# The role of miR-181a/b-1 in thymic development

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 15
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
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aus Perth, Australien

Frankfurt 2019
(D30)

vom Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität als Dissertation angenommen

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Datum der Dissertation: 13/02/2020

# Abstract

**Samantha Winter**
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The balance between peripheral T-cell reactivity and self-tolerance is achieved during T-cell development in the thymus. During thymic development T-cell sensitivity to self-antigens drives their selection and is dynamically regulated via multiple mechanisms. The microRNA miR-181 has been implicated as a post-transcriptional modulator of T-cell sensitivity due to its suppression of several negative regulators of T-cell receptor (TCR) signalling. By tuning developing thymocytes to be exquisitely sensitive to signals transduced through their TCR, miR-181 has previously been shown to be essential for the agonist selection of invariant natural killer T (iNKT) cells. In this thesis, we extend the knowledge on the developmental control elicited by miR-181 in the thymus to cover mucosal-associated invariant T (MAIT), regulatory T (Treg) and conventional T cells. Using a germline knock-out of mature miR-181a/b-1, we could show that all agonist-selected T cell populations are critically dependant on miR-181a/b-1, noting an absence of MAIT and a reduction of thymic-derived Tregs in miR-181a/b-1-deficient mice. Furthermore, we provided evidence that miR-181 is also required for the negative selection of conventional T cells, with miR-181a/b-1-deficient mice presenting with a near absence of apoptotic markers. Therefore, by heightening the TCR sensitivity to self-antigens, miR-181a/b-1 aids in the detection and subsequent elimination of autoreactive thymocytes. In addition, we characterised the murine primary miR-181a/b-1 transcript, which surprisingly has a transcription start site (TSS) more than 70kB upstream of the mature miRNA sequences. This shall hopefully lead to future research aimed at deciphering the upstream regulatory networks that promote dynamic miR-181a/b-1 expression in developing thymocytes. In summary, we present here a single miRNA subset with broad implications in T-cell development. In disagreement with central dogma that individual miRNAs generally provide weak to moderate modulation over cellular pathways, we showcase the miR-181 family subset, miR-181a/b-1, as an efficient regulator of TCR signalling pathways. Due to the sensitive nature of TCR signalling during thymocyte selection, miR-181a/b-1 elicits gross effects, which are essential for agonist selection, central tolerance and generating a functional self-tolerant peripheral T cell repertoire. We therefore conclude that miR-181a/b-1 is fundamental in T-cell development as a whole.

Keywords: miRNA, miR-181, T-cell development, MAIT cells, Treg cells, Conventional T cell selection, Positive selection, Negative selection, Agonist selection, Pri-miRNA

# Acknowledgements

First and foremost, I'd like to express my sincere gratitude to my supervisor and mentor Prof. Dr. Andreas Krueger. Your kindness, patience and immense knowledge has helped shape me into the researcher I am today, and I am beyond thankful. I could not have envisaged a better advisor and I wish you all the best for the future.

To my thesis advisory committee members, Michaela Müller-McNicoll and Kathi Zarnack. Thank you so much for you time, advice and intellectual discussions on this project. An additional thanks goes to all members of the SFB902 who provided a whole lot of entertainment, collaborations and a great scientific atmosphere.

To my fellow lab members both past and present. The fun and support that you guys provided has greatly enhanced the experience as our little lab grew over the years. Thanks for everything you've done for me, from trips to the Ausländerbehörde, to last minute thesis translations and just generally being you. You guys are great, and I hope we all meet each other again to resolve any outstanding debate over the Dreikorn challenge winner.

Young-man. My first true friend in Germany and my fellow international-buddy. I'm going to miss you a lot! Thanks for always being there for me and teaching me so much culturally. I wish the best for you and your family's future, wherever it takes you.

To my adopted sister, Ayesha. I couldn't have thought of a better person to share this experience with. Through personal ups and downs, our friendship has grown even stronger and I can't wait to reminisce about this in 10 years with you.

Mum, dad and Aly. Thanks for encouraging me to have the confidence in my abilities to pursue new and exciting things. Your endless support and late night/early morning phone calls through the ups and downs enabled me to succeed. I love you all more than anything and I can't wait to be home with you again.

And lastly, Heike. You know I could not have made it through without your friendship, laughter and emotional support. I will forever cherish our short time we had together as PhD-buddies and it's so sad to leave knowing that we will be half a world away. I'm going to miss your improvised karaoke, chickpea salad and always having your shoulder to lean on. When you can take a break from Edu-Brdu optimisation, come visit me for a holiday in the sun.

Samantha Winter
February 2019
Frankfurt, Germany

# Zusammenfassung

T-Zellen sind ein zentraler Bestandteil der adaptiven Immunabwehr. Während einer Infektion durchlaufen die verschiedenen T-Zelltypen klonale Expansion, Aktivierung und funktionale Programmierung für gezielte Immunantworten, welche essentiell für die Eindämmung und Bekämpfung vieler Pathogene ist. Andere T-Zellen besitzen regulatorische Rollen und sind verantwortlich für die Balance der Immunantwort und die Unterbindung von Autoimmunität. Sämtliche dieser Subtypen entstehen durch einen genau definierten Entwicklungs- und Selektionsprozess im Thymus. Hämatopoetische Stammzellvorläufer wandern aus dem Knochenmark in den Thymus, ab welchem Punkt sie der T-Zelllinie angehören. Die Entwicklung der Zellen im Thymus findet stufenweise statt und kann grob anhand der Co-Rezeptoren CD4 und CD8 beschrieben werden. Die Thymozyten in den frühen Stufen des Reifungsprozesses besitzen weder den CD4 noch CD8 Co-Rezeptor und werden daher doppelt negativ (DN) genannt. Diese DN Zellen entwickeln sich zu doppelt positiven Zellen (DP), welche sowohl CD4 als auch CD8 besitzen. In der nächsten Stufe regulieren die Zellen einen der beiden Co-Rezeptoren herunter und entwickeln sich dadurch zu einfach positiven (SP) Zellen bevor sie den Thymus in Richtung Peripherie verlassen.

In den späten DN-Stufen bilden die Thymozyten ihren T-Zell-Rezeptor (TCR) durch die somatische Rekombination, was zu einem T-Zell Reservoir mit immenser TCR Diversität führt. Dieses Reservoir geeigneter T-Zellen zu schaffen ist unerlässlich, um eine adaptive Immunantwort zu gewährleisten, die sowohl Bedrohungen von außen (z. B. Pathogene), als auch von innen (z. B. Krebs) erkennt und gleichzeitig Autoreaktivität vermeidet. Diese komplizierte Balance wird während der Entwicklung im Thymus durch einen Prozess, bekannt als thymische Selektion, erreicht. Diese Selektion wird durch zwei Mechanismen gewährleistet. Zuerst werden TCR positive Zellen durch schwache Interaktionen mit allgegenwärtigen Antigenen im thymischen Cortex positiv selektiert, während Zellen, welche keinen funktionalen TCR ausgebildet haben keine Überlebenssignale erhalten und damit durch einen Prozess namens „Tod durch Vernachlässigung“ eliminiert werden. Außerdem findet parallel die negative Selektion statt, welche bis zur SP Phase andauert in der die Zellen in die Medulla wandern. Junge SP Thymozyten sind in der Medulla einer großen Vielfalt an Antigenen peripherer Gewebe ausgesetzt. Starke Reaktionen der Zellen resultieren hier in einer Form der Apoptose, bekannt als klonale Deletion, wodurch potentiell autoreaktive Zellen entfernt werden. Thymozyten, die erfolgreich positive und negative Selektion überleben, maturieren, wandern als naive T-Zellen in die Peripherie und sorgen dort für eine funktionale Immunantwort. Allerdings gibt es auch T-Zellen, welche während ihrer Selektion ein starkes TCR-Signal empfangen und sich zu sogenannten unkonventionellen T-Zellen entwickeln. Diese Zellen, entwickeln sich z.B. zu „invariant natural killer T cells“ (iNKT), „mucosal-associated invariant T cells“ (MAIT) oder regulatorischen T-Zellen (Treg).

Viele Faktoren sind an der Regulation der T-Zell-Entwicklung beteiligt. Ein Beispiel hierfür sind MicroRNAs (miRNA). Diese kleinen, nicht-kodierenden RNAs sind etwa 18-22 Nukleotide lang und wurden in den letzten Jahren mit der Regulation einer immer größer werdenden Anzahl biologischer Signalwege in Verbindung gebracht. Sie sorgen für eine Art der post-transkriptionalen Genregulation, in welcher kleine miRNA Spezies mRNA Transkripte binden und die Translation blockieren oder einen Abbau der mRNA induzieren. MiRNAs können simultan hunderte von mRNA-Transkripten modulieren, wobei die metabolischen Kosten für die Zelle relativ gering bleiben. T-Zellen exprimieren hunderte von verschiedenen miRNAs mit individuellen Expressionsniveaus, die im Verlauf der T-Zell-Entwicklung bis um das 1000-fache variieren können. Eine bestimmte Untergruppe von miRNA, miR-181a/b-1, wird im Verlauf der Thymozytentwicklung sehr dynamisch exprimiert und erreicht im DP-Stadium ihre höchste Anreicherung, die von keiner anderen miRNA-Familie während der gesamten T-Zell-Entwicklung übertroffen wird. Im DP Stadium durchlaufen Thymozyten die Selektion, deren Ausgang stark von den Signalen abhängt, die vom TCR transduziert werden. Der TCR-Signalweg umfasst eine komplexe Kaskade an Phosphorylierungen und Dephosphorylierungen. Es wurde gezeigt, dass miR-181a/b-1 die Expression mehrerer negativer Regulatoren des TCR-Signalwegs moduliert und somit die Selektion von T-Zellen kontrolliert. In Abwesenheit von miR-181a/b-1 ist diese Hemmung dereguliert und es kommt zu einer Abschwächung der TCR-Signale und verändertem Ausgang der Selektion im Thymus.

Besonders die agonistisch selektionierte Population der iNKT-Zellen ist stark von einer Abwesenheit von miR-181a/b-1 betroffen. Dies führte zu der Annahme, dass miR-181a/b-1 eine Schaltstelle des TCR -Signalwegs darstellt und somit auch die Selektion der Agonist-T-Zellen im Thymus maßgeblich beeinflusst, die auf ein starken TCR-Signal angewiesen sind.

Für die Erweiterung des derzeitigen Kenntnisstandes über die Rolle von miR-181a/b-1 im Zusammenhang mit der T-Zell-Entwicklung im Thymus, haben wir ein Maus-Modell verwendet, welches eine Keimbahn-Deletion des miR-181a/b-1 *locus* beinhaltet. Für MAIT-Zellen, die einem ähnlichen Entwicklungsweg wie iNKT-Zellen folgen, war eine Untersuchung einerseits durch das seltene Vorkommen in konventionellen Maus-Modellen und andererseits durch einen Mangel an unterscheidbaren Antikörpern erschwert. Fortschritte in der MR-1-Tetramer-Technologie haben uns allerdings erlaubt MAIT-Zellpopulationen zuverlässig zu identifizieren und die Rolle von miR-181a/b-1 in der Agonisten-Selektion von MAIT-Zellen zu charakterisieren. Durch eine Kombination von MR1-Tetramer-Anreicherung und einem Mausstamm mit miR-181a/b-1-Deletion, waren wir in der Lage zu zeigen, dass miR-181a/b-1 dazu benötigt wird, die Entwicklung von MAIT-Zellen im Thymus in einem frühen Stadium zu propagieren. Außerdem lieferte die Analyse von MAIT-Zellen in miR-181a/b-1-defizienten Mäusen, die $Rag1^{GFP}$, eine molekulare Stoppuhr, co-exprimierten, neue Erkenntnisse in

die Entwicklungsdynamik von MAIT-Zellen sowie ihrer funktionellen Reifung. In MAIT-Zellen spiegelt der Phänotyp des Verlustes von miR-181a/b-1 den Phänotyp des Verlustes aller miRNA in T-Zell-spezifischen Drosha-defizienten Mäusen wieder. Daher ist es plausibel zu schlussfolgern, dass miR-181a/b-1 ein wichtiger Akteur im miRNA Netzwerk ist, das MAIT-Zell Entwicklung kontrolliert.

Als nächstes haben wir die Rolle von miR-181a/b-1 in der Treg-Zell-Entwicklung und -Funktion untersucht. Hierbei konnten wir zeigen, dass die intrathymische Generierung von Treg-Zellen ausreichender Expression von miR-181a/b-1 bedarf, um mit starken TCR-Signalen zu reagieren, die für die Treg-Entwicklung notwendig sind. Ein reduziertes thymisches Treg-Reservoir wirkte sich auch auf die T-Zellen in der Peripherie aus, die vor kurzen den Thymus verlassen haben, obwohl eine homöostatische Expansion die Anzahl der peripheren Treg-Zellen ausgleichen konnte. Die Folgen der gestörten Entwicklung von miR-181a/b-1-defizienten Treg-Zellen resultierte in einer intrinsischen post-transnationalen Hochregulation von CTLA-4 über einen unbekannten Mechanismus. MiR-181a/b-1-defiziente Treg-Zellen zeigten gesteigerte suppressive Fähigkeiten *in vivo*, aber Anstrengungen die Wirkungsweise der Supprimierung zu charakterisieren waren nicht erfolgreich. Insgesamt wirkt miR-181a/b-1 als eine Schaltstelle für TCR-Signale während der frühen Treg-Entwicklung, durch die eine abweichende CTLA-4-Expression auf post-transnationaler Ebene kontrolliert wird.

Aufgrund widersprüchlicher vorangegangener Studien, die die Rolle der miR-181a/b-1 in der konventionellen Selektion beschreiben, war das Ziel dieser Arbeit diesen Widerspruch aufzulösen. Unter Verwendung eines transgenen TCR-Models in Kombination mit veränderten Peptidliganden konnten wir anhand der gezielten Untersuchung von Apoptose, welche durch klonale Deletion hervorgerufen wurde, ermitteln, dass miR-181a/b1-1 auch für die negative Selektion im Thymus benötigt wird.

Ein Verlust der miR181-a/b-1 reduzierte die Anzahl der Zellen, die durch ihren TCR Signale nach Inkubation mit einer geeigneten (oder erhöhten) Konzentration an Peptiden erfahren konnten, die normalerweise zur Induktion von Selektion führen. Sobald Zellen starke Signale erhalten, kommt es zur Einleitung der negativen Selektion, in Abwesenheit von miR-181a/b-1 allerdings zu einer verminderten Aktivierung von Apoptose-Signalwegen. Zusammenfassend ist es wahrscheinlich, dass Veränderungen in der Selektion durch den Verlust von miR-181a/b-1 zu einem veränderten peripheren T-Zellrepertoire führen, bis hin zu einer erhöhten Autoreaktivität.

Daher ist miR-181a/b-1 als essentiell in der Abmilderung des TCR-Signalniveaus während der konventionellen Selektion anzusehen, was zur Entwicklung eines funktionellen, selbst-toleranten peripheren T-Zell Repertoires führt. Schlussendlich fokussiert sich die Untersuchung von miRNAs auf

die Modulierung regulatorischer Netzwerke durch einzelne miRNAs. Darüber hinaus ist es wichtig, vorgeschaltete Signalwege zu identifizieren, die für die Expressionsregulation der miRNA selbst verantwortlich sind. Für ein initiales Verständnis ist eine Charakterisierung der primären miRNA-Transkripte, die der Reifung der miRNA vorangeschaltet sind, notwendig. Im Falle von miR-181a/b1 konnten wir die Existenz eines langen Primärtranskripts zeigen, welches sich ca. 70 kB aufwärts vor der reifen miR-181a-1 und b-1-Transkripte befindet.

Ohne die Prozessierung reifer miRNA kommt es zu einer Akkumulierung der pri-miR-181a/b-1. Diese entspricht dem bereits publizierten Expressionsniveau der reifen miR-181a/b-1, basierend auf qPCR Daten, in sich entwickelnden Thymozyten.

Zusammenfassend konnten wir das pri-miR-181a/b-1-Transkript identifizieren, welches die Grundlage für weitere Untersuchungen und Experimente bildet, um die dynamische Regulation während der T-Zellentwicklung genauer zu untersuchen. Wir zeigen die essentiellen Auswirkungen einer miRNA auf die Entwicklung von T-Zellen. Abweichend vom zentralen Dogma, dass einzelne miRNAs zelluläre Signalwege generell nur schwach bis moderat beeinflussen, präsentieren wir hier ein miR-181-Mitglied, miR-181a/b-1, als einen effizienten Regulator des TCR-Signalweges. Aufgrund der sensitiven Natur des TCR-Signals während der Selektion von Thymozyten hat miR-181a/b-1 globale Auswirkungen, die essentiell für die Entwicklung zentraler Toleranz und der Entwicklung eines funktionellen selbst-toleranten peripheren T-Zell-Repertoires sind. Unsere Studien zeigen, dass miR-181a/b-1 die TCR-Sensitivität in sich entwickelnden Thymozyten erhöht. Dies führt dazu, dass mehr der sich entwickelnden T-Zellen den benötigten Signalstärke-Schwellenwert überschreiten, um sich zu Agonist selektionierten T-Zellen zu differenzieren. Durch die Erhöhung der TCR-Sensitivität gegenüber Selbst-pMHC unterstützt miR-181a/b-1 in der Detektion und anschließenden Eliminierung autoreaktiver Thymozyten. Aus diesen Ergebnissen folgern wir, dass miR-181a/b-1 fundamentale Auswirkungen auf die gesamte T-Zell-Entwicklung hat.

*Translated by Z. Grewers, N. Verheyden & H. Kunze-Schumacher.*

# Contents

# List of publications

This thesis contains material from the following papers. The rights have been granted by publishers to include the material in this dissertation. Some passages have been quoted verbatim from the following sources:

**I.** **Winter SJ**, Kunze-Schumacher H, Imelmann E, Grewers Z, Osthues T, Krueger A (2019). MicroRNA miR-181a/b-1 controls MAIT cell development. *Immunology and Cell Biology* 97 (2), pp. 190–202.

II. Łyszkiewicz M*, **Winter SJ***, Witzlau K, Föhse L, Brownlie R, Puchałka J, Verheyden N, Kunze-Schumacher H, Imelmann E, Blume J, Raha S, Sekiya T, Yoshimura A, Frueh JT, UllricH E, Huehn J, Weiss S, Gutierrez MG, Prinz I, Zamoyska R, Ziętara N and Krueger A (2019). miR-181a/b-1 controls thymic selection of Treg cells and tunes their suppressive capacity. *PLOS Biology* 17 (3), e2006716.
**ML and SJW contributed equally to this work*

III. **Winter SJ**, Krueger A (2019). Development of Unconventional T Cells Controlled by MicroRNA. *Frontiers in Immunology* 10, p. 2520.

IV. **Winter SJ**, Krueger A (2020). Magnetic Bead-Based Enrichment of Murine MAIT Cells. *Methods in Molecular Biology* (Clifton, N.J.) 2098, pp. 299–305.

