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Title:

MR1: a multi-faceted metabolite sensor for T cell activation

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Abstract:

The major histocompatibility complex class I-related molecule MR1 captures and presents small metabolites to MR1-restricted T cells including Mucosal Associated Invariant T (MAIT) cells. The first MR1 ligands discovered were intermediates of microbial riboflavin synthesis, antigens presented to alert inflammatory MAIT cells to bacterial infection. Recent advances have expanded the range of MR1 ligands to include extracellular metabolites released by the commensal microbiome, and yet undefined antigens presented by cancer cells to mediate MR1-dependent anti-tumor activity. MR1 thus exhibits a multifaceted ability to display a diverse range of ligands for immune surveillance in a variety of contexts. The mechanisms of antigen presentation by MR1 are of central importance to understanding metabolite-mediated immune homeostasis, immunity to infection and tumor surveillance.

Highlights:

- MR1 captures a conserved metabolite signature of a diverse range of microbes and activates MAIT cells for inflammation and immunity.
- Commensal microbes at barrier tissues secrete metabolites that are captured and presented by MR1 in distant tissues such as the thymus.
- MR1 displayed by a various cancer cells elicit activation of MR1-restricted T cells for immune control of cancers.
- The trafficking of MR1 to sample its varied metabolite cargo requires a unique pathway among antigen-presenting molecules.

Introduction

The presentation of foreign antigen (Ag) by Major Histocompatibility Complex (MHC) molecules is a pivotal step in adaptive immunity, which allows the activation, expansion and effector functions of T lymphocytes. These cells possess a highly specific T cell receptor (TCR) that recognizes diverse classes of Ag in the context of specialized MHC molecules. Conventional T cells recognize peptides presented by classical MHC molecules, whereas innate-like T cells recognize conserved non-peptidic Ag presented by non-classical MHC molecules, such as the monomorphic MHC class I-related protein 1 (MR1). MR1 is highly conserved among mammals[1], and captures and presents small metabolites to MR1-restricted T cells[2], the largest population of which are Mucosal Associated Invariant T (MAIT) cells. The best characterized Ag presented by MR1 are transient metabolites derived from the microbial synthesis of vitamin B2, riboflavin, which are produced by many microbes but not mammals[3]. The riboflavin-precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) combines with methylglyoxal to form the MR1-binding derivative 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)[2]. Unlike protein Ag which can mutate and drift, metabolites are highly conserved and are essential bacterial 'building blocks' produced by a diverse range of microbes. Therefore, MR1 presents a metabolite signature to allow the detection of pathogenic microbes by the immune system for their subsequent clearance.

Recent discoveries that have expanded the roles attributed to MR1 metabolite Ag presentation (Figure 1): (i) surveillance of not only pathogenic but also commensal microbes; (ii) presentation of a cancer metabolite signature for the immune recognition of tumors. The mechanism by which MR1 presents this unique class of Ag is of central importance to understanding the whole range of functions of the conserved MR1-MAIT cell axis. This review outlines these areas and proposes future directions for the field.

MR1 presents microbial metabolites for immunity and barrier homeostasis

MAIT cells are classed as innate-like T cells. They are abundant in mucosal and barrier tissues and are considered antigen-experienced, tissue-resident cells [4,5]. MAIT cells rapidly secrete pro-inflammatory cytokines upon recognition of their MHC-presented cognate antigen, specifically interferon-γ, tumor necrosis factor-α and interleukin-17A [5-8], and this inflammatory potential somewhat defines MAIT cells. During infection they have also been shown to recruit conventional T cells and secrete granulocyte-macrophage colony-stimulating factor which differentiates monocytes into dendritic cells, together arming the adaptive response [9,10]. Furthermore, MAIT cells can directly kill infected cells presenting MR1-metabolite complexes [11,12]. Many bacterial pathogens produce the riboflavin-related metabolites presented by MR1, and much of the early work that

addressed the function of the MR1-MAIT cell axis focused on its role in immunity against intracellular bacteria [13]. For example, in mice MAIT cells have been shown to be active and/or protective against a range of bacterial infections including tuberculosis [9,10,14-18] whereas in humans MAIT cells are stimulated and enriched in the airways of tuberculosis-infected patients [19,20] and activated in many other bacterial diseases [11,21].

