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Title: MicroRNA-managing the development of MAIT cells

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Graphical Abstract: Winter *et al.* identifies the microRNA-181a/b-1 pair as intrinsic regulators of MAIT cell development.

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ICB News & Commentary: **MicroRNA miR-181a/b-1 controls MAIT cell development**

Mucosal-associated invariant T (MAIT) cells respond to antigens derived from the riboflavin biosynthesis pathway, which occurs in bacteria and yeast but not mammalian cells. These riboflavin-derived antigens are presented to MAIT cells in association with the molecule MR1, an MHC class-I-like antigen-presenting molecule that is highly conserved between

mammals [1]. Upon activation, MAIT cells rapidly produce proinflammatory cytokines, suggesting that the MR1-MAIT cell axis is an important component in host immunity for detecting bacterial metabolism and infection [2]. MAIT cells branch from the mainstream T cell developmental pathway within the thymus, but the factors that control their development are not well-understood. In this issue, Winter *et al.* [3] demonstrate that microRNA 181a/b-1 plays a key role in regulating the early differentiation of MAIT cells.

MAIT cells share similar unconventional, innate-like attributes with the more extensively-studied CD1d-lipid-reactive natural killer T (NKT) cells. They both utilize semi-invariant T cell receptors (TCR)s to recognise non-peptide antigens presented by MHC-I-like molecules (MR1 and CD1d respectively), and are selected by $CD4^+CD8^+$ cortical thymocytes [4-6]. This channels them along a developmental program in the thymus where the expression of the transcription factor PLZF distinguishes them from conventional peptide-MHC-reactive T cells. The development of MR1-tetramer reagents loaded with the riboflavin-derivate antigen 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) has enabled direct studies of MAIT cells in mice and humans, leading to rapid advances in our understanding of the biology of these cells [7, 8]. This included developmental studies that revealed a three-step maturation pathway in humans and mice. In mice, these steps are defined as immature stage 1 ($CD24^+CD44^-$), intermediate stage 2 ($CD24^-CD44^-$) and mature stage 3 ($CD24^-CD44^+$) MAIT cells (Figure 1) [8, 9]. One factor that was found to be essential for MAIT cell development was the enzyme Drosha [8]. Drosha, along with Dicer, are RNAseIII enzymes responsible for the generation of canonical mature micro-RNAs (miR)s [10]. In the absence of Drosha, MAIT cell development reached stage 1 but did not efficiently progress beyond this point, suggesting a key role for miRs in the intrathymic maturation of these cells.

miRs are non-coding post-transcriptional gene regulators involved in diverse biological events ranging from immune development to carcinogenesis, and act predominantly by destabilising mRNA or inhibiting protein translation [11]. Knowing that Drosha was important for intrathymic MAIT cell development [8], Winter *et al.* sought to examine which miRs were controlling this process. Based on previous findings that a specific pair of miRs, miR181a/b-1, were instrumental for intrathymic NKT cell development [12-14], they examined the role of these same miRs in MAIT cell development and function [3]. Using a toolbox of miR-181a/b-$1^{-/-}$, $RAG1^{GFP}$ reporter, and bone marrow chimeric MAIT TCRα transgenic x miR-181a/b-$1^{-/-}$ mouse models, they showed that miR-181a/b-1 is intrinsically

required for MAIT cell development. Deletion of miR-181a/b-1 mirrored the MAIT cell developmental defect in Drosha$^{-/-}$ mice (Figure 1). Thus, as previously reported in Drosha$^{-/-}$ mice, in miR-181a/b-1$^{-/-}$ mice there was a major defect in MAIT cell development but some residual MAIT cells, primarily immature stage 1 cells, were still detectable in the thymus. These were present in similar numbers between the Drosha$^{-/-}$, miR-181a/b-1$^{-/-}$ and wildtype mice, indicating that miR-181a/b-1 are not required for the initiation of the MAIT cell differentiation pathway. While stage 2 MAIT cells were reduced in miR-181a/b-1$^{-/-}$ mice, the greatest impact was on stage 3 MAIT cells, which suggests that miR-181a/b-1 is important for maturation from stage 1 to 2 and from stage 2 to 3. The authors also showed that MAIT cells normally begin intrathymic proliferation at stage 2, and that by stage 3 these cells have divided extensively (Figure 1) [3]. Interestingly, in miR-181a/b-1$^{-/-}$ mice, few of the residual stage 2 MAIT cells had proliferated, while all of the residual stage 3 MAIT cells had proliferated, notwithstanding the fact that they were mostly depleted in miR-181a/b-1$^{-/-}$ thymus. This suggests that different stimulatory pathways regulate proliferation at different stages of MAIT cell development, consistent with earlier observations that immature MAIT cells can develop intrathymically in germ-free mice but fail to expand and populate the periphery in the absence of microbial products [1, 8].