## Additional contributions

Additional scientific contributions during doctoral studies:

**V.** Kunze-Schumacher H, **Winter SJ**, Imelmann E, Krueger A (2018). miRNA miR-21 Is Largely Dispensable for Intrathymic T-Cell Development. *Frontiers in Immunology*. 5(9):2497

VI. Blume J, Ziętara N, Witzlau K, Liu Y, Sanchez OO, Puchałka J, **Winter SJ**, Kunze-Schumacher H, Saran N, Düber S, Roy B, Weiss S, Klein C, Wurst W, Łyszkiewicz M, Krueger A (2019). miR-191 modulates B-cell development and targets transcription factors E2A, Foxp1, and Egr1. *The European Journal of Immunology*. 49(1):121-132

VII. Amado T, Amorim A, Enguita FJ, Romero PV, Inácio DP, Miranda MP, **Winter SJ**, Simas PJ, Krueger A, Schmolka N, Silva-Santos B*, Gomes AG* (2020). MicroRNA 181a regulates IFN-γ expression in effector CD8+ T cell differentiation. *Journal of Molecular Medicine* 98, pp. 309–320.
*BSS and AGG contributed equally to this work

# Abbreviations

| | |
|---|---|
| 181-/- | miR-181a/b-1-deficient mice |
| AIRE | Autoimmune regulator |
| APC | Antigen presenting cell |
| APL | Altered peptide ligand |
| BM | Bone marrow |
| CHX | Cyclohexamide |
| CMJ | Corticomedullary junction |
| cTEC | Cortical thymic epithelial cell |
| DC | Dendritic cell |
| DN | Double negative ($CD4^-CD8\alpha^-$) cells |
| EAE | Experimental autoimmune encephalistis |
| ETP | Early thymic progenitors (also DN1) |
| FACS | Fluorescent activated cell sorting |
| Foxp3 | Forkhead box protein 3 |
| FTOC | Foetal thymic organ culture |
| GvHD | Graft vs host disease |
| IEL | Intraepithelial lymphocyte |
| IELp | Intraepithelial lymphocyte precursor |
| iNKT | Invariant nature killer T cell |
| KO | Knock-out |
| MHC | Majorhistocompatibility complex |
| MAIT | Mucosal-associated invatiant T cell |
| miRNA | MicroRNA |
| mTEC | Medullary thymic epithelial cell |
| NK | Natural killer cell |
| OT-I | $H2K^b$-restricted ovalbumin-specific TCR transgenic mice |
| OVA | Ovalbumin |
| pMHC | peptide - majorhistocompatibility complex |
| PTA | Peripheral tissue antigen |
| pTreg | Peripherally induced Treg |
| RNAi | RNA interferance |
| RTE | Recent thymic emigrant |
| SCF | Stem cell factor (c-Kit) |
| SP | Single positive (in terms of CD4 or CD8 co-receptor) |
| Tconv | Conventional T cell |
| TCR | T-cell receptor |
| Treg | Regulatory T cell |
| TSP | Thymic seeding progenitor |
| tTreg | Thymic-derived Treg |
| WT | Wild-type mouse |

# 1 Introduction

MicroRNA (miRNA) plays a fundamental role in the regulatory landscape orchestrating thymic T-cell development. By simultaneously targeting a multitude of transcripts, individual miRNAs can modulate multiple cellular pathways at defined stages of development. During thymocyte development, cells undergoing selection possess a heightened sensitivity to signals transduced through their T-cell receptor (TCR). Small alterations in TCR sensitivity at these stages can result in drastically different selection outcomes. A miRNA family, miR-181, has previously been reported to elicit control over multiple members of the TCR signalling cascade[1]. As a result, miR-181 is responsible for tuning TCR sensitivity during selection. The importance of this miRNA was confirmed through loss-of-function studies that revealed a complete deficit of a subset of T cells that require strong agonist TCR signals during thymic selection[2,3]. These cells, invariant natural killer (iNKT) cells, are an unconventional T cell population which share developmental similarities with a handful of T cell subsets that also require strong TCR signalling for their successful selection. Mucosal-associated invariant T (MAIT), regulatory T (Treg) and intraepithelial lymphocyte precursor (IELp) cells are among these subsets and the control elicited by miR-181 during their differentiation remains elusive. Efforts to validate the function of miR-181 during conventional selection *in vivo* have provided perplexing results[4], calling for further investigation to provide a link between apparent inconsistencies. Furthermore, the regulatory network of mature miR-181 in the thymus is reasonably well understood yet is it unknown what provokes the dynamic upregulation of miR-181 itself during thymocyte development. The primary miRNA (pri-miRNA) that encodes the mature miR-181a/b-1 subset has been speculated but remains scientifically uncharacterised. In the following pages, we attempt to provide a deeper understanding of the role of the miR-181a/b-1 subset during thymocyte development and selection and address these gaps in current knowledge. In doing so, we present miR-181a/b-1 as a universal regulator of all T cells at the double positive (DP) stage of development by setting thresholds for positive and negative selection. This project contributes to our basic understanding of fundamental immunobiology, specifically in the knowledge of T-cell development and selection in the thymus.

## 1.1 Adaptive immunity & T cells

The mammalian immune system is roughly separated into two arms: innate and adaptive immunity. The innate immune system is comprised of cells (e.g., neutrophils, macrophages, monocytes, natural killer (NK) cells, dendritic cells (DCs)) and receptors that are wired towards rapid recognition of pathogens and initiating rather non-specific pro-inflammatory responses. Inflammation generated by innate immune cells helps to initially contain an infection and instructs the expansion and differentiation of functional adaptive immune cells appropriate for the immunological challenge at

hand (e.g., B cells, αβ T cells and γδ T cells). Upon clearance of an infection, most adaptive cells die, leaving behind a small pool of memory cells. Memory cell subsets have superior protective functions, amounting rapid and strong immune responses upon re-infection of pathogens. The adaptive immune response therefore has major advantages: the ability to generate a specifically tailored immune response to individual pathogens and to form pools of memory cells which exist for years, providing strong protection in the case of reinfection. Interestingly, rapid pathogen evolution surpasses these benefits of adaptive immunity, causing researchers to speculate about the functional relevance of the adaptive immune system. In doing so, a hypothesis has been put forth proposing that our adaptive immune system may have co-evolved with commensal bacterial species. The human body contains an ecosystem of symbiotic commensal bacteria that not only provides homeostatic benefit to the host but poses a unique challenge to the immune system. Commensals and pathogenic bacteria bare no difference in molecular patterns and researchers predict that the process in which the immune system distinguishes friend from foe is one of education. Therefore, the ability of the immune system to learn to tolerate commensal bacteria may in fact be the major advantage of adaptive immunity[5].

T cells are central to the adaptive immune response. Many cell-types fall under the T cell umbrella, due to their shared development within the thymus and expression of a TCR. Upon infection, various T cell subsets undergo clonal expansion, activation and functional programming to elicit targeted immune responses. T cell subsets are also responsible for memory formation. Some T cells, known as Treg cells, function to supress or control aberrant immune activation, protecting the body against autoimmunity. An interesting branch of T lymphocytes is known for its innate-like characteristics and appear to function on the border of both innate and adaptive immunity[6]. Innate-like T cells, such as iNKT, MAIT, IEL and γδ T cells, respond rapidly to challenge, producing large amounts of inflammatory cytokines. At the same time, they are known for their tissue-resident properties, residing in peripheral tissues for prolonged periods, where they contribute to immune homeostasis and local immune protection. To obtain such a wide diversity of subsets and functions, T cells go through sophisticated developmental processes. T-cell development and selection takes place in the thymus, giving rise to a huge repertoire of functionally diverse T cells.

## 1.2 T-cell development

The major organ involved in T-cell development is the thymus. T cells undergoing development in the thymus, termed thymocytes, go through several well-characterised stages of development and selection before appearing in the periphery. To have a clearer understanding of thymic T-cell development, it is critical to first understand the architecture and microenvironment of the thymus. The thymus is a bi-lobed organ encased by a thin capsule of connective tissue. Each capsule is

divided into two major anatomical zones: the outer cortex and the inner medulla with the junction between the cortex and the medulla being referred to as the corticomedullary junction (CMJ). Thymic seeding progenitors (TSPs) enter the thymus through large venules at the CMJ[7]. Following thymic entry, early stages of T-cell development and TCR gene rearrangement occur in the cortex. The cortex is made up of cortical thymic epithelial cells (cTEC) and blood vessels that are surrounded by DCs and is tightly packed with immature thymocytes[7]. Immature thymocytes proceeding through development migrate to the medulla. The medulla is comprised of medullary thymic epithelial cells (mTEC), DCs, macrophages and B cells, which all interact with the developing thymocytes. Upon complete maturation, mature thymocytes egress from the thymus via venules at the CMJ. Thymic development proceeds in a step-wise manner that can be roughly defined by expression of the TCR

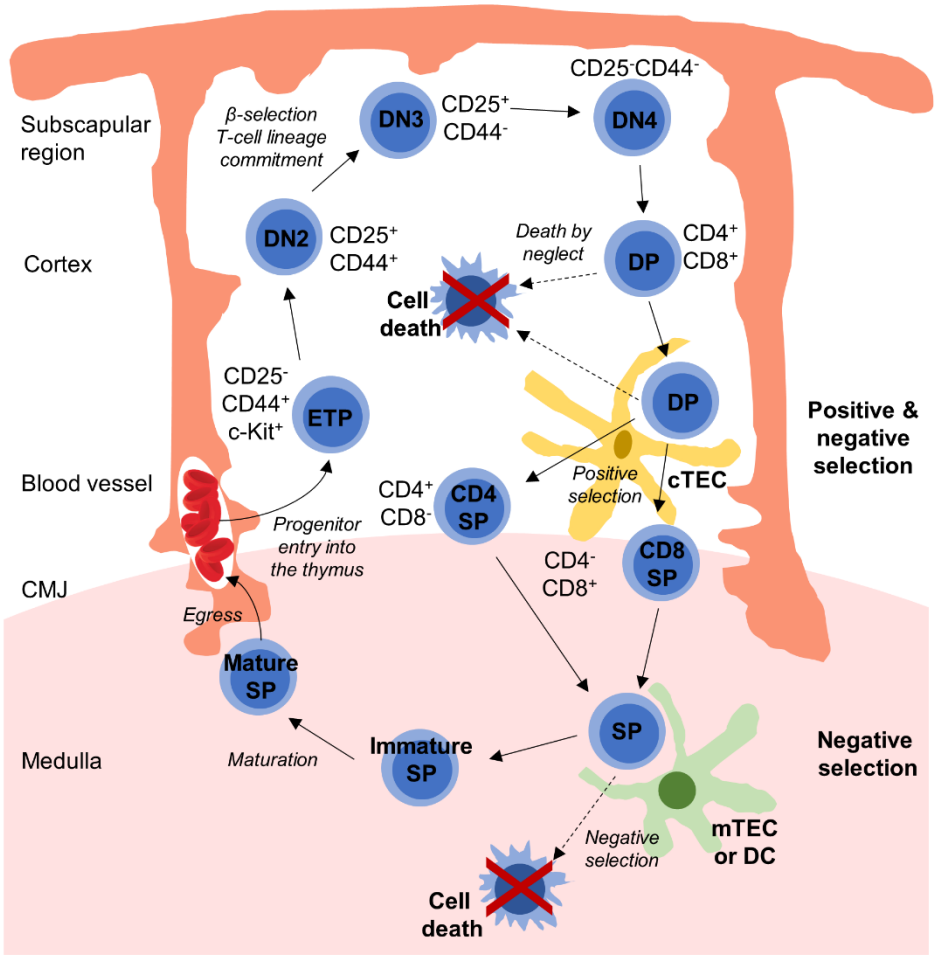


**Figure 1.1 T-cell development and migration through the thymus.** Early thymic progenitors (ETPs) enter the thymus through afferent blood vessels at the cortico-medullary junction (CMJ). ETPs migrate towards the subscapular region in the cortex developing into DN2 cells. DN2 cells undergo β-selection and T-cell lineage commitment as they develop into DN3 cells. DN3 cells downregulate CD25 to develop into DN4 cells which then rapidly upregulate both CD4 and CD8 co-receptors to become pre-selection DP cells. Pre-selection DPs that do not interact with pMHC presented by cortical thymic epithelial cells (cTECs) die by neglect. DP cells that interact with strong affinity to pMHC on cTECs also die through clonal deletion (negative selection). DPs that have weak interactions with pMHC on cTECs in the cortex migrate to the medulla as they down-regulate a single co-receptor, becoming CD4 or CD8 single positive (SP) cells. SP cells then scan self-pMHC on the surface of medullary thymic epithelial cells (mTECs) or dendritic cells (DCs) in the medulla. Strong interactions result in negative selection. Surviving cells travel through the medulla where they mature and eventually egress to the periphery via blood vessels at the CMJ.

co-receptors CD4 and CD8[8]. The most immature thymocytes express neither CD4 nor CD8 co-receptors and are therefore termed double negative (DN) thymocytes. DN cells develop into thymocytes that co-express CD4 and CD8 and are therefore termed double positive (DP) cells. DN and DP cells are mostly found in the cortical regions of the thymus. DP thymocytes that proceed through positive selection in the cortex downregulate a single co-receptor and mature into either CD4 or CD8 single positive (SP) cells. CD4 and CD8 SP cells reside in the medulla for 4-5 days before exiting the thymus and entering the periphery[9]. DN thymocytes account for approximately 2% of total thymocytes, while DP cells account for 80-85% and both CD4SP and CD8SP cells make up an average of 10-15% of the total thymic cellularity.

In the postnatal or adult thymus, thymocyte development begins with the colonisation of bone-marrow derived haematopoietic stem cells termed TSPs[10]. The thymic-seeding niche is surprisingly small, with a normal mouse thymus accommodating approximately 160 TSPs, with only 10 of those niches available for colonising at any one point in time[11]. Inside the thymic niche these TSPs express the delta-like 4 (DL4) ligand which binds the Notch-1 receptor[12,13] as well as stem cell factor (SCF) which binds the c-kit receptor[14], triggering signalling and pushing T-cell specification and thymocyte differentiation. DN thymocytes then proceed through development in a manner distinguishable by the expression of the CD44 and CD25 cell surface proteins. DN thymocytes develop sequentially through the following stages: $CD44^{+}CD25^{-}$ (DN1), $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$ (DN3), and $CD44^{-}CD25^{-}$ (DN4)[15]. As thymocytes proceed through the DN stages they also follow a migratory pattern through the cortex, which continues towards the outer capsule and are interrupted by proliferative bursts, differentiation and TCR gene rearrangement[16]. T-cell commitment begins at the DN2 stage, which is divided into the uncommitted DN2a population and committed DN2b cells. This transition is marked by the downregulation of the c-kit receptor and the upregulation of the T-cell commitment factor Bcl11b[17,18].

Lymphocytes display a large variety of cell surface receptors that can recognise and respond to an unlimited number of antigens – a feature that is the ‘hallmark’ of the adaptive immune system. Strikingly, if the number of different types of receptors present on lymphocytes were encoded by individual genes, the entire human genome would need to be devoted to encoding lymphocyte receptors. To establish the necessary level of diversity without monopolising the whole genome, TCR genes are created by recombining pre-existing gene segments in a process known as TCR gene rearrangement or V(D)J recombination[19,20]. Thus, different combinations of a finite set of gene segments gives rise to receptors that can recognise huge numbers of foreign antigens. This is accomplished by an extremely well-co-ordinated set of reactions starting with cleaving DNA within specific, well-conserved, recombination signal sequences. This highly regulated step is carried out by the lymphocyte-specific recombination activating genes, RAG1 and RAG2[21,22]. The gene segments

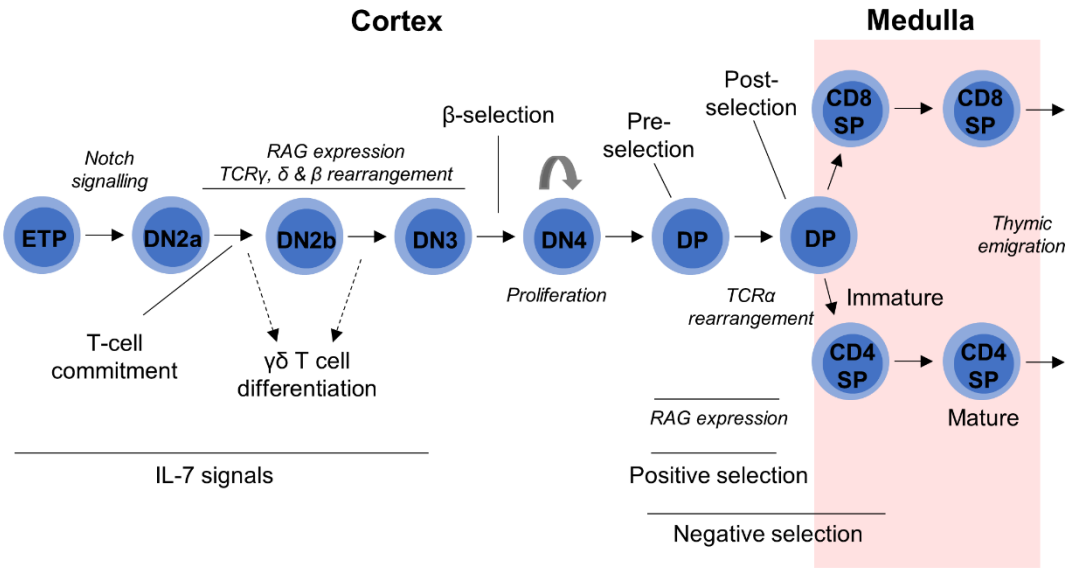


**Figure 1.2 Key features of T-cell development in the thymus.** T-cell development proceeds through an order set of stages. Early thymic progenitors (ETPs or DN1s) require notch signalling to proceed to the DN2 stage. T-cell commitment occurs between the DN2a and DN2b stage. At the DN2 stage RAG1 and 2 expression drives the rearrangement of TCRγ, δ and β. At this stage a portion of cells diverts into the γδ T cell lineage. After TCRβ rearrangement at the DN3 stage cells undergo β-selection. Following successful β-selection, cells proliferate rapidly until they upregulate both CD4 and CD8 co-receptors at the pre-selection DP stage. RAG1 and 2 expression at the DP stage allows for TCRα rearrangement and potential TCRα chains are trialled through positive and negative selection in the cortex. Cells that have undergone positive selection (post-selection DPs) migrate to the medulla and differentiate into either CD4 or CD8 SP cells. SP cells that survive negative selection in the medulla mature and exit the thymus to join the peripheral T cell pool.

are then reassembled using a common cellular repair mechanism. Cells can go through multiple rounds of gene rearrangement until they produce a functional TCRα chain. Upon positive selection, RAG gene expression is abruptly terminated and V(D)J recombination ceases.

Beginning at the DN2 stage and ending at the DN3 stage of development, the rearrangement of the *Tcrb*, *Tcrg*, and *Tcrd* loci which encode the TCRβ, TCRγ and TCRδ chains respectively, occurs[16]. A small portion of thymocytes at this stage diverge into the γδ T-cell lineage after recombining a functional TCRγδ[16,23]. The majority of thymocytes undergo αβ T-cell lineage commitment, where rearrangement of *Tcrb* is essential. Cells that fail to rearrange a functional TCRβ chain are eliminated via apoptosis. Upon expression of a functional TCRβ chain, beta selection commences. β-selection involves forming the pre-TCR, by pairing the developing TCRβ chain with an invariant pre-Tα (pTα) chain. Signalling through the pre-TCR are required for survival and progression to the DN4 and DP stages of development[24].

During beta selection at the DN3 stage, several rounds of proliferation occur and the progressive dilution of CD25 on the cell surfaces signifies the transition to the DN4 stage. Loss of CD25 is followed by a rapid upregulation of both CD4 and CD8 co-receptors, indicating maturation to the DP stage[25]. The massive burst of proliferation occurring in the outer cortex during beta selection[26] is responsible

for approximately 98% of all thymocyte proliferation[25]. DP cells undertake *Tcra* recombination[27] causing cells to cease proliferation and become transcriptionally quiescent until they are positively selected on cTECs and transcription is reawakened[28,29]. Cells that do not undergo positive selection due to possessing non-functional TCRαβ chains exist for approximately 3 days until they die by apoptosis[30]. Developing thymocytes with TCRs having self-reactive properties are deleted during negative selection in the cortex and as they transition into the medulla[31]. Some T cells with self-reactive properties have an alternate fate, receiving survival signals and continuing development in a process termed agonist selection[32]. Positively selected cells transition to the SP stages by downregulating a single coreceptor. After receiving signals through their TCR, developing DPs partially downregulate CD8, creating $CD4^{+}CD8^{int}$ cells, and begin to upregulate their TCR. The partial loss of CD8, corresponds to either sustained or reduced signalling depending on major histocompatibility complex (MHC) restriction. Cells that have reduced signalling are MHC Class I (MHCI) restricted and lose CD4 expression to become CD8SP cells and cells with sustained signalling downregulate their CD8 co-receptor further to become MHC Class II (MHCII) restricted CD4SP cells[33]. These cells egress from the thymus where they enter the peripheral circulation as naïve T cells.