Collectively this has suggested that MR1 evolved as a sensor of microbial, intracellular pathogens [22]. However, two key recent studies have shown that *in vivo,* MR1 can capture soluble metabolites, and not only from pathogenic but also from commensal bacteria. This suggests that MR1 may play a key pivotal role in the crosstalk between microbiota and the mammalian immune system to maintain homeostasis [23,24]. Interestingly, the commensal metabolites presented by MR1 in this scenario do not need to originate within infected cells or in intracellular compartments of phagocytic cells harboring microbes; instead they are bacterial metabolites released to the extracellular environment in peripheral tissues [23,24]. The fate of these soluble compounds is remarkable. It was already known that MAIT cells require commensal flora to colonize peripheral tissues [25], but a recent and elegant study by Legoux et al [24] discovered that the first location where MAIT cells detect that flora is actually the thymus. They showed that 5-OP-RU secreted by commensal microbes in the periphery reaches the thymus, where it is presented to drive positive selection of developing MAIT cells. Purified 5-OP-RU injected intraperitoneally, or administered on the skin or by oral gavage, entered circulation and was likewise captured by thymocytes and presented on MR1. In another unexpected development, Constantinides et al. [23] showed that 5-OP-RU administered on mouse skin induced local accumulation of MAIT cells, another demonstration of presentation of soluble, extracellular 5-OP-RU, in this case in a peripheral tissue. These studies provide direct evidence that cells can be physically separated from commensals that synthesize 5-OP-RU and still capture and present the metabolite on MR1 (Figure 1). There have been numerous studies showing cells readily acquire and present extracellular metabolites *in vitro* [2,3,18,26-29], and Chen et al. [18] showed that extracellular 5-OP-RU along with inflammatory signals can activate MAIT cells *in vivo*, but the studies by Legoux et al [24] and Constantinides et al [23] represent the first evidence of a homeostatic function for this source of MR1 ligands.

Other recent studies also implicate the MR1-MAIT cell axis in responding to and shaping the microbiome; MR1-deficient mice harbored distinct microbiota to wildtype mice [30,31], and antibiotic treatment depleted MAIT cell numbers [32]. Conversely, detection of changes in the microbiome, or invasion of normally sterile tissues by commensal bacteria, may trigger reactions in the host initiated by MAIT cells to restore homeostasis. Constantinides showed that MAIT cells had

wound-healing properties, and other studies have shown that MAIT cells are associated with gut barrier integrity [31,33]. Together, these studies widen the scope of the roles played by the MR1-MAIT cell axis and implicate multi-faceted mechanism for MR1 presentation of microbial metabolites, catering for Ag from intra- and extracellular sources, from both commensal and infecting pathogens (Figure 1), as will be discussed later.

Presenting a metabolite signature of tumors?

The metabolite 5-OP-RU is the canonical microbial MR1 ligand, a conserved antigen produced by a range of microbes spanning different biological kingdoms; yet MR1 is capable of presenting a more diverse range of metabolites. The structural flexibility of its Ag-binding cleft enables MR1 to accommodate a diverse range ligands [34], including drug-like molecules [35] and other microbial metabolites that have not been identified yet [36-38]. Recently, several studies have provided compelling evidence that MR1 can present Ag from tumors for immune surveillance of cancers. Two separate studies found MR1-restricted T cell clones that seem to recognize undefined cancer-specific Ag [39,40]. Crowther et al [39] identified a T cell clone could recognize MR1 complexes presented by cancer cells, but not healthy cells. This clone was highly cytolytic for a diverse range of human tumor cells and it controlled a mouse model of leukemia. Furthermore, primary T cells transfected to express the TCR of this clone could kill both autologous and non-autologous melanomas. These results suggest that the TCR of this clone recognizes an MR1-presented Ag shared by human and murine tumor cells, so T cells expressing this TCR have promise for use as a pan-cancer therapy [41]. Lepore et al. [40] also found MR1-restricted T cell clones that recognized MR1 likely presenting tumor-derived ligands; these clones were stimulated by hydrophilic fractions from the lysates of THP-1 leukemia cell line and murine breast tumors. Interestingly, the T cell clone discovered by Crowther et al [39] did not recognize the canonical ligands 5-OP-RU or the vitamin B9-related metabolite, Ac-6-FP. Further, it did not recognize MR1 with the residue lysine-43 mutated to alanine (K43A). This lysine contains a positively charged side chain that needs to be neutralized by forming a Schiff base bond with ligands lodged into the antigen binding site of MR1 to enable transport of the MR1-ligand complexes from the endoplasmic reticulum (ER) to the cell surface [29]. MR1-K43A molecules cannot form this covalent bond with metabolites although they can still bind non-Schiff base ligands [42]. The fact that the anti-tumor clone described by Crowther et al requires MR1 molecules with the lysine 43 present suggests it does not recognize MR1 alone but in complex with a Schiff base-forming ligand.

