Fig. 2** Synthetic or Mānuka honey-derived methylglyoxal (MGO) enhances antigen-specific activation of mucosal-associated invariant T cells (MAIT cells) in the presence of 5-amino-6-D-ribitylamino uracil (5-A-RU). (A) Representative gating and quantification of activated MAIT cells. (B) Dose-dependent activation of MAIT cells with 5-A-RU in the absence or presence of equimolar concentrations of synthetic or Mānuka-honey derived methylglyoxal. (C) Side-by-side comparison of 5-A-RU-mediated activation of MAIT cells in the presence (upper panels) or absence (lower panels) of Mānuka-honey derived MGO. (D) Impact of MGO-equivalent amounts of MGO™ 30+ and MGO™ 400+ Mānuka honeys on 5-A-RU-mediated activation of MAIT cells. Plots show mean  $\pm$  SEM. Data are representative of two independent experiments conducted using PBMC samples obtained from two donors (two independently-treated technical replicates/donor PBMC). Statistics were calculated using (B) one-way ANOVA with Tukey *post-hoc* tests using 1000 nM data points throughout; or (D) using the non-parametric Mann-Whitney *U*-tests. \*\**p* < 0.01; ns, not significant. Only relevant statistics are shown.





Using the gating and quantification strategy defined above, PBMC subjected to five different treatment regimens were independently analysed for the extent of activation of MAIT cells (Fig. 2B). It is clear, that in the absence of 5-A-RU, treating PBMC with synthetic MGO alone at any concentration (0.5–1000 nM) did not activate MAIT cells to any significant extent (Fig. 2B). This finding suggests that MGO is unlikely to bind MR1 nor activate MAIT cells.

When PBMC were treated with increasing concentrations (0.5–1000 nM) of 5-A-RU, there was a dose-dependent increase in the proportion of activated MAIT cells. While this observation gives the impression that 5-A-RU alone activates MAIT cells, it is well-established that 5-A-RU neither binds MR1 nor directly activates MAIT cells.<sup>28</sup> Instead, 5-A-RU can diffuse into host mammalian cells and form simple adducts with a number of common host cellular metabolites that provide MR1-binding MAIT cell agonists,<sup>26</sup> including endogenous MGO and glyoxal, to form 5-OP-RU and 5-(2-oxoethylideneamino)-6-D-ribitylamino-uracil (5-OE-RU),<sup>18</sup> respectively. Glyoxal is an  $\alpha$ -dicarbonyl metabolite that can arise from glyceraldehyde *via* glycolaldehyde autoxidation.<sup>20</sup> It is important to note that 5-OP-RU remains as one of the most potent agonists of MAIT cells, characterised to-date, and since this metabolite is absent in mammals, it therefore allows host-pathogen discrimination by the mammalian immune system.<sup>12,22,29</sup>

When PBMC were treated with equimolar amounts of exogenously-added, synthetic MGO and 5-A-RU, there was consistently and significantly higher proportion of activated MAIT cells compared to those induced at all corresponding concentrations of 5-A-RU treatment alone (Fig. 2B and C). This finding suggests that exogenous MGO can enhance MAIT cell activation, demonstrating the presence of a bottleneck, rate-limiting conversion of 5-A-RU to MAIT cell agonists, in the absence of any exogenous MGO.

Using Mānuka honey with an MGO content of 72.6 mg kg<sup>-1</sup> (MGO<sup>TM</sup> 30+ Mānuka honey), we demonstrated that when PBMC were treated with various honey dilutions to give a final MGO concentration equimolar (0.5–1000 nM) to that of 5-A-RU, a similar extent of activation of MAIT cells, in a manner consistent with activation by synthetic MGO and 5-A-RU (*i.e. in situ* formation of 5-OP-RU), was observed (Fig. 2B). There was clearly a higher proportion of activated MAIT cells in PBMC treated with increasingly higher equimolar concentrations of 5-A-RU and honey-derived MGO (Fig. 2C).

To confirm that MGO in the honey matrix was the component responsible for controlling the 5-A-RU-dependent activation of MAIT cells, Mānuka honey with 72.6 mg kg<sup>-1</sup> MGO (MGO<sup>TM</sup> 30+ Mānuka honey) was directly compared to Mānuka honey with 451.4 mg kg<sup>-1</sup> MGO (MGO<sup>TM</sup> 400+ Mānuka honey). When both honeys were diluted so that each gave an equivalent final concentration of 1  $\mu$ M MGO, and PBMC independently incubated with equimolar (1  $\mu$ M) 5-A-RU, both treatments gave a similar extent of MAIT cell activation (Fig. 2D), suggesting that 5-OP-RU, is the main component responsible for activating the MAIT cells.