## 1.3 Thymic selection

Following V(D)J recombination, each mature conventional thymocyte expresses a TCR on its surface. From the massive repertoire of TCRs generated each one is capable of recognising and responding in different degrees to an extremely low dose of an appropriate agonist peptide, which is presented via an MHC (peptide-MHC, pMHC) on the surface of an antigen presenting cell (APC). Generating a pool of 'appropriate' receptors is crucial to guarantee an adaptive immune system that can recognise non-self (e.g., pathogens) and modified self-threats (e.g., cancer), without developing autoreactivity against self. This intricate balance is achieved during thymic development of T cells in a process known as thymic selection. Selection occurs through two mechanisms. Firstly, DP thymocytes possessing a functional TCR are positively selected by interactions with ubiquitous antigens in the thymic cortex, whilst cells failing to rearrange functional TCRs do not receive survival signals and are eliminated through a process aptly termed 'death by neglect'. The second phenomena, known as negative selection, occurs alongside positive selection in the cortex[34,35], but also continues as positively selected DP cells mature into SP cells and migrate into the medulla. Nascent SP thymocytes in the medulla sample a large range of peripheral tissue antigens (self-pMHC) on the surface of Aire-expressing medullary thymic epithelial cells or cross presented by DCs. Strong interactions between TCRs and self-pMHC result in clonal deletion, removing potentially autoreactive clones from the repertoire[36]. It has been reported that approximately six times more cells undergo negative selection than positive selection and that only a quarter of negative selection occurs in the medulla, whilst most

occurs at the DP stage in the cortex[35]. Thymocytes that successfully survive positive and negative selection mature and migrate to the periphery as naïve T cells, where they can elicit functional immune responses.

### 1.3.1 Positive selection & death by neglect

Pre-selection DP thymocytes travel through the cortex where they undergo either positive selection or death by neglect. The transition to the DP stage is signified by the largest change in a thymocytes transcriptome throughout T-cell development. This transition accompanies the downregulation of approximately 1,500 genes, leading to a period of metabolic quiescence[28]. In this state, DP cells survive for approximately 3 days before they succumb to apoptosis[37]. During this time DP thymocytes 'audition' their nascent TCRαβ chains by encountering a multitude of pMHC on the surface of cTECs[38]. The cells of the thymic cortex exclusively express the thymoproteasome[39,40], which is responsible for the generation and presentation of ubiquitous antigens on cTECs during both positive and negative selection[39,41–44]. DP cells can go through multiple rounds of *Tcra* rearrangement during this time, allowing them greater opportunity to develop a functional TCRαβ that will be positively selected[45]. When a T cell receives positively selecting signals through their functional TCR, RAG gene expression is acutely terminated and *Tcra* rearrangement subsides[46]. Many DP thymocytes at this stage will not rearrange a functional TCRαβ during this time causing them to undergo death by neglect, which is mediated by an intrinsic apoptotic pathway[47,48]. Imaging studies monitoring DP thymocyte motility through the cortex and intracellular calcium levels revealed that DP cells pause and scan pMHC on several cTECs, receiving multiple transient activation events[49–51]. Basal levels of calcium increase with each TCR triggering event, reducing the length of migratory pauses as positive selection continues[51]. Cortical thymocytes were shown to have low motility and random trajectory until being placed under positively selecting conditions causing them to triple their speed and migrate in the direction of the medulla[52].

Positive selection is accompanied by multiple molecular and biochemical changes in the cell. A major player in positive selection is a protein known as the thymocyte-expressed molecule involved in selection, or more simply, Themis. Themis is phosphorylated rapidly after TCR triggering and makes up part of the TCR signalling cascade[53–56]. Its highest expression is in pre-selection DPs which decreases throughout development, with SP thymocytes and peripheral T cells exhibiting relatively low expression[53,54,57,55,56]. Themis negatively regulates TCR signalling through the phosphatase Shp1[58–61] and in its absence Shp2[62,63], by potentially controlling their access to TCR signalling machinery. Interestingly, the effect of Themis as a regulator of signal transduction is limited to responses toward weak positively selecting ligands[58]. Furthermore, Themis-deficient mouse models have a defect in positive selection, displaying reduced SP thymocytes and peripheral T cells.

Peripheral T cells remaining in Themis-deficient mice have an antigen experienced memory phenotype characteristic to agonist-selected T cells, showing that the control of Themis over signal transduction is not extended to agonist selection[53,64].

Additionally, positive selection is supported by weak but sustained signalling through the Ras-Erk MAP kinase (MAPK) pathway[65–67]. During positive selection ERK is translocated to the nucleus, activating the SAP-1 transcription factor which leads to the expression of genes required for positive selection[68]. Further changes that happen during positive selection involve a switch in pro-survival molecules. The DN to DP transition is marked by upregulation of the pro-survival protein BCL-$X_L$[69]. Cells expressing TCRαβ chains that are positively selected switch their pro-survival reliance from BCL-$X_L$ to BCL-2 and MCL-1[70–72]**.** BCL-2 is another pro-survival protein that is thought to antagonise pro-apoptotic proteins such as BIM, which is also increased after successful TCRαβ ligation during positive selection[48,73]. Moreover, the Schnurri-2 protein acts downstream of TCR signalling to reduce the induction of cell death pathways, promoting positive selection[74,75]. Positively selected thymocytes upregulate CD69[51] as well as the chemokine receptors CCR4 and CCR7 to aid in migration to the medulla. CCR4 is required for medullary entry[76] and CCR7 promotes the chemotaxis of SP thymocytes towards the medulla[77] where they undergo negative selection.

### 1.3.2 Negative selection

Negative selection, also referred to as clonal deletion, generates a repertoire of T cells that are self-tolerant by deleting potentially auto-reactive thymocytes[78,79]. Most thymocytes undergo negative selection alongside positive selection in the thymic cortex[34,80,35]. Additionally, during the DP to SP transition[81–83], developing thymocytes migrate to the medulla where they encounter self-pMHC. At this stage some clones interact with strong engagement through their TCR, triggering apoptotic cell death[78]. The medulla is occupied with mTECs and DCs, two types of APCs that present self-pMHC and are central to negative selection[84–87]. mTECs express a transcription factor known as autoimmune regulator (AIRE). AIRE expression in mTECs causes the transcription of a wide variety of organ specific genes that create tissue-restricted antigens (TRA) that are normally only expressed in peripheral tissues. mTECs are therefore able to express the entire self-peptide repertoire of an individual and developing thymocytes can sample these antigens with their TCR. Thymocytes which interact with moderate to high affinity are deleted through negative selection to prevent the escape of these auto-reactive T cells into the periphery[88,89]. To sample a wide range of TRAs, thymocytes must scan several to test the entire peripheral repertoire[90–93]. Defects in AIRE expression result in autoimmune polyendocrine syndrome type I, which is distinguished by a multi-organ form of autoimmunity[94,88]. In the medulla, negatively selecting conditions reduce thymocyte motility, increasing thymocyte-APC contact and causing self-reactive thymocytes to undergo migratory arrest

and finally, cell-death[95,72]. Migratory arrest of cells going through negative selection is distinct from cells experiencing positive selection and one study showed that only 1 hour of TCR signalling was required to induce negative selection whilst over 36 hours of several transient TCR signalling events was necessary to facilitate positive selection[96].

Medullary thymocytes harbouring MHCII restricted TCRs coupled with high CD24 cell surface protein expression, have a greater sensitivity to negative selection. Mature $CD24^{high}$ SP thymocytes undergo apoptosis in response to antigen while their $CD24^{low}$ counterparts proliferate. Downregulation of CD24 therefore signifies a reprogramming of mature thymocytes in response to their interactions with high affinity self-pMHC ligands[97]. The cell surface protein CD5 and orphan steroid receptor Nur77 have also been implicated in negative selection, by acting as surrogate markers for TCR signalling intensities. The stronger the affinity of TCR-ligand interaction during selection and consequently the stronger the TCR signal generated, results in a correspondingly high expression of both CD5 and Nur77[98–102]. Unlike weak stimulation, strong agonist stimulation through the TCR leads to ERK phosphorylation at the immunological synapse, instead of being translated to the nucleus which is observed during positive selection[103,68].

Thymocyte deletion during negative selection is triggered by the induction of intrinsic apoptotic pathways. BCL-2 has been shown to protect against clonal deletion[104], whist BIM aids in the cleavage of caspase-3 apoptotic pathway under negatively selecting conditions[105]. Co-stimulatory signals through CD28 are also a requirement for inducing apoptosis during negative selection[106,107].

Finally, SP thymocytes that survive both positive and negative selection mature into functionally competent T cells before exiting the thymus and joining the peripheral lymphocyte pool. Thymocyte maturation occurs sequentially, beginning after cells receive positively selecting signals in the cortex. After approximately 4 days[9] mature SP thymocytes egress to the periphery through blood vessels at the CMJ. Egress occurs through upregulation of CD62L, Qa2, CCR7, KLF2 and MHCI and downregulation of CD24, CD69 and CCR9[108,109]. The transcription factors, KLF2, promotes the transcription of S1P1, which is responsible for transendothelial migration out of the thymus[110–113]. Recent thymic emigrants (RTEs) can be visualised using ‘molecular timer’ models. Mice carrying a GFP under reporter elements of RAG genes can be used as a temporal marker for time since abrupt RAG gene transcriptional termination upon a cell undergoing positive selection. GFP is diluted either through half-life or through division, making Rag-GFP expression a measure of pseudo-time since positive selection and a reliable method for identifying RTEs[9].

### 1.3.3 Role of the TCR

The outcome of both positive and negative selection is mediated through the TCR. Minute differences in ligand potencies have demonstrated remarkably different selection outcomes through interaction with the TCR, indicating a narrow window between positive and negative selection[114,29,115]. Despite selection having a switch-like digital output (i.e., survival or death) the input through the TCR that determines this outcome is an analogue system (i.e., continuous and variable). The affinity and kinetics of TCR-pMHC interactions has been studied to fit models of selection-predictability. Groups measuring TCR affinity and half-life using surface plasmon resonance (SPR) saw a general, but not complete, correlation between half-life and selection. TCR-pMHC complexes with high affinity and longer binding kinetics induced negative selection, whilst the opposite favoured positive selection[29,116–118]. Further techniques measuring TCR-pMHC half-life using tetramer dissociation produced similar outcomes[115,119,120]. Using Förster resonance energy transfer (FRET) methods, 2D binding kinetics the correlation was further enforced under conditions of applied force[121]. Interestingly, upon application of force to the TCR-pMHC interaction, the affinity for agonist ligands increases due to the formation of catch bonds, whilst weak ligands form slip bonds and do not display this force-induced increase in affinity[121]. It is therefore evident that multiple factors are involved to achieve particular selection outcomes.

Early experiments used foetal thymic organ culture (FTOC) systems, coupled with either Tap1-, Tap2-, or $\beta_2$-microglobulin-deficient MHCI restricted TCR transgenic mice, which are developmentally arrested at the positive selection checkpoint. Addition of weak peptides was shown to induce positive selection[114,122–124], whilst high concentrations of strong agonists resulted in negative selection. Intriguingly, when using low concentrations of strong agonists, researchers observed the production of CD8αα-expressing cells, due to a process we now know as agonist selection[124,120,125,122,114,126,127]. Further experimental validation revealed the threshold between positive and negative selection to be extremely narrow. Transgenic TCR-specific immunogenic peptides can be modified to create altered peptide ligands (APL). APLs are designed to have single amino acid substitutions at the sites expected to interact with the TCR-MHC, therefore reducing their affinity for the transgenic TCR. Using a library of APLs for the OT-I TCR transgenic system, three ligands with remarkably similar binding kinetics were identified to straddle the threshold of positive and negative selection and result in immensely different biological outcomes[115,128]. Using these peptides with defined selection outcomes, they were able to visualise ERK activation in the cytoplasm or nucleus after stimulation with positive selecting peptides or ERK phosphorylation close to the plasma membrane after incubation with negatively selecting peptides[115]. Further experiments using alternative MHCI- or MHCII-restricted systems has supported the notion of the existence of a threshold affinity or half-life for negative selection[129,130,117].

Experimentally, the discrimination between positive and negative selection is rather cumbersome. There is no explicit way to determine whether defects in transitioning from DP to SP thymocytes is a cause of reduced positive or increased negative selection. Methods to assess clonal deletion are in higher abundance due to the digital 'life or death' output. One method to assess negative selection-mediated deletion is through the absence of antigen-reactive thymocytes or T cells using paired ligand-TCR transgenic systems. In RIP-mOVA mice, which present ovalbumin (OVA) on their mTECs, introduction of OVA-specific TCR transgenic MHCI (OT-I) or MHCII (OT-II) restricted thymocytes leads to massive clonal deletion and a reduction in OT-I/II transgenic T cells in the periphery[131,132]. Measures of apoptosis commitment can also be an indicator of negative selection. Activated caspase-3 is expressed late in the apoptotic cascade and studies have shown that in a normal TCR setting, approximately 25% of thymocytes were both active caspase-3$^+$ and CD5$^+$CD69$^+$, indicating cells which had received strong TCR signals[35]. In the absence of MHC class I or II, negative selection is unable to occur and this population disappears[133]. Furthermore, new techniques involving the use of thymic slices have been instrumental in visualising selection processes. Thymic slices are generated by culturing thin slices of thymi and perfusing with peptides of interest. The outcome of positive or negatively selecting conditions on thymocytes present in the slice or introduction of labelled or transgenic pre-selection DP thymocytes which when overlaid, simply migrate into the slice, can be assessed[134,135,50,136]. TCR/CD3 crosslinking triggers negative selection of CD4$^+$CD8$^+$CD24$^{high}$ thymocytes, providing another way to observe negative selection *in vivo*[97,137]. Despite a seemingly large collection of methods available, there is still a long way to go to fully understand the molecular and mechanical mechanisms that constitute conventional thymocyte selection.

### 1.3.4 Agonist selection

In contrast to conventional selection, agonist selection occurs when developing T cell clones receive moderate-strong signals through their TCR, yet do not undergo negative selection. Specific αβ T cell subtypes arise from agonist selection including the TCR-diverse thymic-derived Tregs (tTregs) and αβ TCR intraepithelial lymphocytes precursors (IELp), as well as the TCR-restricted innate-like iNKT and MAIT cell populations. These cell-types can develop from different developmental stages, be MHC-restricted to either class I, class II or the MHC-like CD1d or MR1 and can express either CD4 or CD8 co-receptors or none. The major unifying factor between the development of each cell-type is their selection by agonist TCR interactions. Other shared characteristics between the cell-types include the possession of an activated/memory phenotype, regulatory immune functions and a requirement for TGFβ receptor (TGFβR) during selection. TGFβ signalling is reported to have an anti-apoptotic function to all thymocytes that receive an agonist TCR stimulus[138]. The absence of TGFβR or downstream factors during development results in reduced numbers of Treg[139] and IELs[140]. iNKT cell lineage expansion, protection against apoptosis and maturation was also shown to be TGFβ

signalling dependent[141]. TGFβ signalling in MAIT cell development is yet to be assessed, but due to their strong parallels to iNKT cells, it is begin to hypothesise that TGFβ signalling is a requirement for all agonist-selected lineages. Unique from conventional selection, further shared commonalities between agonist-selected T cells are their requirement for store-operated calcium entry[142,143], a redundant function of Themis[58] and sensitivity to miRNA-mediated control[144–149].

Additionally, unlike conventional T cells which require CD28 co-stimulation for negative selection, CD28 co-stimulation is necessary for the survival and expansion of agonist selected populations[150–152]. IL-2/IL2R signalling has also been implicated in Treg cell development[153], whilst homotypic interactions between DP thymocytes potentiate SLAM-SAP signalling which is essential for expansion and differentiation of iNKT cells[151].

Lymphocytes with reactivity to self-antigens are beneficial for several reasons. They can promote immunological tolerance, enhance immunity to foreign antigens and contribute to immune homeostasis. Treg cells are a classical example of self-reactive cells that promote immunological tolerance due to their suppressive effects in the periphery[154]. IELs in the gut exist in a partially activated state[155,156] and are presumed to survey the environment, producing cytokines, causing cytolysis or contributing to tissue repair upon receiving danger signals, with reports suggesting αβ IELs prevent the development of inflammatory bowel disease[157]. iNKT and MAIT cells are dubbed innate-like due to their ability to rapidly release an array of cytokines during early infection[158,159] and exhibit tissue-resident signatures[160], remaining at peripheral sights and influencing steady-state immune homeostasis[160–163]. To obtain such a variety of functions, each agonist-selected cell type requires unique cellular and molecular factors to develop, which pertains to the topic of discussion below.

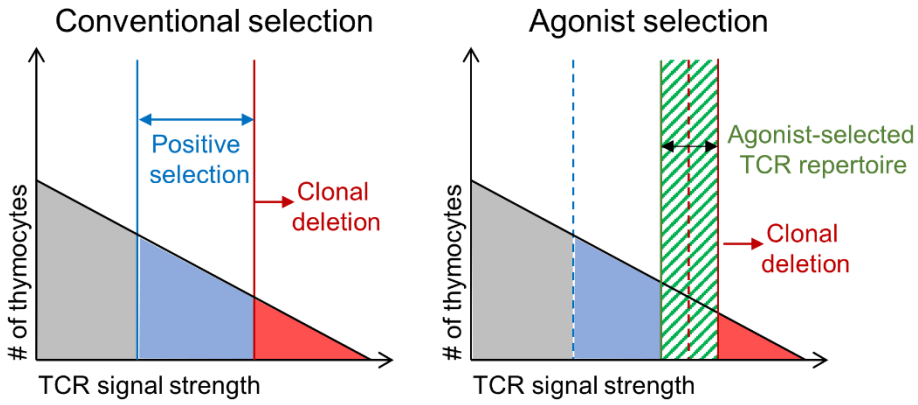


**Figure 1.3 Theoretical model of thymocyte selection thresholds. The** theoretical thresholds for thymic positive and negative selection are displayed. T cell clones with weaker affinity for self-pMHC undergo positive selection whilst strong affinity interactions result in clonal deletion (negative selection). In the case of agonist selection, cells that are successfully selected have a TCR repertoire overlapping with moderate-high TCR signals that are normally on the upper boundary of positive selection or inductive of negative selection.

### 1.3.4.1 iNKT cells

Despite being numerically less abundant than conventional αβ T cells, iNKT cells play an important role in providing protective immunity against pathogens as well as contributing to immune homeostasis[164–167]. They respond to lipid antigens presented by the MHCI like antigen-presenting molecule CD1d[168,169]. These CD1d-lipid antigen complexes are recognised by iNKT cells through a semi-invariant TCR, which consists of an invariant Vα14Jα18 chain coupled with Vβ chains biased toward Vβ8, Vβ7 and Vβ2. iNKT cells are positively selected on cortical DP thymocytes through agonist interactions with high affinity lipids presented by CD1d[170–172] and are also subjected to negative selection[173,174].

The developmental kinetics of iNKT cells are somewhat under debate. Previous studies have suggested distinct development stages, defined by the sequential expression of the markers CD24, CD44 and NK1.1: stage 0 $CD24^{+}CD44^{-}NK1.1^{-}$, stage 1 $CD24^{-}CD44^{-}NK1.1^{-}$, stage 2 $CD24^{-}CD44^{+}NK1.1^{-}$ and stage 3 $CD24^{-}CD44^{+}NK1.1^{+}$[169]. Reports using Rag-GFP reporter mice support the notion that these stages progress sequentially through development. Unfortunately, initial stages are

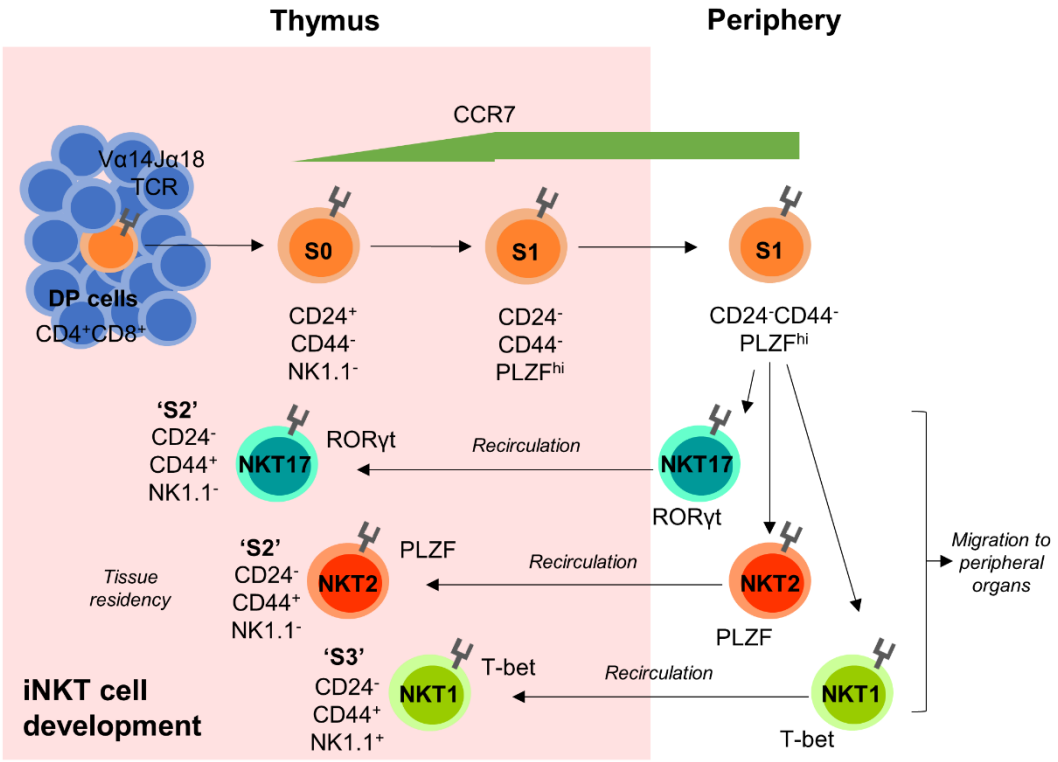


**Figure 1.4 iNKT cell development.** iNKT cell development begins at the DP stage when DP cells expressing a Vα19Jα33 TCR are agonistly-selected on fellow CD1d-restricted DP cells. Stage 0 (S0) iNKT cells express high levels of CD24. As cells progress to stage 1 (S1) they downregulate CD24 and upregulate PLZF as well as the chemokine CCR7. CCR7 induction prompts developing iNKT cell to migrate to the medulla and can be detected in recent thymic emigrants (RTEs). S1 cells emigrate from the thymus and develop into the three effector subsets NKT1, NKT2 and NKT17. These cells are functionally mature and recirculate back into the thymus where they remain resident for long periods of time. NKT1 cells have previously been thought to be more mature than NKT2 or NKT17 cells due to their expression of NK1.1 ('S3'), but this may not be the case.

highly proliferative and completely dilute GFP expression by stage 1-2, inadvertently 'excluding' stage 3, the most abundant stage of iNKT cells, from temporal analysis[161,175]. Using a Nur77-GFP reporter mouse as an immediate-early readout of TCR stimulation, iNKT cells which receive strong TCR signals during selection showed highest Nur77 expression in Stage 0 cells, supporting that they are the population of cells to most recently undergo selection[101].