Despite their impaired development in miR-181a/b-1$^{-/-}$ mice, immature MAIT cells could emigrate from the thymus to periphery in these mice [3], which reflects the previous study showing that stage 2 MAIT cells can leave the thymus [8]. Curiously, while numbers of MAIT cells were much lower in spleen of miR-181a/b-1$^{-/-}$ mice, they were present in normal numbers in lymph nodes, suggesting independent regulation of MAIT cells in these tissues. However, without miR-181a/b-1, most peripheral MAIT cells were functionally immature, based on the lack of PLZF and very limited RORγt and T-bet expression which characterises mature MAIT cells. These data align with the characteristics of residual cells observed from PLZF-deficient and Drosha-deficient mice [8]; and collectively imply that miR-181a/b-1 is necessary for MAIT cells to fully mature and acquire effector function, even in peripheral organs. That said, a small fraction of RORγt^{+} and T-bet^{+} MAIT cells were detected in the periphery of miR-181a/b-1$^{-/-}$ mice, suggesting that some MAIT cells can escape the regulatory control of miR-181a/b-1 and complete their maturation in the periphery [3].

Lastly, the authors showed that the defect in MAIT cell development in the absence of miR-181a/b-1 could be partially overcome by forced expression of a transgenic MAIT TCR-α chain (Vα19Jα33) [3]. This is similar to how transgenic expression of the semi-invariant

NKT TCRα chain (Vα14Jα18) restores NKT development in miR-181a/b-1$^{-/-}$ mice [15] and suggests a similar mechanism by which miR-181a/b-1 regulates both cell types. It is possible that this simply reflects a slightly higher level of transgenic TCR expression that can overcome a signalling defect in the absence of miR-181a/b-1, but regardless, this restoration in MAIT cell numbers was incomplete. While the mature transgenic cells showed increased PLZF expression, they did not recover normal levels of RORγt or T-bet [3]. Collectively, these data suggest that miR-181a/b-1 regulate MAIT cell development at multiple levels.

There are at least two mechanisms that may explain why miR-181a/b-1 are so important in MAIT cell development. miR-181a is known to regulate the TCR signalling threshold by repressing inhibitors such as phosphatases during conventional T cell development [16]. Moreover, miR-181a/b-1 facilitate selection of NKT cells by high affinity antigens and in their absence, NKT cell development is impaired [12]. Another mechanism by which miR-181a influences NKT cell development is the regulation of the phosphatase PTEN, which in-turn regulates PI3K signalling, that is necessary to support the increased metabolic demands when NKT cells undergo intrathymic developmental proliferation [14]. Despite these examples in the NKT cell lineage, the reason why MAIT cell development is so dependent on miR-181a/b-1 remains unclear. It is currently unknown whether MAIT cells undergo agonist-mediated selection, nor whether they have high metabolic demands, although the extensive proliferation as they mature [3] suggests that they might. Indeed, it is possible that both mechanisms of action of miR-181a/b-1 are important for MAIT cell development but unravelling this will require further investigation.

The study by Winter and colleagues [3] adds to growing number of studies elucidating how MAIT cell development is regulated [9], and offers another checkpoint control factor that regulates this important population of T cells. As miR-181s are conserved between mice and humans [17], this study raises the question of whether these regulators also control human MAIT cell development and/or post-thymic maturation and expansion. Given the high degree of variability in human MAIT cell numbers between individuals, further investigations into miR-181 expression in human thymus and blood MAIT cells and their mechanisms of action will be valuable to explore their potential as a tool in manipulating the generation and/or homeostasis of MAIT cells.