These findings altogether demonstrate for the first time, that MGO in Mānuka honey could increase the conversion rate of 5-A-RU into 5-OP-RU. We hereby speculate that this effect is likely to enhance microbial sensing by the immune system through increased activation of peripheral MAIT cells; a speculation needing confirmation in future work.

Free 5-A-RU can be released by bacteria in quantities sufficient to conjugate with exogenously added metabolites that can subsequently bind MR1 and stimulate MAIT cells.<sup>18</sup> 5-OP-RU, externally-administered to the body by cutaneous application or oral gavage, has been shown to cross mucosal barriers to reach peripheral organs, such as the thymus for presentation to resident MAIT cells.<sup>30</sup> While the mechanism(s) of transport of 5-OP-RU across these barriers remain unknown, these findings clearly highlight a novel mechanism by which commensal bacteria in mucosal surfaces can ‘remotely’ contribute to the activation of MAIT cells in systemic sites. In line with the findings of these previous studies,<sup>18,30</sup> it is plausible that methylglyoxal-rich food, such as Mānuka honey, could contribute to the pool of 5-OP-RU at mucosal surfaces *via* condensation of Mānuka honey-derived MGO and microbial 5-A-RU from commensals. The pre-formed 5-OP-RU could then cross mucosal barriers to further enhance MAIT cell activation in systemic sites or potentially support local, gastrointestinal immune homeostasis through the stimulation of intraepithelial MAIT cells.<sup>31</sup>

Another interesting finding in this communication is that, there are clearly other compounds present in Mānuka honey that can activate MAIT cells, albeit only to a relatively limited extent. PBMC treated with MGO<sup>TM</sup> 30+ Mānuka honey dilutions alone, in the absence of 5-A-RU, can activate MAIT cells, up to *ca.* 12% of gated MAIT cells (Fig. 2B and D). Given the plasticity of the antigen-binding cleft of the MR1 protein and its propensity to bind small aromatic, mono- and/or bicyclic molecules,<sup>29</sup> it is not surprising to anticipate a variety of small molecules fitting the above chemical criteria, likely present in Mānuka honey, that may bind MR1 and activate MAIT cells. At present, we do not have an indication on the identity of these compounds.

Future work should aim to identify other potential MAIT cell agonists present in Mānuka honey. Particular emphasis should be put on whether they are of relevance to peripheral MAIT cell activation, as they will need to be demonstrated to cross mucosal sites and reach peripheral sites, like 5-OP-RU, before their potential effects can be conveyed under physiologically-relevant conditions.

## 4. Conclusions

In this communication, we report that Mānuka honey-derived MGO can increase the conversion rate of 5-A-RU to 5-OP-RU, which contributes to the activation of MAIT cells in peripheral blood. We showed that Mānuka honey, a prototypical food source rich in methylglyoxal, can dose-dependently activate MAIT cells in the presence of equimolar amounts of the



microbial metabolite, 5-A-RU, in a manner consistent with activation by synthetic methylglyoxal and 5-A-RU. Based on our findings, we propose that Mānuka honey-derived MGO may play a role in enhancing microbial sensing by MAIT cells, which may in turn assist MAIT cells in controlling microbial infection and systemic immune homeostasis.

## Abbreviations

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
5-D-RP	5,5-Dihydroxy-6-(D-ribitylamino)pyrimidine-2,4 (3H,5H)-dione
MAIT cells	Mucosal-associated invariant T cells
MGO	Methylglyoxal
MHC	Major histocompatibility complex
MR1	MHC-related protein-1
PBMC	Peripheral blood mononuclear cells
RL-7-Me	7-Methyl-8-D-ribityllumazine
TCR	T-cell receptor

## Author contributions

JT reviewed the data and wrote the manuscript, BC provided experimental design and contributed to writing of the manuscript, RA, AM, IH and GFP contributed reagents, contributed to research design and writing of the manuscript. OG performed experiments (with contributions from JT, YL and HvdW), provided experimental design, reviewed the data and co-wrote the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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