The strict definition of stage 2 and 3 iNKT cells are when complications arise. As iNKT undergo functional maturation they can be separated into three terminally differentiated subsets defined as T-bet$^+$ NKT1, ROR$\gamma$t$^+$ NKT17, and PLZF$^{hi}$ NKT2 cells[164]. In disagreement with the linear development model, iNKT cell subsets appear to heterogeneously express CD44 and NK1.1[164,176,158,177–182]. Recent reports have redefined the classification of iNKT cell development by showing that very immature iNKTs express CCR7. Upon expressing CCR7, immature iNKT cells migrate to the thymic medulla where they are exported in an immature state. Once in the periphery, iNKT cells have the capacity to terminally differentiate into their respective effector subsets. In the thymus the majority of 'stage 2 or 3' functionally mature cells are actually long-term residents, expressing the tissue-resident marker CD69[161]. Tissue-residency programs are transcriptionally defined and different subsets of mature iNKTs preferentially home to certain organs[160]. Interestingly, reports have also showed that a small fraction of iNKT cells are not DP in origin, instead arising from DN cells that bypass the DP stage in

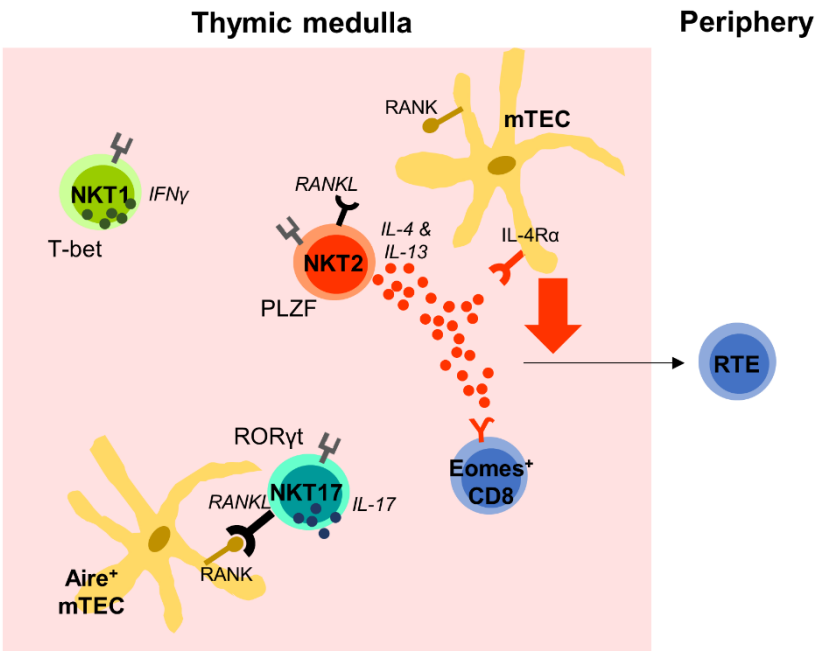


**Figure 1.5 iNKT effector subsets modulate immune homeostasis in the thymic medulla**. Recirculating iNKT effector subsets predominantly reside in the thymic medulla where they contribute to immune homeostasis. Interactions between RANK ligand on the surface of NKT2 and NKT17 cells with RANK on the surface of medullary thymic epithelial cells (mTECs) promotes Aire expression. NKT2 cells that have been activated through TCR stimulation secrete IL-2 and IL-13, which influences mTECs to promote emigration of mature thymocytes out of the thymus. IL-4 produced by NKT2 cells also causes CD8 SP thymocytes to upregulate Eomes and adopt a memory-like phenotype and function.

a process referred to as the 'DN pathway'. Cells arising through this pathway exhibit altered cell functionality and preferential homing[183].

The strict definition of stage 2 and 3 iNKT cells are when complications arise. As iNKT undergo functional maturation they can be separated into three terminally differentiated subsets defined as T-bet$^+$ NKT1, ROR$\gamma$t$^+$ NKT17, and PLZF$^{hi}$ NKT2 cells[164]. In disagreement with the linear development model, iNKT cell subsets appear to heterogeneously express CD44 and NK1.1[164,176,158,177,180,179,178,181,182]. Recent reports have redefined the classification of iNKT cell development by showing that very immature iNKTs express CCR7. Upon expressing CCR7, immature iNKT cells migrate to the thymic medulla where they are exported in an immature state. Once in the periphery, iNKT cells have the capacity to terminally differentiate into their respective effector subsets. In the thymus the majority of 'stage 2 or 3' functionally mature cells are actually long-term residents, expressing the tissue-resident marker CD69[161]. Tissue-residency programs are transcriptionally defined and different subsets of mature iNKTs preferentially home to certain organs[160]. Interestingly, reports have also showed that a small fraction of iNKT cells are not DP in origin, instead arising from DN cells that bypass the DP stage in a process referred to as the 'DN pathway'. Cells arising through this pathway exhibit altered cell functionality and preferential homing[183].

Several factors are known to be involved in early iNKT cell development. Both PLZF and ROR$\gamma$t are essential for development. PLZF is expressed in very immature post-selection iNKTs and is required for their effector program[184,185]. Through fate mapping experiments, iNKT cells were shown to derive from ROR$\gamma$t$^+$ DP cells[172]. ROR$\gamma$t expression supports DP survival through regulating Bcl-xL expression, permitting optimal Vα14Jα18 rearrangement[186]. Similarly, c-Myb promotes the long half-life of DP thymocytes supporting iNKT cell development[187], whilst c-Myc controls the maturation of iNKT cells[188,189]. In addition to strong TCR signals, iNKT development requires secondary SLAM-SAP signalling for positive selection, which is also promoted by c-Myb[190,187].

#### 1.3.4.2 MAIT cells

MAIT cells are a highly conserved population of innate-like T cells that make up the largest group of antigen-specific αβ T cells in the human immune system[191–195]. Unlike conventional αβ T cells, MAIT cells recognise microbial metabolites from the riboflavin biosynthesis pathway presented on MHC class 1-related (MR1) molecules, through their highly conserved and semi-invariant TCRs[196–200]. In fact, MAIT cells are mostly defined by their invariant TCRα chain comprising of Vα19Jα33 in mice, which preferentially pairs with Vβ6 or Vβ8[201–203]. Human MAIT cells show higher heterogeneity than their murine homologs, predominantly expressing Vα7.2 with Jα33, Jα20 or Jα12 combined with either Vβ2 or Vβ13[201,204,202,199]. Initial observations of their presence in gut lamina propria, lead to MAIT cells being coined 'mucosa-associated'[196], but further studies have observed MAIT cells in all sites where

conventional T cells are located[203,205,192]. Despite making up to 10% of peripheral human T cells in blood and up to 50% in liver[206,207,192,208,194,191,195], common strains of laboratory mice have exceptionally low numbers of MAIT cells impeding their biological characterisation[209]. Discovery of riboflavin derivatives as MAIT cell antigens[198,197], followed by the production of MR1 tetramers employing these antigens[201] has brought forth greater opportunity to study the development and function of MAIT cells.

MAIT cells lie on the threshold between innate and adaptive immunity. Analogous to iNKT cells, MAIT cells can be activated by recognition of antigens through their TCR or cytokines, such as IL-12 and IL-18. Activated MAIT cells rapidly secrete the proinflammatory cytokines IL-17, IFNγ and TNFα[146,192,210,211]. To shed light on the role of MAIT cells in disease, many studies have reported changes in MAIT cell frequencies in patients infected with a myriad of microorganisms.

Early MAIT cell development and selection occurs intrathymically through interactions with MR1[196,202,194,212]. Unlike conventional T cells which are selected via interactions with thymic epithelial cells, MAIT cells are selected on MR1-expressing DP cortical thymocytes[212]. Currently the antigens involved in MAIT cell selection are unknown as well as the extent to which their TCR repertoire is

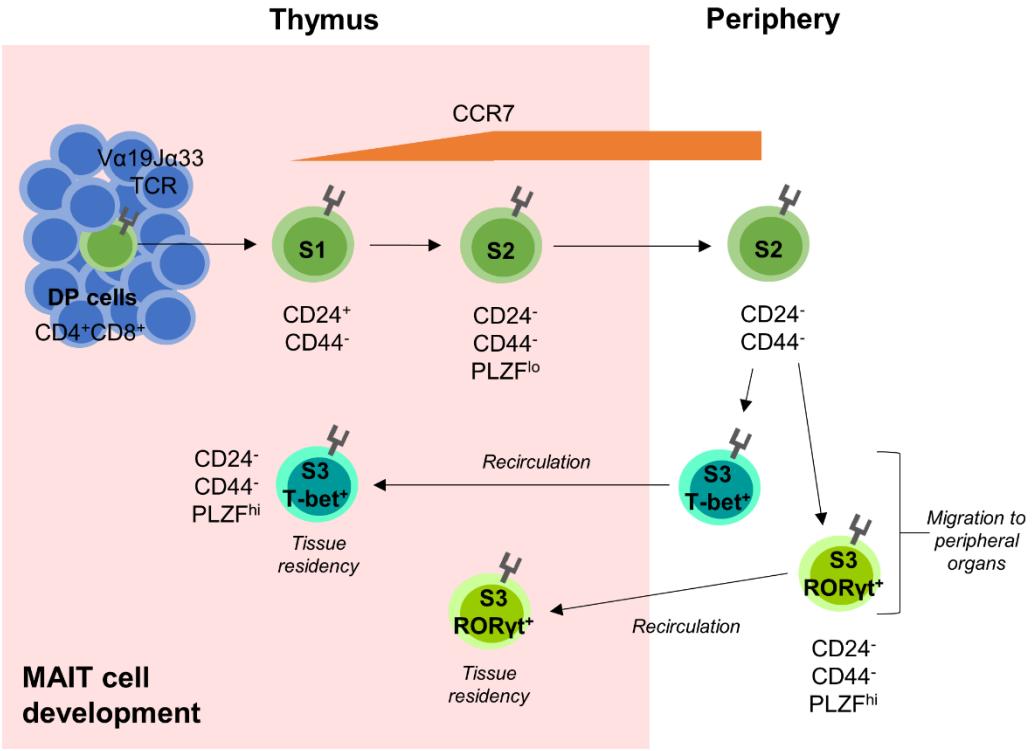


**Figure 1.6 MAIT cell development**. MAIT cell development begins at the DP stage when Vα19Jα33 expressing DP cells are selected on MR1-restriced DP thymocytes. The earliest distinguishable MAIT cell precursors express the MAIT cell TCR combined with high CD24 expression ad are known as stage 1 (S1). The downregulation of CD24 and upregulation of CCR7 corresponds with stage 2 (S2) of development and CCR7 drive the migration of these cells into the medulla which is following by thymic emigration. Recent thymic emigrants (RTEs) are CCR7 positive and give rise to the $CD44^{+}PLZF^{hi}$ stage 3 (S3) effector subsets. Effector subsets either express the transcription factors T-bet (MAIT1) or RORγt (MAIT17) and migrate to peripheral organs or recirculate back to the periphery where they remain resident.

shaped by negative selection. What has been recently described, is a three-stage developmental process in the thymus. After undergoing selection at the DP stage, MR1-5-OP-RU-reactive MAIT cells proceed through the following stages: stage 1 $CD24^{+}CD44^{-}$, stage 2 $CD24^{-}CD44^{-}$ and stage 3 $CD24^{-}CD44^{+}$[146]. CCR7 also defined a precursor pool for effector subset differentiation, when early MAIT cell development was visualised with a Rag2-GFP reporter mouse[161]. Progression from stage 1-3 is dependent on MR1 expression, while development between stage 2 and 3 requires both PLZF[146] and commensal microbiota[146]. Similarly, human MAIT cells progress through the following three stages: stage 1 $CD27^{-}CD161^{-}$, stage 2 $CD27^{+}CD161^{-}$ and stage 3 $CD27^{lo/+}CD161^{+}$[146]. Progression to stage 3 coincides with the upregulation of IL-18R and CD69, a marker for tissue residency[161]. Following PLZF expression, stage 3 MAIT cells can be separated into two functionally distinct subsets. The major population of MAIT cells comprises of $ROR\gamma t^{+}$ IL-17 producing cells, whilst a minor population express T-bet and produce $IFN\gamma$[146,203]. These two populations share functional similarities with NKT17 and NKT1 cells, respectively[160]. Diverging from typical iNKT cell development, MAIT cells appear to not require SLAM/SAP/Fyn signalling[194,190,213–215], nor do they express PLZF early on in development, with PLZF upregulation only occurring in late stage MAIT cell development[146].

Evidence for the extrathymic development of MAIT cells has been shown in both mice and humans. Stage 2 MAIT cells were detected in PLZF-null mice, indicating early thymic emigration[146]. The existence of $CD8\alpha\alpha^{+}$ peripheral MAIT cells, despite $CD8\alpha\beta^{+}$ co-expression in the thymus, also suggest these $CD8\alpha\alpha^{+}$ homodimers are derived from $CD8\alpha\beta^{+}$ MAIT cells extrathymically[201,146,216,208]. Furthermore, recent studies suggest MAIT cells exit the thymus before functional maturation[161] and then remain resident in tissues for extended periods of time[160].

#### 1.3.4.3 Treg cells

Tregs are a polyclonal subset of CD4 T cells that mediate peripheral tolerance. Their tolerogenic properties stem from high reactivity to self, which is in part attributed to agonist selection in the thymus. The peripheral Treg compartment consists of two subsets which arise through varying mechanisms. Thymus-derived Tregs (tTreg) develop through selection processes in the thymus and account for approximately 80% of all Tregs[217,218]. In the presence of agonist self-ligands, peripheral CD4 T cells can develop into Tregs through lineage diversion, generating peripherally-induced Tregs (pTregs)[219]. The tTreg subset will remain the focus for the remainder of this thesis, unless otherwise indicated. The expression of forkhead box protein P3 (Foxp3) transcription factor is the hallmark of all Treg cells[220]. In humans and mice alike, Foxp3-deficiency results in defective development of Tregs coupled with unrestrained lymphoproliferation and multi-organ autoimmunity[221]. In addition to Foxp3, mature Tregs express the IL-2Rα chain, CD25[222,223]. During thymic development, Foxp3 expression

is seen mostly in the medulla and it has become clear that DP cells are not a prerequisite for Treg differentiation[224,225].

Diversion into the Treg lineage begins at the CD4SP stage of development with studies revealing that the intrathymic transfer of antigen-naïve CD4SP cells could efficiently generate Tregs[226]. Conventional CD4SP cells that have strong self-reactivity and would normally undergo clonal deletion are diverted into the Treg lineage. Selection of Treg cells is mediated by TRA presentation by mTECs through temporally persistent TCR signals[227]**.** Treg selection varies from both iNKT and MAIT cell selection which propose the existence of sharply demarcated TCR signalling thresholds surrounding negative selection and iNKT or MAIT cell development. For Treg selection, the TCR signal strength model may not be so simple and may underscore findings that Treg development appears to be in the range of both above and below negative selection thresholds. Experiments have shown that Treg TCR clones can overlap with conventional T cell TCRs, revealing that some Treg cell selection lies beneath the negative selection boundary[228,229,219,230]. Contrastingly, models of clonal deletion using TCR-transgenic systems where neo-antigen expression in the thymus drives massive clonal deletion, has also shown to support Treg cell development, therefore suggesting some Treg clones lie above the negative selection threshold[231–234]. Furthermore, it appears that Treg development itself can still be restrained by negative selection. Studies showed that low thymic concentrations of self-peptides favoured Treg cell development, whilst higher concentrations of self-peptide resulted in massive clonal deletion[234], therefore suggesting that negative selection may bias Treg cell repertoire towards low abundance self-peptides. Precise understanding of what drives Treg development in the thymus still requires further investigation.

Proceeding agonist selection, Tregs develop through two distinct pathways characterised by the timing and expression of Foxp3 and CD25. The first developmental pathway recognised by scientists involves the initial upregulation of CD25 in response to strong TCR signals, which in turn promotes the upregulation of Foxp3[235,236]. Approximately 20% of these $CD25^{+}Foxp3^{-}$ precursor cells give rise to mature $CD25^{+}Foxp3^{+}$ Tregs in this two-step process[236–238] and they will forthright be referred to as $CD25^{+}$ precursor 1b Tregs. The alternative route in the bifurcated Treg developmental pathway appears to have less of an early requirement for IL-2 signalling due to delayed expression of CD25. These cells upregulate Foxp3 before inducing CD25 and are denoted $Foxp3^{+}$ precursor 1a Tregs[238,237] and this subset is the major contributor to the Treg pool. *In vivo* molecular timer studies have also confirmed the precursor-prodigy relationship between both $CD25^{+}Foxp3^{-}$ (1b) and $CD25^{-}Foxp3^{+}$ (1a) precursors and mature $CD25^{+}Foxp3^{+}$ Tregs[238,227,239]. TCR signalling plays a role in the development of both precursor populations, but the ongoing progression in their two-step pathway to mature $CD25^{+}Foxp3^{+}$ Tregs is largely TCR signalling independent[238]. Both precursors have high expression

of GITR, which has shown to correlate with TCR signal strength. Mature Tregs express higher GITR suggesting Tregs with high GITR may have a selective advantage during development[240].

The biological relevance of having multiple Treg developmental pathways is still unclear but a handful of differences have emerged between the two. Firstly, the developmental kinetics of each pathway varies with molecular timer studies showing the rapid appearance of the CD25$^+$ precursor 1b population, whilst Foxp3$^+$ precursor 1a cells developed slower with similar dynamics to the appearance of mature Tregs[238,239]. CD25$^+$ precursor 1b cells require strong persistent TCR signals during development to upregulate Foxp3, whilst Foxp3$^+$ precursor 1a cells require weaker and/or less sustained TCR signals to develop into mature Tregs[239,236,238,227]. The higher TCR signalling intensities observed in the CD25$^+$ precursor 1b population suggests that these cells lie on the border of negative selection and their rapid developmental kinetics, despite no evidence of onward progression towards the mature Treg phenotype, may hint towards either clonal deletion or reversion to a CD25$^-$ state[227,238,236,237]. In alignment with this, a recent study found that these CD25$^+$ precursor 1b cells expressed higher apoptotic markers than their 1a counterparts on both the protein and transcriptomic level and selective expansion of the CD25$^+$ precursor 1b population occurred following inhibition of negative selection pathways[239]. Furthermore, a distinction can be made between the two precursor populations both transcriptomically and in their clonality. TCR repertoire sequencing revealed that very little overlap in TCR clones exists between the two precursors and imaging of Foxp3$^+$ precursor 1a cells in the thymus showed that a small portion localise within the cortex, therefore sharing features with conventional T cells undergoing positive selection[239].