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FIGURE CAPTION

Figure 1. microRNA 181a/b is a checkpoint regulator for MAIT cell development between stage 1 and the highly proliferative stage 2, and is required for MAIT cells to reach full maturation. The image was created with Biorender.

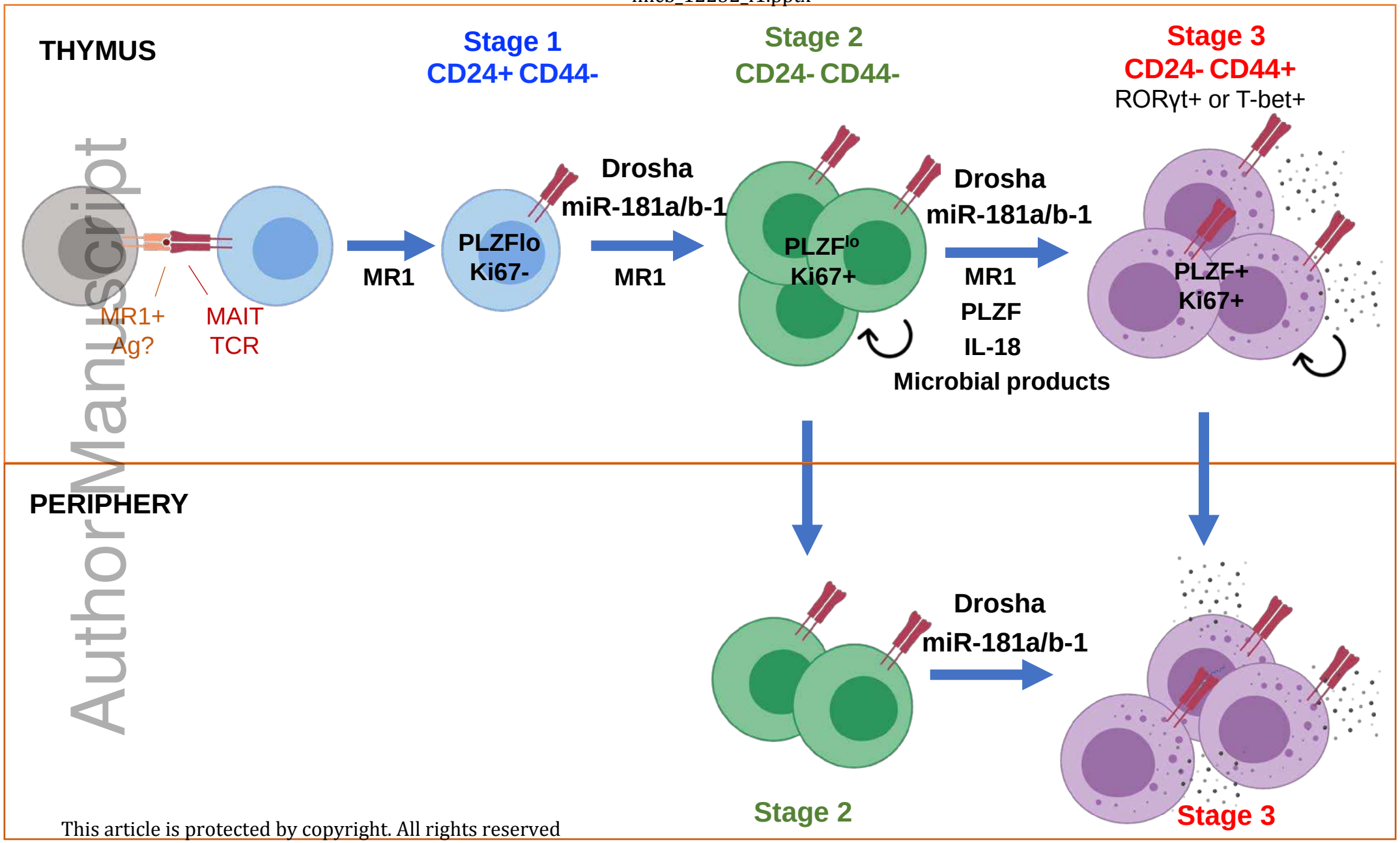

Figure 1. microRNA 181a/b-1 is a checkpoint regulator for MAIT cell development between stage 1 and the highly proliferative stage 2, and is required for MAIT cells to reach full maturation (refs 3, 8).