These studies suggest a new role for MR1; not only to present microbial Ag but to display a metabolic signature of cancer, which is potentially a unique metabolite from within transformed cells. The identity of such ligands is thus a crucial question.

How does MR1 present these diverse metabolites signatures?

The picture that has emerged from studies of MR1 ligands is that this molecule can sample metabolite Ag from within cells, derived from intracellular bacterial infections or produced by cancer cells themselves. In addition, MR1 can present Ag derived from extracellular sources, produced by commensal or pathogenic organisms. This versatility sets MR1 apart from other antigen presenting molecules. MHC class II molecules utilize a presentation pathway that enables sampling the proteome that reaches the lumen of endosomal compartments, including extracellular proteins. The pathway used by MHC class I molecules is adapted to present mostly the cytosolic proteome, although in some cells MHC class I can also present Ag sampled from endosomes via cross-presentation [43,44]. The various human CD1 subclasses employ distinct trafficking routes to survey the full range of lipids that occur in different compartments [45]. What are the mechanisms that enable MR1, the product of a single, monomorphic gene [46], to achieve its multifaceted display of so many different metabolite ligands?

This is an ongoing area of research and we still do not understand all the nuances of MR1 presentation. In all cell types examined, there is a pool of pre-synthesized MR1 that is poised to capture Ag from either extracellular sources or intracellular infection [28,29]. The majority of this pool is located inside the ER in a ligand-receptive conformation, with a small portion at the cell surface or in endosomes [29,47-50]. Metabolite Ag binding to MR1 causes it to traffic to the cell surface for presentation [2,3,27,29,51]. Logic dictates that the location where the majority of ligand-receptive MR1 resides is the compartment where the Ag is captured. In support of this, we found that the binding of ligands to MR1, accompanied by covalent binding to the K43 side chain, causes the release from the ER. The mechanism is hypothesized to be the neutralization of the K43 charge, allowing the stable folding of MR1 [29]. We and others support an ER-binding model as the primary mode of presentation of extracellular metabolites [22,28,29,52,53]. But is this the only way that MR1 acquires its cargo?

There is substantial evidence that MR1 molecules already expressed on the cell surface can be used to present Ag from exogenous or intracellular Ag. The first evidence comes from the recycling of MR1 molecules that acquired ligands in the ER and then trafficked to the cell surface. Most of these molecules are endocytosed, delivered to lysosomes and degraded [29], but a small percentage (~5%) returns to the surface after internalization [29]. We showed that MR1 pre-loaded with the ligand, 6-

formylpterin (6-FP) and expressed on the cell surface could replace this ligand with 5-OP-RU during transit through endosomes and recycle back to the surface [29]. Karamooz et al [28] extended this further and showed that cell lines incubated overnight with high concentrations of 6-FP enabled more efficient presentation of subsequently added exogenous ligands, but not of ligands derived from intracellular infections. The implication was that the recycling pathway could be used by ligands derived from extracellular sources but not from intracellular bacteria, the latter relying on the ER-loading pathway. Further indications of the ability of MR1 to capture endosomal ligands through a recycling pathway comes from a recent study describing a modified form of the ligand, 5-A-RU that requires processing in endosomes [54]. This 5-A-RU *prodrug* could not be presented by a mutant MR1 that could not recycle, indicating this was its preferred presentation route.

The second evidence that MR1 employs more than one trafficking pathway comes from the knockdown of several proteins in the secretory pathway. The silencing of VAMP4 and Rab6 inhibited MR1 presentation of intracellular Ag without affecting extracellular 6-FP presentation [27], while Syntaxin 4 silencing reduced the presentation of intracellular but not extracellular Ag [28]. One interpretation of these experiments is that either MR1 employs different pathways in the same cell to survey various compartments, hence the interruption of specific endosomal pathways can block one mode of presentation over another. However, an alternative explanation is that the metabolite Ag itself is captured and trafficked by different pathways, depending on its source; extracellular or from within a phagosome. Future studies are needed to conclusively decide on the answer.