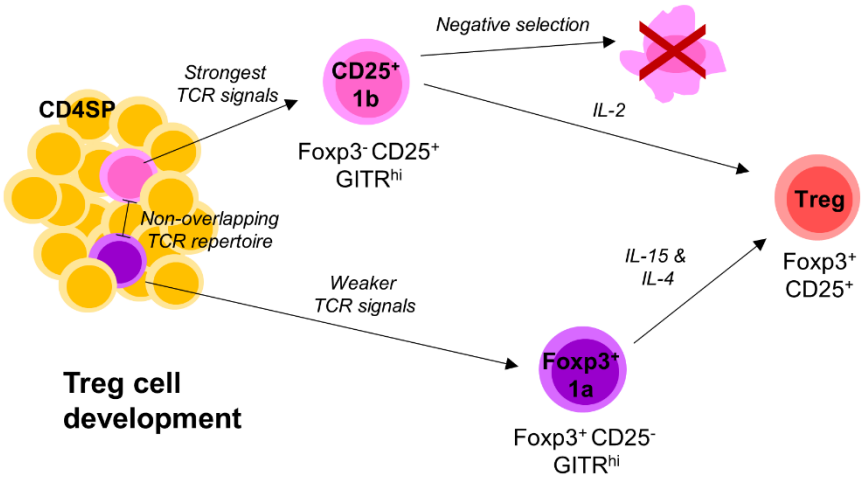


**Figure 1.7 Thymic Treg development.** Thymic-derived Tregs develop via two pathways beginning at the CD4 SP stage of development. The first Treg precursors to arise are the CD4$^+$CD25$^+$GITR$^{hi}$ 1b population. These precursors develop from strong TCR signals and this population is subjected to negative selection. CD25$^+$ 1b cells also require IL-2 signalling to mature into CD25$^+$Foxp3$^+$ Tregs. The second population to develop is the Foxp3$^+$ 1a precursors. These precursors develop from comparably weaker signals to CD25$^+$ 1b precursors and share little TCR repertoire overlap. As they mature into Tregs they upregulate CD25 and require both IL-4 and IL-15 signalling. Mature Tregs emigrate from the thymus to join the peripheral T cell pool.

Extrinsic cues are also involved in the onward progression of each precursor into mature Tregs. Differential requirements for cytokine signalling during development exist with the CD25$^+$ precursor 1b population having enhanced sensitivity to IL-2 and the Foxp3$^+$ precursor 1a population being dependent on IL-15 signalling[238,235,236]. IL-4 produced by iNKT cells has also been implicated in conversion of Foxp3$^+$ precursor 1a cells into mature Tregs[236,239]. Paradoxically, both precursor populations are dependent on co-stimulatory signals through CD28, despite co-stimulation inducing negative selection in conventional thymocytes[238,239,152]. Additionally, TGFβ in the thymus appears to restrain clonal deletion, whilst also supporting Treg cells development[139,138,241]. Lastly, a recent study showed that mature Tregs generated from the different precursors had different suppressive capacities. Upon induction of experimental autoimmune encephalomyelitis (EAE), mature Tregs derived from CD25$^+$ precursor 1b cells exhibited superior suppressive capacities, reducing EAE onset and severity. Tregs derived from Foxp3$^+$ precursor 1a cells appeared to exhibit no protection to EAE progression and mimicked disease severity of mice receiving no cells at all[239]. Currently, the mechanism behind this increased suppressive capacity of CD25$^+$ precursor 1b derivatives is unknown.

Central tolerance in the thymus through deletion of self-reactive TCRs is imperfect. Occasionally T cell clones with the potential to cause harm escape clonal deletion and make their way into the peripheral T cell pool. Tregs are a key player in peripheral tolerance by controlling and suppressing said autoreactive escapees. Due to Foxp3-mediated expression[242–246], cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is constitutively expressed on the surface of Tregs[247–249] and plays a vital role in the immunosuppressive functions of Tregs. Naïve T cell activation requires signals through both the TCR from pMHC engagement and the costimulatory receptor CD28 through the costimulatory ligands CD80 and CD86. CTLA-4 functions in a cell-extrinsic manner by removing the costimulatory ligands CD80 and CD86 from neighbouring cells via trans-endocytosis and therefore impairing T cell responses[250,251].

#### 1.3.4.4 γδ T cells

γδ T cells are an agonist-selected T cell subset that elicit rapid innate-like immune responses to a variety of molecules, including non-peptide antigens[252]. They are among the first T cells to appear during ontogeny and their foetal development proceeds in a wave-like manner. Each wave of development is characterised by either a defined γ-chain variable (Vγ) regions usage that preferentially home to barrier epithelial tissues[253,254] or by polyclonal Vγ chain usage that arises during a temporal window of commitment[255]. Depending on the sub-type, γδ T cells preferentially reside in tissues such as skin, liver, lung, intestine, tongue and reproductive tract. After birth, γδ T cell development continues in the thymus and requires even further differential TCRγ chain usage.

Development in the thymus proceeds in a slightly different manner to other conventional and agonist-selected T cell subsets. Commitment to the γδ T cell lineage commences after TCR expression[256]. Rearrangement of *Tcrg* and *Tcrd* loci begins at the DN2 stage encoding the γδ TCR[16]. Therefore, by the DN3 stage a small subset of cells successfully rearranges a γδ TCR and diverts into the γδ lineage[16,257]. TCR-dependent mechanisms play a role in γδ T cell commitment with strong γδ TCR signals promoting linage differentiation of γδ T cells over αβ T cell fate[258,257]. Additionally, within the thymic γδ T cell compartment, strong-ligand induced signals activate the ERK/MAP kinase pathway and preclude IFNγ-producing γδ T cell development, while slightly weaker signals instruct the IL-17A-producing lineage fate[259–263]. The pre-TCR can act in concert with the γδ TCR to promote γδ T cell lineage fate in the presence of weak TCR signals[263,264]. Additionally, thymic γδ T cell development has been shown to undergo negative selection[265].

Developing γδ T cell subtypes are rare in the thymus in comparison to conventional T cells, which has led to slow progression into the understanding of their thymic differentiation. The earliest defined γδ T cell subset is the 'pre-selection' $CD25^{+}CD24^{+}$ stage[266,267,262,259]. Proceeding selection CD25 is downregulated, giving rise to a $CD25^{-}CD24^{+}$ post-selection population, which is the checkpoint at which IL-17A-producing γδ T cell lineage specialisation begins[268]. IL-17A effector lineage differentiation ends with $CD24^{-}CD44^{hi}CD45RB^{-}$ cells that express RORγt and are $CD27^{-}$[262,268,267]. Alternatively, terminally differentiated IFNγ-producing cells are typically $CD24^{-}CD44^{+}CD45RB^{hi}$, expressing the transcription factor T-bet and can be differentiated through their high expression of the CD27 costimulatory receptor[262,268,267]. Sox13 was the first transcription factor identified to promote the γδ T cell lineage[269], with recent studies more precisely mapping its function mainly to IL-17A-producing subtypes[270,271]. The transcription factor c-MAF was also recently identified as a key regulator of IL-17A-producing γδ T cells. C-MAF drives *Rorc* expression (RORγt) through suppression of TCR signalling[259,268]. Downstream of c-MAF, the transcription factor HEB is selectively essential for IL-17A-producing γδ T cell differentiation of the Vγ2+ subset[270,272], while the PLZF controls the development of IL-17-producing Vγ4+ γδ T cells[273]. Furthermore, Notch and TGFβ signalling support weak γδ TCR signals to differentiate into IL-17A-producing lineage, whilst Id3 and TCF1 expression promote IFNγ-producing γδ T cell development[257,263,274,270,275,276]. NKT-like IL-4-producing γδ T cells can also arise through expression of RORγt and PLZF[277,278,259].

A recent study uploaded to BioRxiv performed single-cell RNA sequencing analysis on sorted pre/post-selection and $CD24^{+}$ immature/$CD24^{-}$ mature γδ thymic T cell subsets. Their research confirmed the trajectory of γδ T cell development whilst identifying 6 subsets of immature $CD24^{+}$ γδ T cell compartment. At the $CD24^{-}$ mature end of development, 4 subsets were split into either $ROR\gamma t^{+}$ and $ROR\gamma t^{+}PLZF^{+}$ IL-17A-producing cells or T-$bet^{+}$ and $Eomes^{+}$ IFNγ-producing γδ T cells (Sagar 2018). Effector programming into mature cytokine producing γδ T cells was shown to be molecularly

distinct from γδ selection[268]. Prior to thymic egress, γδ T cell subsets are functionally pre-programmed and exhibit only limited plasticity once they reach the periphery[267]. Upon migration into the periphery γδ T cells regulate early immune responses after activation through secretion of cytokines and cytotoxic molecules, recruiting neutrophils during infection, hypersensitivity and autoimmunity[279,280]. Their localisation in epithelial tissues also sees their involvement in tissue repair and wound healing following injury[281].

## 1.4 miRNA

MicroRNAs (miRNA) are a form of small noncoding RNA. In spite of their title, miRNAs have demonstrated profound effects on an ever-increasing number of biological pathways. They provide a form of post-transcriptional gene regulation in which tiny miRNA species bind to and destabilise mRNA transcripts, blocking translation and promoting mRNA decay[282]. The ability to simultaneously modulate hundreds of mRNA transcripts at a relatively low metabolic cost to a cell is a major advantage of miRNA-mediated gene regulation[283]. Mature miRNA is derived from long primary miRNA (pri-miRNA) transcripts that go through two major processing steps[284]. First, RNA polymerase II transcribes pri-miRNAs containing a stem-loop structure[285]. This stem-loop structure is recognised by the Microprocessor complex, which consists of the RNase III enzyme Drosha[286] and its cofactor DGCR8[286,287]. Upon recognition, the Microprocessor complex cleaves the pri-miRNA, generating approximately 65 nucleotide long precursor miRNA (pre-miRNA). Pre-miRNA is then translocated to the cytoplasm by Exporting 5[288], where it undergoes its second major processing event. In the cytoplasm, the RNase III enzyme Dicer[289] and its cofactor TRBP[290], cleaves pre-miRNA. The resulting small RNA duplex is then loaded in an AGO protein where the strands of the duplex unwind, and one is degraded, leaving behind a single mature 18-22 nucleotide miRNA[291]. The AGO protein loaded with a miRNA is referred to as an RNA-induced silencing complex (RISC)[292]. RISCs scan mRNA transcripts within a cell, searching for a sequence match. The exact targeting mechanisms by which the RISC operates are still incompletely understood, but some conditions enhance the likelihood of targeting. Target recognition by a RISC is aided by the miRNA ‘seed sequence’, which entails 6-8 nucleotides at the 5’ end of the miRNA[293]. Due to most miRNA-mRNA pairing being dictated by such short sequences, it is understandable that most of the coding genome falls subject to miRNA regulation, with conservative estimates suggesting that at least 60% of mRNAs are targets of miRNA[283]. Following target recognition, the RISC initiates a complex process that ultimately leads to destabilisation of the mRNA by deadenylation and/or inhibition of translation initiation[294].

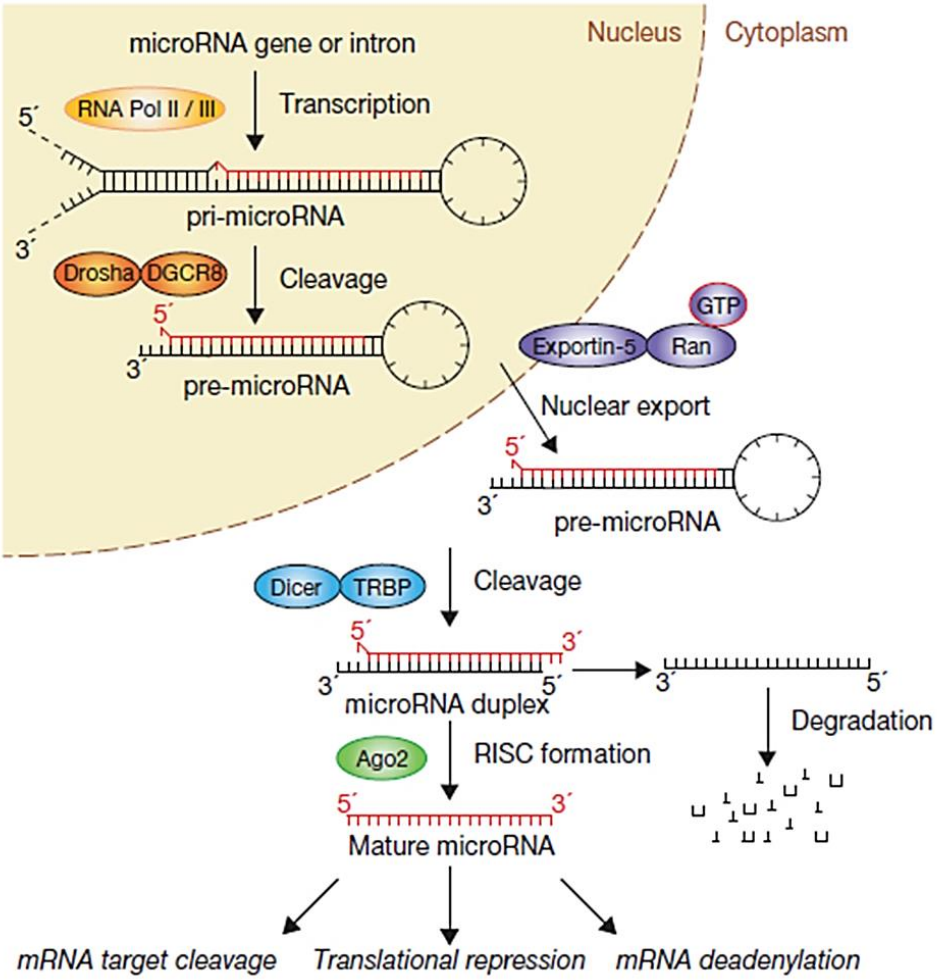


**Figure 1.8 The canonical pathway of miRNA processing**. Primary miRNA (pri-miRNA) transcription is commenced through RNA polymerase II or III and these pri-miRNAs are cleaved by the Drosh-DGCR8 microprocessor complex in the nucleus. The remaining precursor hairpin (pre-miRNA) is exported from the nucleus via Exportin-5-GTP. Upon entry into the cytoplasm, the double stranded RNA binding protein TRBP and the RNase Dicer form a complex and cleave the pre-miRNA to generate a mature miRNA duplex. The non-functional strand of the miRNA is degraded whilst the functional strand is loaded into the RNA-induced silencing complex (RISC) with Argonaute (Ago2) proteins. The RISC guides the mature miRNAs to target mRNAs, where mRNA silencing occurs through mRNA cleavage, translational repression or deadenylation. Extracted from Winter J. et al. (2009).

### 1.4.1 miRNA in T-cell development

T cell express over 600 different miRNAs, with individual miRNA expression levels differing as much as 1000-fold throughout T-cell development. Dynamic mRNA composition as T cells advance through development, alters the availability of miRNA recognition sites and therefore the miRNA 'targetome'[295]. Therefore, many miRNAs may target one mRNA, while one miRNA may target many mRNAs. Furthermore, recent reports suggest that miRNAs exhibit different targeting hierarchies in different cell types[296]. To make matters even more complicated, an interesting study revealed that most cells contain reservoirs of low molecular weight RISCs (LMW-RISC) that are not associated with mRNAs and are therefore inactive. Upon T-cell activation, signal transduction pathways were

demonstrated to increase the assembly of high molecular weight RISCs (HMW-RISC), which prompted miRNA-mediated repression, despite there being no net increase in miRNA expression[297]. From this it is clear that the study of individual miRNA functions in T cells and all biological pathways for that matter, are unsurprisingly multifaceted due to their interconnectedness and dynamic targeting capacities. Current methods to interrogate miRNA functions during thymocyte development involve the manipulation of the miRNA biogenesis pathway to achieve almost full absence of miRNA as well as single miRNA-deficient models to assess to role of individual miRNAs at the various stages of thymocytes development. The amount of total RNA per cell widely varies depending on the stage of thymocyte development. The higher end of the scale the largely proliferative DN4 subset has approximately 6.8pg/cell, which decreases to approximately 0.7pg/cell in DP thymocytes undergoing selection[298]. Similarly, the miRNA pool varies from a maximum of 33,000 copies per cell in DN4 cells to a minimum of 5,000 copies in DP cells. The estimated size of the miRNA pool varies with total RNA levels during thymocyte development suggesting that the total miRNA pool tightly corresponds to the level of ribosomal and messenger RNA within a cell[298]. When observing the relative degree of enrichment or depletion of predicted targets of prominent miRNAs during development, generally only subtle effects on gene expression are observed. Most miRNAs expressed during thymocyte development have a pattern of constant expression with transient enrichment, whilst stage-specific depletion is exceedingly rare. This may explain why miRNAs only modulate gene expression at an intermediate level as they are likely exerting post-transcriptional control constantly, with stages of significant depletion or enrichment resulting in more robust effects. Furthermore, miRNAs were shown to play an active role in the stages in which they were transiently expressed as well as downstream stages of development, implying their control extends past their temporal window of expression[298].

### 1.4.2 miRNA-deficient models and T-cell development

Developing thymocytes show a dynamic expression of miRNA profiles[295,298]. Before hypothesising which individual miRNAs have key functional roles, researchers first determined which stages of development were most sensitive to total miRNA-deficiency. Deletion of all miRNAs generated through conventional pathways was achieved through manipulating enzymes of the miRNA biogenesis pathway. Due to embryonic lethality of germline knockout models, Dicer, Drosha or DGCR8 deficiency has been evaluated using conditional Cre-*loxP* knock-out methods. Using the *Cre-loxP* system, miRNA-deficient mouse models were created for various stages of T-cell development. Depletion of the miRNA pool during early thymocyte development using Lck-mediated excision of Dicer, revealed a large defect in thymic cellularity and problems in the DN-DP transition[149]. Unfortunately, these results should be interpreted with caution, with later studies revealing that expression of the Lck-Cre alone causes a similar phenotype[299]. Using CD4-Cre mediated Dicer excision, developing thymocytes have reduced miRNA expression at the later DP stage of

development. In this mouse model a reduction of CD4 and CD8SP thymocytes was seen, which extended to the periphery, suggesting either a defect in selection and/or in late T-cell differentiation[300]. Drosha-deficiency at the same checkpoint produced the same phenotype, regardless of the presence of handful of Drosha-independent miRNA[301].

Developing iNKT cells display a dynamic miRNA expression profile substantially different from conventional T cells. A strong defect in iNKT development has been demonstrated with *Dicer*$^{fl/fl}$ or *Drosha*$^{fl/fl}$ mice crossed to *Tie2*-Cre, *hCD2*-Cre and *CD4*-Cre transgenic mice. These three Cre-transgenic mouse models have a tissue-specific miRNA deficiency from the haematopoietic progenitor, DN and DP stage of thymic development, respectively[146,145,144]. Each show a near absence of iNKTs, just slightly above background as confirmed by CD1d$^{-/-}$ mice[145]. Both *Tie2*-Cre and *Cd4*-Cre models revealed cell-intrinsic early stage blocks in development. A defect was seen in *Tie2*-Cre *Dicer*$^{fl/fl}$ cytokine production and CD69 upregulation following stimulus with α-GalCer, which argued that miRNA deficiency leads to a defect in iNKT TCR signalling [145,144]. An unbiased approach to identify miRNAs responsible for the defect in iNKT cell development in *Cd4*-Cre *Dicer*$^{fl/fl}$ mice was used, by comparing transcriptional signatures from Dicer-deficient iNKTs to S1-2 and S3 WT iNKTs. This alternate approach identified several miRNAs that have been experimentally validated to play a role in iNKT cell development, such as miR-181 and members of the miR-17~92 cluster[302].

The role of miRNAs in MAIT cell development was briefly addressed through analysis of Drosha$^{fl/fl}$CD4-Cre mice[146]. Due to Drosha deficiency, these mice have a substantial reduction of miRNAs in all T cells from the DP thymocyte stage. In thymi of Drosha deficient mice, the relative abundance of thymic and peripheral MAIT cells was significantly reduced with most thymic MAIT cells exhibiting a stage 1 phenotype due to a heavy reduction in stage 2 and 3 cells, in comparison to littermate controls. This data provided us with evidence that MAIT cell development beyond stage 1 is heavily dependent on miRNAs[146].

In multiple mouse models, deletion of both Dicer and Drosha resulted in Treg defects. Lineage specific deletion of miRNAs in Tregs phenocopies loss of Foxp3, resulting in fatal multi-organ autoimmunity. Deletion in all T cells showed a reduced suppressive capacity of Tregs and diminished homeostatic potential. Upon challenge, Tregs generated from mice lacking Dicer in all T cells showed a complete lack of suppressor capacity[147–149].

### 1.4.3 miRNAs in iNKT cell development

So far, many individual miRNAs have been implicated in the developmental processes of iNKT cells. Interestingly, few miRNAs have shown to be readily involved in early conventional T cell development, while agonist-selected T cell lineages have demonstrated to be quite sensitive to miRNA-mediated

regulation. Some of the more notable miRNAs that have been found to modulate developmental processes in unconventional T cell populations are detail below.

#### 1.4.3.1 Let-7

The let-7 family of miRNAs is the most abundant family of miRNAs in the genome and play an important role in iNKT cell development. Foetal haematopoietic cells express the LIN28A and LIN28B proteins which bind to let-7 precursor molecules, promoting their degradation and preventing the biogenesis of functional let-7 miRNAs. This depression of functional let-7 miRNA expression shapes foetal lymphopoiesis [303]. Exploiting this knowledge, researchers generated a mouse model with a significant reduction in most let-7 family members from the DN stage of thymocyte development onwards by transgenically overexpressing LIN28 proteins under the control of a *hCD2*-Cre[304]. Loss-of-function studies using this model led to evidence that dynamic upregulation of endogenous let-7 expression throughout thymic iNKT differentiation results in a downregulation of PLZF, through direct targeting of *Zbtb16* mRNA. This let-7-dependent decrease in PLZF directs iNKT cells to terminally differentiate into IFNγ-producing NKT1 cells. In contrast, inhibiting the upregulation of let-7 miRNAs allowed iNKT cells to maintain high PLZF levels and favoured the development of IL-4-producing NKT2 and IL-17-producing NKT17 cells. Dynamic upregulation of let-7 miRNAs during iNKT cell development was triggered by exogenous stimuli in the thymic medulla, including IL-15, vitamin D and retinoic acid. Targeting of the lineage specific transcription factors PLZF during NKT cell development is a prime example of the potential power of developmental regulation by miRNA in the thymus[304].

#### 1.4.3.2 miR-17~92 cluster

Comparisons between WT and Dicer-deficient developing iNKT cells revealed an increase in the expression of TGFβ receptor II (TGF-βRII) mRNA in the absence of miRNA[302]. TGFβRII is a subunit of the heterodimeric receptor for TGFβ, a cytokine known to protect against clonal deletion and promote the development of iNKT cells and other agonist-selected lineages[177,139,140,138]. During stage 1 of iNKT cell differentiation, TGFβ signalling through TGFβRII sustains iNKT cell expansion. Upon progression through development stage 2 iNKT cells downregulate TGFβRII, which is a requirement for normal progression to stage 3. In Dicer-deficient mice, lack of regulation by miRNAs led to unconstrained TGFβRII expression and therefore TGFβ signalling, as well as an accumulation of early stage iNKTs[302]. The miR-17~92 cluster was experimentally validated to have two target sites in the Tgfbr2 3'UTR, which coincided with and inverse relationship between the expression of miR-17~92 cluster miRNAs and TGFβRII expression. Analysis of miR-17~92 cluster-deficient mice revealed an increase in TGFβRII expression and therefore TGFβ signalling, while simultaneously demonstrating early iNKT cell defects at stage 2. Interestingly, double-deficiency of Dicer and TGFβRII did not rescue

frequencies or numbers of iNKT cells but appeared to aid cells stuck at stage 2 of development to differentiate into stage 3. Frequencies of stage 1 iNKTs remained the same in Dicer and Dicer–TGF-βRII double-deficient mice, revealing that potential regulation of TGFβRII expression by miR-17~92 cluster miRNAs, promote progression of stage 2 iNKTs to Stage 3[302].

#### 1.4.3.3 miR-150

The role of miR-150 in iNKT cell development has been addressed by two independent groups. miR-150 expression is low in stage 1 iNKTs and progressively increases through to stage 3. miR-150-deficiency resulted in an overall decrease in thymic iNKTs, appearing to have a mild defect in the transition from stage 2 to 3, despite normal levels of stage 1 iNKT cells[305,306]. Ubiquitous ectopic expression of miR-150 also resulted in an overall reduction in iNKT cells, displaying a developmental block due between stage 1 and 2, through increased numbers of stage 1 iNKT cells[**305**]. Perhaps an explanation between the similar phenotypes between miR-150-deficiency of transgenic ectopic expression lies in the need for specific constraints in miR-150 expression at specific stages of iNKT cell development. For example, it could be the case that early iNKT cells require low levels of miR-150, explaining the normal progression of iNKT cells to stage 2 in miR-150-deficient mice followed by a block in any further development due to a potential requirement for elevated levels of miR-150. Conversely, transgenic overexpression of miR-150 is likely to result in constitutively upregulated miR-150 at each iNKT developmental stage, which may downregulate genes or pathways that are needed to move through development normally. If low expression of miR-150 was a requirement during early iNKT cell development, then this would explain the block seen in miR-150-transgenic mice. Alternatively, huge proliferative expansion of iNKT cells in the early stages of development[171] could also explain the larger reduction in iNKT numbers in miR-150-transgenic mice. These mice have a defect at stage 1 in comparison to miR-150-deficient mice, which are halted in their development at stage 2, after massive iNKT cell expansion has already occurred. Increased production of IFNγ by splenic iNKTs in miR-150-deficient mice[306], may be a result of skewed iNKT differentiation towards the iNKT1 subset. C-myb was identified as a potential target of miR-150[306,305,307], with heterozygous C-myb-deficiency showing a reduction in splenic iNKTs in mixed BM chimeras, similar to transgenic expression of miR-150[305]. Further evidence is required to confirm the relationship between C-myb and miR-150 in the regulation in iNKTs. This combined deficiency and overexpression data suggests that a dynamic and tightly regulated expression of miR-150 is required for normal iNKT cell development.

#### 1.4.3.4 miR-155

miR-155 was shown to have a mild effect on iNKT cell development. Germline deletion of miR-155 shows relatively normal development and function of iNKT cells[308]. On the other hand, transgenic

overexpression of miR-155 using a Lck-miR-155-tg mouse resulted in a substantial block in development at stage 2, coupled with a decrease in the peripheral iNKT cell compartment[309]. miR-155 expression was identified to progressively decline throughout iNKT cell development, ending with lowest expression in stage 3 cells[309], suggesting that tightly controlled miR-155 expression is required for normal iNKT cell development. The Ets1 and ITK transcripts, modulators TCR signalling and maturation respectively, were brought forth as potentially relevant targets of miR-155 via *in vitro* luciferase assays. Mouse models deficient in Ets1 and ITK show a correlation in iNKT developmental phenotypes to miR-155 transgenic mice[310–312]. Interestingly, miR-155 transgenic mice showed upregulation of both targets in stage 1 and 2 cells, followed by a downregulation in stage 3, suggesting that increased expression of miR-155 in the earlier stages of iNKT development does not render Ets1 and ITK vulnerable to miR-155-mediated control[309]. More evidence is needed to validate the miR-155/Est1/ITK axis of iNKT differentiation.

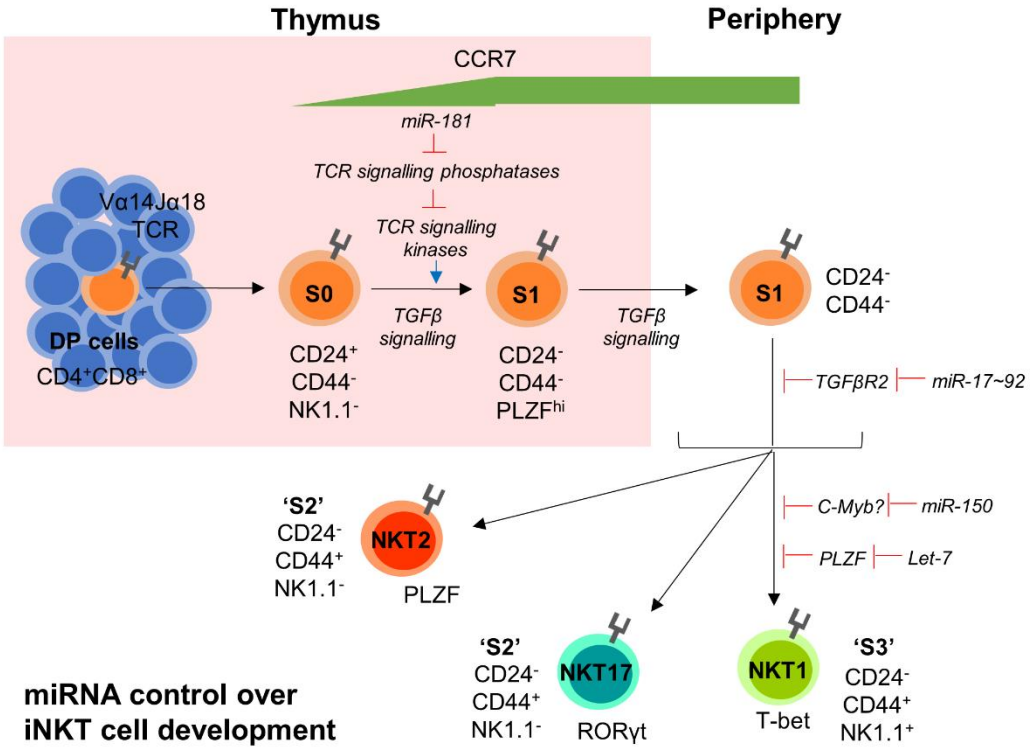


**Figure 1.9 miRNA-mediated control over iNKT cell development.** Several miRNAs are involved in the process of iNKT cell development. Firstly miR-181 family members have been shown to negatively regulate TCR signalling phosphatases that in turn negatively regulated TCR signalling kinases which are essential for the heightened TCR sensitivity required during thymic selection. Secondly, TGFβ signalling through TGFβRII promotes the maturation and expansion of iNKT cells at stage 1 (S1). TGFβ signalling is dampened at stage 2 through miR-17~92 expression, promoting normal effector subset differentiation. miR-150 may also downregulated C-Myb, promoting NKT1 cell differentiation.

#### 1.4.3.5 miR-21

miR-21 was identified to be strongly upregulated in the iNKT cell compartment, prompting it to be a potential candidate for iNKT cell regulation[145]. Studies from our lab have shown that despite dynamic expression of miR-21 during early thymocyte development, miR-21 appears to be phenotypically redundant for both conventional and unconventional T cell development, including MAIT (unpublished observations), iNKT, Treg and γδ T cells when analysing miR-21$^{-/-}$ mice[313].

### 1.4.4 miRNAs in Treg development

#### 1.4.4.1 miR-155

To date, the major miRNA shown to play a role in Treg development is miR-155. miR-155 is interesting in itself as reports have shown that Foxp3, binds to the promoter of miR-155's host gene *Bic*, thereby regulating its expression[244]. Impaired fitness coupled with inferior Foxp3 expression and stability was observed upon deletion of miR-155[314]. miR-155 deficient mice show reduced Treg numbers[315] and upregulated suppressor of cytokine signalling 1 (SOCS1)[314]. SOCS1 is a negative regulator of STAT5 signalling, which is downstream of the IL-2R complex (CD25), which is consequently diminished in miR-155-deficient mice[314,316]. It has been suggested that during Treg cell development, induction of Foxp3 drives high expression of miR-155, which maintains the competitive fitness of Tregs[314].

### 1.4.5 miRNAs in γδ T cell development

Despite dynamic miRNA expression in γδ T cells and their subsets, not much is known about specific miRNAs that modulate their development or function. Co-regulation of miR-133b and miR-206 with the IL-17 locus Il17a/f was shown in γδ subsets. Unfortunately, it is still unclear as to whether these Il17a/f co-regulated miRNAs are involved in specific mRNA silencing during IL-17-specfic γδ lineage differentiation or whether they are epigenetically involved in regulating the Il17a/f locus[317].

#### 1.4.5.1 miR-146

Recently, miRNA miR-146 was implicated in γδ T cell differentiation[318]. Using combined gain-of-function and loss-of-function approaches, miR-146 was demonstrated to act as a posttranscriptional brake on IFNγ production through targeting *Nod1* mRNA. This inhibition of IFNγ production by miR-146 is responsible for restricting the plasticity of CD27$^{-}$ γδ T cells. Microarray analysis of CD27$^{+}$ and CD27$^{-}$ γδ T cells revealed that miR-146a/b was the only upregulated miRNA subset in in CD27$^{-}$ γδ T cells isolated from the spleen and LNs. Functional plasticity of γδ27- cells is driven by inflammatory signals in the periphery, which result in IFNγ coproduction by IL-17 single producers[319]. Following Listeria infection, germline deletion of miR-146 led to the accumulation of double producing

IL-17$^+$IFN$\gamma^+$ cells *in vitro* and *in vivo*, resulting in a protective memory response against the bacteria[318]. The miR-146 target Nod1 was identified using a combination of retroviral overexpression of miR-146 in transgenic Argonaute 2 (Ago2) flag-tagged CD3$^+$ T cells followed by unbiased Ago2 RNA immunoprecipitation and deep sequencing (Ago2 RIP-seq). This was validated through *in vitro* Dual-luciferase assays, and an inverse relationship between Nod1- and miR-146-deficient mice in their susceptibility to Listeria infection. Furthermore, a rescue experiment involving double deficiency of both Nod1 and miR-146 prevented the accumulation of CD27$^-$ dual cytokine producing $\gamma\delta$ T cells, providing evidence for Nod1 being the dominant target of miR-146 in regulating CD27$^-$ $\gamma\delta$ T cell plasticity[318].

## 1.5 miR-181: The governor of T-cell development and selection

Six members of the miR-181 family have been identified in mice and humans. The family is divided into three subsets, miR-181a/b-1, a/b-2 and c/d, each of which is encoded on a separate chromosome. Mature miR-181a-1 and a-2 as well as b-1 and b-2 share an identical sequence. Mature miR-181a-1 and b-1 are encoded by murine chromosome 1 and are situated ~150bp apart. High expression of the miR-181a/b-1 subfamily is observed in the thymus, accounting for ~98% of all the miR-181 species[2].

miR-181a/b-1 is dynamically expressed throughout thymocyte development and accounts for the largest enrichment of a miRNA family at any stage of development[298]. This enrichment occurs at the DP stage, during which thymocytes undergo selection[298,295]. Transcripts containing predicted seed matches to miR-181 family members are depleted at the DP stage and are contrastingly enriched at

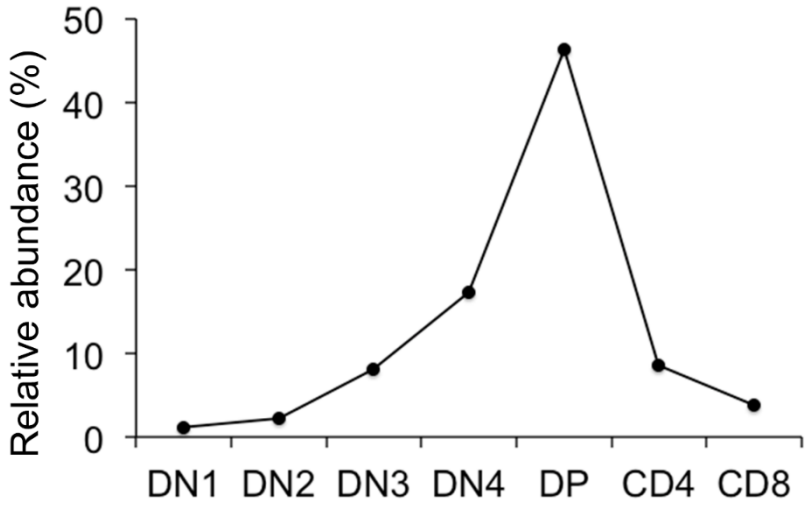


**Figure 1.10 Relative abundance of miR-181a during thymic development**. The relative abundance of miR-181a in comparison to all other miRNA in developing thymocytes was extracted from Kirigin *et al.* 2012. At its peak, miR-181a makes up over 40% of all miRNAs expressing in DP cells.

the DN3 stage of development[298]. So far, miR-181a/b-1 has been implicated in TCR signalling and thymocyte selection during these developmental stages[2,1,4,320].

TCR signalling requires a multitude of coordinated phosphorylation and dephosphorylation events within a complicated network of intracellular signalling molecules. T cells express over 40 different negative regulators of TCR signalling including multiple phosphatases for each of the fundamental TCR signalling kinases, such as Lck, Zap70 and ERK[321]. For a cell to tune its internal selection or activation thresholds, it must simultaneously modulate multiple signalling molecules which consist of minimal sequence homology. In 2007, Li Q-J *et al*.[1] identified miR-181a as modulator of TCR sensitivity by controlling multiple intracellular signalling molecules[1]. Only modest regulation of the phosphatases PTPN22, SHP-2 and DUSP6 by miR-181a was required to yield substantial differences in both activation and selection thresholds. Using retroviral overexpression, negative regulation of these phosphatases by miR-181a was confirmed via multiple methods including luciferase assay for target validation, and western blot and qPCR for visualisation of reduced protein and mRNA levels, respectively in the presence of increased miR-181a. An increase in both Lck and ERK phosphorylation could be seen in the presence of increased miR-181a[1], which is consistent with their reported dephosphorylation by PTPN22 and DUSP5/6, respectively[322,323]. Moreover, restoring DUSP6 expression to normal levels by co-expressing miR-181a and *Dusp6* mRNA with mutated miR-181a target sites, reduces basal Lck serine phosphorylation to background levels[1]. In addition to miR-181a overexpression studies, Li Q-J *et al*. also performed loss-of-function analyses using miR-181a antagomirs. Simulating thymocyte development *in vitro* using foetal thymic organ culture (FTOC) in the presence of miR-181a antagomirs and TCR antigens revealed a large reduction in negative selection and an impairment in positive selection. Upon antagomir treatment in DP thymocytes, both mRNA and protein levels of PTPN22, SHP-2, DUSP5 and DUSP6 were elevated, along with a decrease in ERK activation. Therefore miR-181a was identified as an intrinsic modulator of TCR sensitivity through targeting multiple negative regulators of TCR signalling. This study provided us with the *in vitro* framework for the mechanism behind miR-181a's involvement in thymocyte selection.

Another *in vitro* study that identified a number of endogenous positively selecting peptides in the thymus also provided evidence that miR-181a sets a threshold for selection in the thymus[320]. CD4SP T cells that matured in miR-181a antagomir treated foetal thymic organ culture (FTOC) and were subsequently incubated with APCs produced higher levels of proinflammatory cytokines and had higher expression of CD69, therefore showing a higher degree of self-reactivity. Furthermore, FTOC studies also showed that an elevated number of cells with moderate affinity to self-peptide developed from miR-181a antagomir treated pre-selection DPs in comparison to controls and these cells were

able to expand in the presence of their cognate ligand. From this it seems that miR-181a is necessary to tune the selection of TCRs that are reactive to self-pMHCs with moderate affinity[320].