Since MR1 can capture ligands in endosomal compartments via recycling, it is reasonable to assume that any MR1 molecules already expressed on the plasma membrane prior to infection might be used to present extracellular Ag. Indeed, a small amount of MR1 is found on the surface of cell lines or primary cells in the absence of ligands [29]. To explain the origin of these molecules, we proposed that empty ER-resident MR1 is maintained in a folding equilibrium between a partially misfolded, predominant conformer that cannot leave the ER, and a minor one that can traffic to the cell surface [53]. However, we believe this cohort of MR1 molecules is unlikely to play a prominent role in Ag presentation because they represent a very small proportion compared to the ER-resident pool, and of these only a small number (~5%) would be able to reach endosomes, escape transfer to lysosomes, and return to the cell surface bound to ligands. It cannot be discarded that empty MR1 molecules may recycle more efficiently than MR1-ligand complexes. However, it appears more likely that the recycling pathway supplements the ER-binding route to enable presentation of ligands that cannot reach the ER by employing the recycling pathway, but only once a sufficient number of MR1-Ag complexes have been recruited from the ER to the plasma membrane (Figure 1). This would add

another layer of versatility to MR1 presentation pathway, enabling display of diverse ligands with distinct intracellular trafficking properties.

Future directions

The MR1 presentation pathway is multifaceted; it can display metabolites from commensal and pathogenic microbes and cancer, either distant to the presenting cell or from within. Understanding how it manages this will require to answer numerous questions. Are there specialized mechanisms for preservation and transport of extracellular MR1 ligands in blood or tissues? Their description might improve vaccination protocols. What is the contribution of MR1 outside the ER to T cell activation? To date this has not been addressed in a physiological setting and obtaining the answer may need novel tools. Which cells present MR1-Ag complexes *in vivo*? MR1 is expressed by a diverse range of cell types [53], but their relative roles in different scenarios of homeostasis or infection *in vivo* remain unknown. Another complication is that the cells that capture and present for commensal monitoring may be different from those presenting during acute infection. Recently Wang et al. [15] used bone marrow chimeras to show that the cell type required to express MR1 for MAIT cell expansion was dependent on the type of infection: *Salmonella* infection required MR1 on non-bone marrow-derived cells, whereas for *Legionella longbeachae* infection MR1 expression was required on bone-marrow-derived cells. This reinforces the idea that a range of professional and non-professional APCs can perform MR1 presentation and their relative contribution depends on the context. Understanding this is important to develop therapeutic strategies to arm MAIT cells in the right setting and location without inducing side-effects. Finally, what is the identity of the cancer ligands presented by MR1? The identity of these ligands would allow a novel approach for cancer therapy and allow the creation of tetramers to study cancer-specific MR1-restricted T cells in healthy donors or cancer patients.

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Figure legends

Figure 1. The multifaceted presentation of metabolites by MR1 *in vivo*.

Commensal microbes secrete MR1 ligands (5-OP-RU) at barrier tissues such as the skin (1). These extracellular ligands are transported to the thymus by an unknown mechanism where they are acquired by thymocytes (2) and presented for positive selection of MAIT cells. The commensal-derived 5-OP-RU can also be acquired locally by an antigen presenting cell (APC; 3) for loading onto MR1 molecules in the APC's endoplasmic reticulum (ER) and then presented at the cell surface. During disease, such as the breach of a barrier (4), APCs may phagocytose microbes or be infected by pathogens (5). The ligands in this situation may be sampled by MR1 in the ER or in endosomes (5). Finally, MR1 expressed by tumor cells may present cancer metabolite ligands to T cells for cancer surveillance (6).

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Papers of interest:

Outstanding interest

** Legoux et al. *Science* 2019

Discovered that 5-OP-RU at barrier tissues was presented by thymocytes to enable the selection of MAIT cells. This is compelling evidence that MR1 can survey commensal bacteria at distant sites for training immune system.

** Constatinidies et al *Science* 2019

Showed that MAIT cells expand in skin by recognising soluble 5-OP-RU from commensal bacteria, implying that MR1 captures extracellular ligands for monitoring commensal organisms.

** Crowther et al. *Nature Immunology* 2020.

Identified a T cell clone that recognises MR1 presented by cancerous cells, not healthy cells, and is cytolytic for a range of diverse tumors. This is solid evidence that MR1 presents tumor metabolites.

Special interest

* Karamooz et al. *Scientific Reports* 2019

Showed that cell-surface MR1-ligand complexes can be re-used to present extracellular ligands but not those from infecting intracellular bacteria.

* Wang et al. *Science Immunology* 2019

Found that the type of cells that present MR1 for efficient MAIT cell activation and proliferation was dependent on the type of infection.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1