A study published in 2015 by Schaffert *et al*. attempted to characterise the *in vivo* effect of miR-181a/b-1 on conventional T cell selection and therefore the downstream peripheral T cell pool but came up with some confusing results[4]. On the one hand, they demonstrated an increase in the intrinsic self-reactivity of naïve T cells from miR-181a/b-1-deficient mice due to alterations in thymic selection thresholds. On the other hand, miR-181a/b-1-deficient mice displayed no signs of spontaneous autoimmunity in the steady-state and even experienced a delayed induction of experimental autoimmune encephalitis (EAE) in comparison to controls. Upon immunization with MOG, T cells reactive for MOG from miR-181a/b-1-deficient mice were twice as reactive as WT mice, suggesting that a MOG-specific repertoire with increased reactivity escaped clonal deletion during selection. Analysis of MOG-specific CD4 T cells in the CNS and periphery of miR-181a/b-1-deficient mice revealed that CNS CD4 T cells were less activated than controls although being present in higher numbers in the periphery. This led researchers to hypothesise that miR-181a/b-1 also influenced

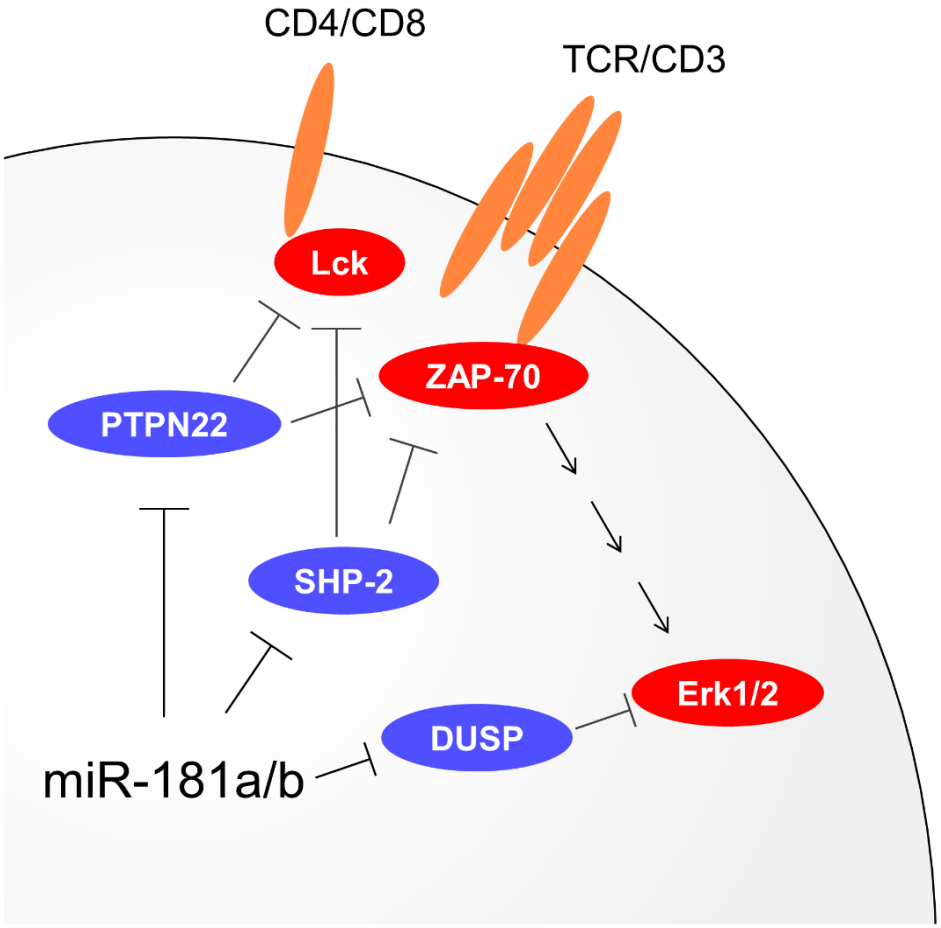


Figure 1.11 miR-181a/b-1 targets negative regulators of TCR signalling. miR-181a/b-1 has been shown to repress the phosphatases PTPN22, SHP-2, DUSP5 and DUSP6. These negative regulators of TCR signalling dephosphorylate multiple kinases downstream of the TCR and therefore dampen TCR signalling. Loss of miR-181a/b-1 results in the derepression of these phosphatases, resulting in more phosphorylation of the TCR signalling kinases and therefore decreased TCR signalling.

migration of cells through potential targeting of S1PR1 mRNA[4]. To make matters more complex, studies involving the onset of graft-vs-host disease (GvHD) showed that in the presence of elevated miR-181a, no GvHD was detectable[324]. Additionally, miR-181a/b-1-deficient mice exhibited a more rapid onset of GvHD[324]. This incongruity between models plus the alterations in selection and the self-reactivity of the peripheral T cell pool in miR-181a/b-1-deficient mice leaves open some unanswered questions, which we attempt to resolve in this thesis.

miR-181 has shown to be crucial in the development of iNKT cells. The mechanism behind miR-181's involvement in iNKT cell development has been controversial. A study published by Henao-Mejia et al. concluded that targeting of *Pten* by miR-181 was responsible for dramatic loss of iNKTs due to altered global metabolic fitness[325]. They showed that germline deletion of the miR-181a/b-1 cluster using an *E2a*-Cre, displayed elevated PTEN levels in the DN4 and DP subsets and a simultaneous reduction in PI3K signalling, leading to the dysregulation of thousands of genes[325]. Despite the fact that the largest increase in PTEN levels was seen in DN4 subsets of miR-181a/b-1-deficient mice compared to WT, total miRNA-deficiency studies showed that Dicer ablation both before and after the DN4 stage led to a similar iNKT phenotype[145,144]. By crossing miR-181a/b-1 mice to a *Cd4*-Cre, which depletes miR-181a/b-1 from the DP stage onwards, Henao-Mejia et al. also witnessed the same phenomenon, showing that the regulation over iNKT cell development by miR-181 begins at the DP stage or later. A rescue experiment involving the simultaneous loss of PTEN and miR-181a/b-1 also appeared to rescue the phenotype of miR-181 deficiency[325]. Henao-Mejia *et al.* therefore concluded that modulation of PTEN by miR-181a/b-1 was responsible for regulation of iNKT cell development.

Alternatively, researchers from our lab have provided evidence towards the existing hypothesis that miR-181 controls multiple negative regulators of TCR signalling and therefore iNKT cell development[1]. An additional group also saw that increased miR-181 led to decreased phosphatase activity[4]. Our mouse model developed by Ziętara et al. carries an ~250bp germline deletion of miR-181a/b-1[2]. This model shows the same gross phenotype as Henao-Mejia et al. 2013, with a massive reduction in the iNKT cell compartment, due to a block in the transition between stage 0 and 1, coupled with a reduced proliferation capacity. Ziętara et al. also observed a decreased capacity to transmit signals through the TCR, through $Ca^{2+}$ flux and a rescue of iNKT cell development through administration of supraphysiological levels of cognate α-GalCer ligand**[2]**. miR-181a/b-1-deficient iNKTs displayed an alteration in Vβ chain usage as well as altered effector subsets[2,3]. Vα-transgenic mice were shown to rescue the defect in iNKT cell development, which was also observed in Henao-Mejia et al. despite arriving at an alternate conclusion, and surprisingly transgenic TCR expression rescued both Vβ chain usage and effector subset differentiation. We saw slight but non-significant increased levels of PTEN in miR-181a/b-1-deficient mice on the protein level, which were not observed in Vα-transgenic miR-181a/b-1-deficient mice suggesting that the defect seen in iNKT cell development is most likely

a result of a defect in TCR signalling, with alterations in metabolic fitness being a minor or secondary effect[3]. Undoubtedly Henao-Mejia et al. provide evidence that metabolic fitness is altered in their model, but the discrepancy between mouse models (250bp vs 950bp deletion) is something that should be carefully considered before proceeding with future research. Henao-Mejia et al. also saw defects in peripheral T cell homeostatic proliferation and early B-cell development, which have not been reported in either of the additional two mouse models[4,2,3].

Certain populations of γδ T cells develop from strong TCR signals. It was therefore hypothesized that miR-181, a modulator of TCR signalling, may influence the development of γδ T cell subsets. γδ T cell development was therefore examined in the absence of miR-181a/b-1 and found to be largely dispensable[326,2]. Despite a relatively normal phenotype, an increase in liver γδ NKT cell department was observed, attributable to homeostatic expansion in the absence of αβ iNKT cells[326]. Interestingly, the pool of γδ NKTs in the liver of miR-181a/b-1-deficient mice showed a reduced capacity to secrete IFNγ, perhaps hinting to a yet undefined role of miR-181 in IFNγ regulation in $CD27^+$ γδ T cells. Recent data show that both miR-181a and miR-181d are upregulated in $CD27^+$ compared to $CD27^-$ cells, which hints towards their role in γδ T cell differentiation [326]. In regard to this, it is important to note that normal miR-181d expression levels are present in miR-181a/b-1-deficient mice and this could have an obscuring effect on 181a-deficiency.

# 2 Methods and Materials

## 2.1 Methods

### 2.1.1 Mice

miR-181a/b-1 knockout mice (B6.*Mirc14*$^{tm1.1Ankr}$; miR-181a/b-1$^{-/-}$; or 181$^{-/-}$) were generated as described in Zietara *et al*[2] and bred at the Zentrale Forschungseinrichtung (ZFE), Goethe University Frankfurt. C57BL/6N mice (WT) were purchased from Charles River Laboratories, Germany. *Rag1*$^{GFP}$ mice[327] were crossed to both miR-181a/b-1$^{-/-}$ and miR-181a/b-1$^{+/-}$ genotypes and bred at the ZFE, Goethe University Frankfurt. T-cell development in miR-181a/b-1$^{+/-}$ mice is comparable to WT littermates as described in Zietara *et al*[2]. R*ag*1$^{GFP}$ x miR-181a/b-1$^{+/-}$ mice carrying a homozygous insertion of *GFP* in the *Rag1* locus (*Rag1*$^{GFP/GFP}$) were treated as *Rag1*$^{-/-}$ x miR-181a/b-1$^{+/-}$ mice and were crossed to OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) obtained from The Jackson Laboratory. These mice, OT-I x *Rag1*$^{-/-}$ x miR-181a/b-1$^{+/-}$ (OT-I RAG1$^{-/-}$) were bred and housed at the ZFE, Goethe University Frankfurt. B6.SJL-*Ptprc*$^{a}$*Pepc*$^{b}$*/BoyJ* mice (CD45.1) and C57BL76N x *Ptprc*$^{a}$*Pepc*$^{b}$*/BoyJ* F1 mice (CD45.1/CD45.2 heterozygous) were bred at the ZFE, Goethe University Frankfurt. OT-I transgenic TCR mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) were also crossed with miR-181a/b-1$^{-/-}$ mice and bred at ZFE Frankfurt (OT-I 181$^{-/-}$). Foxp3$^{hCD2}$ × Rag1$^{GFP}$ mice were obtained by crossing Foxp3$^{hCD2}$ mice[228,328] with Rag1$^{GFP}$ mice[327]. These mice were crossed to miR-181a/b-1$^{-/-}$ and miR-181a/b-1$^{+/-}$ mice and bred the ZFE. *Rag1*$^{-/-}$ mice and B6 CD45.1 used in *in vivo* suppression assay were maintained at the Hannover Medical School and Goethe University Frankfurt. All mice were used between 7-16 weeks of age and maintained under specific pathogen-free conditions. All experiments were performed in accordance to German law on care and use of laboratory animals and have been approved by the Regierungspräsidium Darmstadt, Abteilung Veterinärwesen.

### 2.1.2 Flow cytometry

Phycoerythrin (PE)- and allophycocyanin (APC)-conjugated MR1-5-OP-RU and MR1-Ac-6-FP tetramers as described in Reantragoon *et al*[201] and APC-conjugated CD1d-PBS-57 and CD1d-unloaded tetramers as described in Liu *et al*[329], were provided by the NIH Tetramer Core Facility. Cells were analyzed using a BD FACSCanto II and data was processed using FlowJo software (Treestar). Dead cells were removed from analysis using the Zombie Aqua Fixable Viability kit as per manufacturer's instructions and doublets were excluded. For all panels used to identify MAIT cells, B220$^{+}$ CD19$^{+}$ CD11b$^{+}$ CD11c$^{+}$ NK1.1$^{+}$ cells (Lin$^{+}$) were excluded. MAIT cells were defined as Lin$^{-}$ TCRβ$^{+}$ MR1-5-OP-RU$^{+}$ cells. For all panels used to identify iNKT cells, B220$^{+}$ CD19$^{+}$ CD11b$^{+}$ CD11c$^{+}$

cells ($Lin^+$) were excluded. iNKT cells were defined as $Lin^-TCR\beta^+CD1d\text{-}PBS\text{-}57^+$ cells. All experiments were analysed on a BD FACSCanto II.

### 2.1.3 Dual tetramer staining

To quantify absolute numbers, samples were stained simultaneously with APC-MR1-5-OP-RU and PE-MR1-5-OP-RU tetramers or APC-MR1-5-OP-RU and PE- MR1-Ac-6-FP for 40 mins at room temperature, followed by surface antibody staining and flow cytometric analysis.

### 2.1.4 Cell viability staining

Live/dead cells were discriminated using Zombie Aqua dye (BioLegend) in all flow cytometric experiments. As a final stage before fixation or analysis, cells were washed in PBS to removed traces of FCS. Samples were then stained with Zombie Aqua dye in PBS for 15 mins at room temperature in the dark. Cells were then washed with PBS (when proceeding to fixation and intracellular staining) or FACS buffer when proceeding to flow cytometric analysis.

### 2.1.5 Absolute number determination

Absolute number determination was performed by harvesting lymphoid organs into a single cell suspension and counting the total cell number of each. Lymph node counts represent the 6 axillary, brachial, and inguinal nodes. For spleen lymphocyte counts, RBCs were lysed first (see Cell preparations). Percentages of gated lymphoid populations, analysed via flow cytometry were then used to calculate the average number of cells, per population, per organ.

### 2.1.6 Magnetic-bead enrichment of MAIT cells

For analysis of MAIT cells, organs pooled from 3 mice were homogenised into single-cell suspensions. Cells were then stained with biotinylated CD19 and B220 antibodies. B cells were then depleted using streptavidin microbeads as per the manufacturer's instructions (Miltenyi Biotec). Cells were then stained with Fc block on ice for 10 mins followed by APC- or PE-MR1-5-OP-RU tetramers for 40 mins at room temperature. Magnetic-bead enrichment was performed using Miltenyi Biotec anti-APC or anti-PE microbeads as per the manufacturer's instructions. One independent enriched sample therefore represents organs pooled from three mice. Following isolation, MAIT cells were stained with surface or intracellular markers and analysed via flow cytometry. To identify intrathymic MAIT cell development stages, CD24 and CD44 surface antibodies are required. We discovered that CD24 antibodies will not stain in the presence of EDTA. Considering most MAIT cell isolation procedures require Miltenyi Biotec MR1-tetramer-based enrichment, which suggests the use of buffers containing EDTA to prevent cell clumping, we recommend either multiple washing steps after

MAIT cell isolation (and before CD24 staining) with FACS buffer (PBS + 3% FCS) to remove the EDTA, or simply using de-gassed FACS buffer for the whole enrichment and subsequent staining procedures.

### 2.1.7 Cell preparations

Single-cell suspensions of whole lymphoid organs (thymus, spleen, lymph nodes) were obtained by crushing organs through a 70µm filter. For all experiments, only the axillary, brachial and inguinal lymph nodes were harvested. Red blood cells (RBCs) were lysed (spleen only) using Qiagen RBC Lysis Solution for 10 mins on ice with intermittent vortexing. For lymphocyte isolation from the lung and liver, mice were euthanized, and liver/lungs were immediately perfused with phosphate-buffered saline (PBS). For cell samples isolated from all organs except thymus, Fc receptors were blocked for 15 mins on ice before proceeding with antibody staining.

### 2.1.8 Mouse lung and liver perfusion

To remove circulating blood cells from organs of interest the circulation must be perfused. To specifically perfuse liver and lung samples mice must be euthanized and the chest cavity opened immediately. Scissors are then used to cut open the right atrium and 10mL of PBS is injected into the left ventricle. As the blood is removed from the lungs and liver the organs should become pale. Successfully perfused organs can be harvested and used form downstream lymphocyte isolation procedures.

### 2.1.9 Lung lymphocyte isolation

To isolate lymphocytes from the lung, the circulation is perfused, and lungs are harvested. Lungs are then placed into a petri dish and roughly chopped into small pieces with a scalpel. Lung pieces are then transferred into a MACS C tube with 4mL of 10mM HEPES, 1mL of Collagenase D stocks (final conc. 2mg/mL) and 20µL of DNase I stocks (final conc. 80U/mL). Using a genetleMACS Dissociator (Miltenyi Biotec), the MACS program m_lung_01_02 is run. Samples are then incubated for 30 mins in a 37°C shaking incubator. After incubation the MACS program m_lung_02_01 in run. Samples are then centrifuged and resuspended in FACS buffer. Cell suspensions are filtered through 70µM mesh before tetramer and surface antibody staining and downstream flow cytometric analysis.

### 2.1.10 Liver lymphocyte isolation

Lymphocyte isolation from the liver was carried out using the Liver Dissociation Kit (Miltenyi Biotec). To begin, livers were perfused and harvested. Gallbladders were resected, and connective tissue removed before dicing the liver into small pieces and transferring into a MACS C Tube containing a

dissociation mix buffer (provided by company). Using a genetleMACS Dissociator (Miltenyi Biotec), the gentleMACS Program m_liver_03 was run. Samples were then incubated for 30 mins at 37°C. After incubation, the gentleMACS Program m_liver_04 was run. Tubes were then detached, and the digested sample were strained through a 100µM filter. Samples were washed and then stained with tetramers and surface antibodies for downstream applications flow cytometry analysis.

### 2.1.11 iNKT cell isolation

iNKT cells were stained with Fc block for 10 mins on ice followed by staining with APC-CD1d-PBS-57 tetramers for 30 mins at room temperature. Following tetramer staining, cells were stained with surface antibodies to further identify iNKT cells. For all panels used to identify iNKT cells, $B220^+$ $CD19^+$ $CD11b^+$ $CD11c^+$ cells ($Lin^+$) were excluded. iNKT cells were defined as $Lin^-TCR\beta^+$CD1d-PBS-$57^+$ cells.

### 2.1.12 Ki67 staining

To detect the proliferation marker Ki67, samples were washed with PBS and 3mL of -20°C ethanol was added dropwise to the cell pellet whilst vortexing. Samples were then incubated at -20°C for 1 hour. Cells were then washed with FACS buffer (PBS + 3% FBS) and incubated with Ki67 antibody for 30 mins at room temperature.

### 2.1.13 Intracellular staining

To analyse transcription factor or intracellular protein expression in lymphocytes, cells were isolated and stained for surface markers and viability. Samples were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Samples were incubated in Fix/Perm buffer for 30 mins at room temperature. Cells were then washed twice in 1x Permeabilization buffer (eBioscience) before being stained overnight at 4°C with intracellular antibodies in 1x Permeabilization buffer. To preserve GFP staining in samples, an alternative intracellular staining method was used. Cells were fixed in 2% paraformaldehyde for 10 mins at room temperature. Samples were then washed twice with 1x Permeabilization buffer (from eBioscience Foxp3/Transcription Factor Staining Buffer Set) and then stained overnight at 4°C in the dark.

### 2.1.14 Generation of retrovirus encoding the Vα19Jα33 TCRα chain

Retroviral transduction was used for stable expression of a prearranged Vα19Jα33 TCR in mouse T cells. To generate this construct, we subcloned the Vα19Jα33 TCR sequence into the *SalI-ClaI* site of the MSCV-based retroviral vector, MigR1[330]. The empty vector MigR1 served as a control and an enhanced GFP (eGFP) served as a reporter gene. Functional Vα19Jα33 cell surface expression was

validated by transducing the mouse hybridoma cell line BW58α-β+ and confirming cell surface presence of TCRβ by surface antibody staining and flow cytometry.

### 2.1.15 TCRα overexpression BM chimeras

Bone marrow was isolated from the femur and tibia of miR-181a/b-1$^{-/-}$ (CD45.2) and WT (CD45.2). On day 1 lineage negative cells were depleted using the Lineage Cell Depletion kit, as per manufacturer's instructions (Miltenyi Biotec). Lineage negative cells were cultured overnight at a density of 1 x $10^6$ cells/mL (24-well plate) in DMEM containing 10% FBS, mouse IL-6 (20 ng/ml), mouse IL-7 (25 ng/ml), mouse Flt-3L (25 ng/ml) and mouse SCF (50 ng/ml; all obtained from PeproTech). On day 2, cells were transduced by adding Vα19Jα33-encoding or control retrovirus supernatant supplemented with polybrene (8 µg/ml) and centrifuging at 700 *g* for 1.5 hours at room temperature. Cells were further incubated with virus in a 37°C incubator before virus containing medium was removed and replaced with fresh medium as described for day 1. After 48 hours transduction efficiency was determined via GFP expression. CD45.1 or CD45.1/CD45.2 recipient mice were then lethally irradiated (9 Gy) and cells were intravenously injected (5 x $10^5$ per recipient). Thymi and spleen samples were analyzed 8 weeks after transfer. Each sample was individually enriched for MR1-5-OP-RU$^+$ MAIT cells, as described above (without B cell depletion).

### 2.1.16 Competitive BM chimeras

To generate competitive mixed BM chimeras, BM was isolated, and lineage negative cells were depleted and cultured as described in (2.1.15 TCR overexpression BM chimeras). Immediately prior to injecting into lethally irradiated recipients, bone marrow from either WT of miR-181a/b-1-deficient mice was mixed in a 1:1 ratio. BM mixtures were then injected into WT recipients and left to reconstitute for 8 weeks.

### 2.1.17 Multiplexing flow cytometry samples

Due to weak antibody staining or only slight differences in expression between WT and KO cells, some samples were multiplexed to account for small differences in staining intensities between these groups. To do so, WT and KO samples were harvested separately and then one of the samples was stained for a congenic marker (e.g., CD45.2). After washing, WT and KO samples were then mix and stained for additional markers within the same tube. Upon flow cytometric analysis, WT and KO samples could be distinguished by their expression of stained congenic markers.

### 2.1.18 RNA flow cytometry

RNA flow cytometry was performed using the PrimeFlow RNA Assay (Invitrogen). For each sample, between 1-5 x $10^6$ cells were resuspended and stained with surface antibodies and viability dye. Samples were washed once in FACS buffer and the fixed in PrimeFlow RNA Fixation Buffer 1 for 30 mins at 4°C. Samples were washed twice with 1x PrimeFlow RNA Permeabilization Buffer with RNase Inhibitors and then resuspended in 1x PrimeFlow RNA Fixation Buffer 2 for 60 mins at room temperature. Fixed samples were then washed twice with PrimeFlow RNA Wash Buffer followed by Target Probe hybridization for 2 hours at 40°C. Samples were washed once more with PrimeFlow RNA Wash Buffer and then PreAmp hybridization was performed for 1.5 hours at 40°C. Samples were washed 3 times with PrimeFlow RNA Wash Buffer before Amp hybridization for 1.5 hours at 40°C. Samples were again washed twice with PrimeFlow RNA Wash Buffer and Label Probe hybridization was performed at 40°C for 1 hour. Samples were then washed twice with PrimeFlow RNA Wash Buffer before being washed once in FACS buffer and analysed on the flow cytometer.

### 2.1.19 Treg enrichment

Regulatory T cells were enriched using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Cells harvested from thymi, spleen or lymph nodes were homogenised and filtered through a 30µM filter. Filtered cells were then incubated with MACS $CD4^+CD25^+$ Regulatory T Cell Biotin Antibody Cocktail (Cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8a, CD11b, CD45R, CD49b, and Ter-119) and incubated for 10 mins at 4°C. Following incubation, samples were incubated with Anti-Biotin Microbeads and CD25-PE antibodies for 15 mins at 4°C. After 15 mins, magnetically labelled samples were loaded onto a LD MACS column and non-$CD4^+$ T cells were depleted. After flushing column with buffer, the unlabelled flow through cells were collected, centrifuged and stained with Anti-PE MicroBeads for 15 mins at 4°C. Samples were then directly applied to a MACS LS column and the unlabelled flow through fraction was discarded. LS columns were removed from the magnetic field and the labelled fraction was purged by plunging 5mL of MACS buffer through the column. These cells contained the enriched $CD4^+CD25^+$ fraction and were used for downstream high purity cell-sorting.

### 2.1.20 Treg protein degradation assay

To assess CTLA-4 protein degradation in Tregs, Tregs were first enriched from spleens and lymph nodes of miR-181a/b-$1^{+/-}$ and miR-181a/b-$1^{-/-}$ mice. In order to monitor protein degradation cycloheximide (CHX) was added and incubated with cells for 15, 30, 60, and 120 mins at 37°C. Samples were then washed and stained with surface antibodies. Stained samples were fixed and then stained for intracellular CTLA-4 and analysed via flow cytometry.

### 2.1.21 *In vitro* Treg stimulation assay

Enriched Tregs from spleens and LNs of miR-181a/b-1$^{+/-}$ and miR-181a/b-1$^{-/-}$ mice were stimulated for 30, 60, 120 and 180 mins at 37$^{o}$C in the presence of plate-bound αCD3 (17A2, 2.5µg/ml) and soluble αCD28 (37.51, 2.5µg/ml). Samples were then stained with surface antibodies, fixed and then stained for intracellular CTLA-4 and analysed via flow cytometry.

### 2.1.22 Inhibition of lysosomal degradation in Treg cells

CD4$^{+}$CD25$^{+}$ Treg cells were enriched from spleens and LNs of miR-181a/b-1$^{+/-}$ and miR-181a/b-1$^{-/-}$ mice using MACS isolation kit (Miltenyi Biotec). Cells were incubated in the absence or presence of 50nM Bafilomycin (InvivoGen) and collected after 30, 60, 120 and 180 mins at 37°C. Samples were then stained with surface antibodies, fixed and then stained for intracellular CTLA-4 and analysed via flow cytometry.

### 2.1.23 Naïve CD4 T cell isolation

Naïve CD4 T cells were isolated via MACS enrichment. Cell suspensions from combined spleen and lymph nodes were stained with Biotin-Antibody Cocktail (containing antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, Ter-119, and TCRγδ) and stained for 5 mins at 4°C. After incubation samples were simultaneously stained with Anti-Biotin MicroBeads and CD44 MicroBeads for 10 mins at 4°C. Cells were then washed with MACS buffer and loaded onto a LS MACS column. The unlabelled flow through was collected as the enriched naïve CD4$^{+}$ T cell fraction and used for downstream *in vivo* suppression assays.

### 2.1.24 CellTrace Violet labelling

CellTrace Violet (CTV) reagent was diluted with DMSO to make a 5µM stock solution. Cells were washed in PBS to remove all traces of buffer-derived proteins. Samples were warmed to 37°C and 1µL of CTV was added whilst gently vortexing to each 1mL of cell suspension. Samples were then incubated for 37°C for 5 mins. Following incubation, a minimum of 5x the total sample volume of FCS containing buffer (FACS buffer) was added to the sample and immediately centrifuged. Labelled cells were then used for downstream *in vivo* suppression assays.

### 2.1.25 *In vivo* suppression assay

To analyse the function of Treg cells *in vivo*, enriched populations of naïve CD45.1$^{+}$ (4x10$^{5}$ cells) CD4 T cells were stained with CTV proliferation dye and then mixed in a 2:1 ratio with sorted CD45.2$^{+}$ miR-181a/b-1$^{+/-}$ or miR-181a/b-1$^{-/-}$ Treg cells (2x10$^{5}$). Cell mixtures were then injected intravenously into Rag1$^{-/-}$ recipients and spleen/lymph nodes were harvested and analysed after 5 days. Before

analysis of T cell subsets, lineage$^+$ cells were removed by using a B220$^+$CD11b$^+$CD11c$^+$CD19$^+$ dump channel.

### 2.1.26 Peptide loading assay

T2K$^b$ APCs were incubated with peptides at various concentration for 2 hours at 37°C. Pre-selection DP cells (CD4$^+$CD8α$^+$CD69$^-$CD62l$^+$) from OT-I TCR transgenic mice (from miR-181a/b-1-sufficient or deficient backgrounds) were sorted via FACS and added in a 2:1 ratio to peptide pulsed APCs. Cell mixtures were then incubated overnight at 37°C. Following incubation cells were washed and stained with surface antibodies and then analysed via flow cytometry.

### 2.1.27 Cleaved caspase-3 staining

To stain for caspase-3, cell suspensions were washed and resuspended in 1mL of cold PBS. For fixation, 2mL of 4% PFA was added dropwise whilst vortexing (to avoid clumping) and cells were incubated for 20 mins at room temperature. Samples were then washed twice with PBS to remove traces of PFA, before being washed in 1mL of IFA-Tx buffer (4mL FBS, 10mL HEPES, 100µL Triton X-100, 0.1µg Sodium Azide and 86mL sodium chloride (0.9%)). Cell pellet was resuspended in primary antibody mix and incubated for 30 mins at room temperature. Cells were then washed with IFA-Tx buffer and incubated with secondary antibody mix for 30 mins at room temperature. Cells were washed a final time, before being analysed via flow cytometry.

### 2.1.28 Cell lines

The T2K$^b$ cell line was used as an APC during peptide loading assays. T2K$^b$ cells were first described in EZ Wolpert et al.[331]. T2K$^b$ cells are derived from the transporter associated with antigen processing (TAP)-deficient cell line T2 and are unable present endogenous antigens. The T2 cells were transfected with the MHC class-I molecule H2-K$^b$ (K$^b$) to generate a cell line that is only able to present exogenous peptides on the surface of MHC Class-I.

### 2.1.29 Small RNA-seq

Enriched Treg cells (1x10$^5$) sorted from 3 pooled WT and miR-181a/b-1$^{-/-}$ thymi were stored in RNAprotect Cell Reagent (Qiagen). SmallRNA sequencing was performed by Admera Health (South Plainfield, NJ, USA) using the SMARTer smRNA-Seq Kit (Takara). Adapters were trimmed with Flexbar 3.4 and rRNA was removed using Bowtie2. The remaining reads were aligned using STAR aligner and counted using HTSeq. Differential expression analysis was performed in R using the DESeq2 package. Three biological replicates for each genotype were analysed. Bioinformatics analysis was performed by Nikita Verheyden.

### 2.1.30 Cell sorting

Fluorescent activated cell sorting (FACS) was used to isolate cell populations with high purity. First cells were harvested and staining with surface antibodies (no live/dead discrimination or intracellular staining). Cells were then sorted for >95% purity on a BD Aria Fusion. Cells were sorted into sterile FACS buffer and then used for downstream assays or RNA-Seq analysis.

### 2.1.31 Thymic slices

To generate thymic slices, thymi from mice were gently harvested and the two thymic lobes were separated. Individual lobes were dried on paper towel and embedded in tissue molds containing molten low-melt agarose (2% low melt agarose in HBSS). Thymi-containing agarose blocks were then sliced into 400µM thick slices using a vibratome set to 0.225 mm/sec speed, 100 Hz frequency, and 5° angle and RPMI-10% media bath cooled to -4°C. Using a bent spatula agarose-thymi slices were collected from the media bath and transferred onto a cell culture insert in a 6-well plate containing RPMI-10% media. Peptides were then resuspended in RPMI-10% at desired concentration and overlaid onto thymic slices in a volume of no more than 8µL and incubated at 37°C for 4 hours. After incubation remaining peptides were washed away with media and sorted pre-selection DP cells were overlaid onto slices in a volume of no more than 8µL. After 24-48 hours, migration of congenically labelled sorted cells into the slice can be analysed by mashing the agarose-thymi section through a 70µM filter, stained with surface antibodies and then analysed via flow cytometry.

### 2.1.32 cDNA synthesis

Total RNA samples were incubated with dNTPs and random hexamer primers for 5 mins at 65°C then placed directly on ice. M-MLV buffer and the RNase inhibitor RNAsin was then added to samples and incubated for 2 mins at room temperature before transferring samples back onto ice. Solutions were then halved and separated into 2 tubes. One tube received M-MLV Transcriptase and one received $H_2O$ (non-reserves transcribed control). Samples were briefly mixed and then run for the following program: 25°C for 10 mins, 42°C for 50 mins and 7°C for 15 mins in a PCR machine, before placing back on ice or storing at -20°C.

### 2.1.33 Chromosome walking and RT-qPCR

Total RNA was isolated from thymi using the NucleoSpin RNA isolation kit (Machery Nagel) as per manufacturer's instructions. Reverse transcription was then performed as described (2.1.32 cDNA synthesis). Primer pairs were designed using Primer3 (v 0.4.0) at homologous regions upstream and downstream of the mature miR-181a/b-1 sequences on mouse chromosome 1. SYBR Green RT-qPCR was then performed using each primer pair. cDNA template was mixed with a forward and

reverse primer from each primer pair as well as 2x SYBR Green PCR Master Mix and analysed using a QuantStudio RT-qPCR machine (ThermoFischer). Fold differences were calculated using the $\Delta C_t$ method and either normalised to the 'middle' primer pair (between miR-181a-1 and b-1 transcripts) or to TATA sequence binding protein (TBP) in the case of comparing WT and miR-181a/b-1-deficient RNA. All RT-qPCR samples were run alongside -RT and NTC controls to detect inefficient reverse transcription or contamination.

### 2.1.34 **5' RACE**

5' Rapid amplification of cDNA eds (RACE) was performed using the 5´ RACE System for Rapid Amplification of cDNA Ends (ThermoFischer). First strand cDNA was synthesised from total RNA using a gene specific primer downstream of the putative TSS and Superscript II Reverse Transcriptase (Invitrogen). mRNA was then digested using an RNase Mix (including RNase H to remove RNA:DNA heteroduplexs and RNase T1). Unincorporated dNTPs, primers, and proteins were then separated from cDNA using a SNAP Column. A homopolymeric tail was then added to the 3'-end of the cDNA using TdT and dCTP. Samples were then amplified using Taq DNA polymerase and a second nested gene specific primer that anneals to a site before the TSS within the generated cDNA molecule, in conjunction with a deoxyinosine-containing anchor primer. Following amplification, products were visualised on an agarose gel, purified and cloned into a sequencing TOPO TA vector. Vectors were then sent for sequencing at SeqLab (Germany) and insert fragments represented experimentally validated TSS.

### 2.1.35 **Statistical analysis**

All analysis was performed using GraphPad Prism software (version 7). Data are represented as mean plus or minus SD. When no *P*-value is indicated, samples were not significant.

## 2.2 Materials

### 2.2.1 Antibody list

| Antibody | Clone | Company |
|---|---|---|
| CD4 | GK1.5 | Biolegend |
| CD5 | 53-7.3 | eBioscience |
| CD8a | 53-6.7 | Biolegend |
| CD11b | M1/70 | eBioscience |
| CD11c | N418 | eBioscience |
| CD19 | 6D5 | Biolegend |
| CD24 | M1/69 | Biolegend |
| CD25 | PC61.5 | eBioscience |
| CD44 | IM7 | Biolegend |
| CD45.1 | A20 | eBioscience |
| CD45.2 | 104 | Biolegend |
| CD45R (B220) | RA3-6B2 | Biolegend |
| CD62L | MEL-14 | eBioscience |
| CD69 | H1.2F3 | Biolegend |
| CD152 (CTLA-4) | UC10-4B9 | eBioscience |
| CD197 (CCR7) | 2B8 | eBioscience |
| CD279 (PD-1) | J43 | eBioscience |
| Foxp3 | FJK-16s | eBioscience |
| Ki67 | 16A8 | BioLegend |
| NK1.1 | PK136 | BioLegend |
| Nur77 | Klon 12.14 | eBioscience |
| PLZF | Mags.21F7 | BioLegend |
| RORgT | B2D | eBioscience |
| T-bet | O4-46 | eBioscience |
| TCRß | H57-597 | BioLegend |
| Cleaved caspase-3 | Asp175 | Cell signalling |
| MR1 5-OP-RU | | NIH Tet Core Facility |
| MR1 6-FP | | NIH Tet Core Facility |
| CD1d PBS-57 | | NIH Tet Core Facility |
| CD1d unloaded | | NIH Tet Core Facility |

### 2.2.2 Primer list for chromosome walking

| Primer Name | Sequence (5' →3') | Position on Chr1 | Product Size (bps) |
|---|---|---|---|
| Primer 1 FOR | GAGGCAGAGATGGAGTTAGGTAA | 137 889 093 | 147 |
| Primer 1 REV | GCAAAGTCTCAATGGCTAGAACT | 137 889 239 | 147 |
| Primer 2 FOR | TTTGCCCTGAATAAACCATGTGT | 137 894 747 | 227 |
| Primer 2 REV | GCACACTACATACAAAGCCTCTT | 137 894 973 | 227 |
| Primer A FOR | TCCTACAGTCATTCTTCCACACA | 137 898 457 | 131 |
| Primer A REV | ATCCTATTGGCCACAGAACCATA | 137 898 588 | 131 |
| Primer 3 FOR | TTCCGAGTGACTTTAGGAAATGC | 137 898 641 | 224 |
| Primer 3 REV | CAAATCTTCGAGTATTTGGGGCT | 137 898 864 | 224 |
| Primer 4 FOR | CCCTTTCATTGCCTTTAAAACCG | 137 900 481 | 150 |
| Primer 4 REV | CCTCAAATATTCTGCCAGGATCC | 137 900 630 | 150 |
| Primer 5 FOR | TTGTGGTTGAGATTCCCATTCTG | 137 905 429 | 172 |
| Primer 5 REV | AGAGTCATTGCTACGTTTCACAG | 137 905 600 | 172 |
| Primer 6 FOR | TAGGGAACATGACGAATCTGTCT | 137 911 603 | 201 |
| Primer 6 REV | CTCTCTGTTGCTTTGAAACTTGC | 137 911 803 | 201 |
| Primer 7 FOR | GTGACAAACCCAAGTATGAGAGG | 137 917 735 | 166 |
| Primer 7 REV | CAAGGGGAAACGGCTACTTATTT | 137 917 900 | 166 |
| Primer 8 FOR | GCTCTTGTTATGAGGCAGACAAT | 137 923 491 | 168 |
| Primer 8 REV | GCCCTGCACTGAACTAACAATTA | 137 923 658 | 168 |
| Primer 9 FOR | CTGAGGGGAAAGGATGTGAGATA | 137 929 615 | 151 |
| Primer 9 REV | GAAGAACACACACCCATAGCTAC | 137 929 765 | 151 |
| Primer 10 FOR | AAGCTGGTAGTGATTTCGATTGG | 137 935 873 | 124 |
| Primer 10 REV | TTTTCTGTGTCGTAGTCTCAAGC | 137 935 960 | 124 |
| Primer 11 FOR | GAGAAGAGCAGTGAACTACGAAG | 137 941 865 | 115 |
| Primer 11 REV | AAGAGCCTAGACTCTGAACACAT | 137 941 979 | 115 |
| Primer 12 FOR | CACACACACAGCCAAATCTAGAT | 137 947 167 | 177 |
| Primer 12 REV | GTTGATGGAATGACTAGGGTTGG | 137 947 343 | 177 |
| Primer 13 FOR | GCTCTAGGTTCTTCTCTGGGTTA | 137 953 115 | 141 |
| Primer 13 REV | ACTTTCCATTTCCACAAAGCTGA | 137 953 255 | 141 |
| Primer 14 FOR | AGTCACTCTAGCCTTCACAAGAT | 137 959 061 | 215 |
| Primer 14 REV | TGACCTGTTGCCTAATGATCCTA | 137 959 275 | 215 |
| Primer F FOR | ATCAGGAATAATCACCGTCCTCA | 137 961 705 | 127 |
| Primer F REV | CGGTGATGGCTACGTAAAAGTAA | 137 961 832 | 127 |
| Primer 15 FOR | CTGGCCTGAATATGATAGTGCAG | 137 965 070 | 151 |
| Primer 15 REV | GTGGCTTCCTTTGAGATAGCTTT | 137 965 220 | 151 |
| Primer G FOR | GTTTCTGCTTGATTCTGCATGAC | 137 966 161 | 230 |

| Primer Name | Sequence (5' →3') | Position on Chr1 | Product Size (bps) |
|---|---|---|---|
| Primer G REV | GCTGTGTCAAAGAAAAGAATCGG | 137 966 391 | 230 |
| Primer H FOR | CTCTCTCCCAATTGTGCAGATTT | 137 968 111 | 171 |
| Primer H REV | ATCAATGAAATAGAGGCCAGTGC | 137 968 282 | 171 |
| Primer I FOR | CAAGTCAAATTCTGGGCAATTGG | 137 969 728 | 159 |
| Primer I REV | GGCTTGAAACAAAACACAGCAAT | 137 969 887 | 159 |
| Primer 16 FOR | AGCAAAATGACAGTGTGACAAGA | 137 971 555 | 115 |
| Primer 16 REV | CATCAGGGTGCAGATCTGAATTT | 137 971 669 | 115 |
| Primer 17 FOR | ATGTCTGCAGCCTCTAACTATCA | 137 977 155 | 180 |
| Primer 17 REV | ATTAACTGCCGGCTTTGTGAATA | 137 977 334 | 180 |
| Primer J FOR | GCATACCAAAGACACAGAAGACA | 137 984 713 | 241 |
| Primer J REV | AAGAGTTGCCTAGGTCATGGTAT | 137 984 954 | 241 |
| Primer C FOR | GAGAGCTGAAACAAATGACTCGA | 137 989 138 | 230 |
| Primer C REV | AAAAGACTGGGCTTATGATCTGC | 137 989 368 | 230 |
| Primer D FOR | GCTGTATGTTCTAGGCCAATGTT | 137 995 627 | 230 |
| Primer D REV | GCATTTTCTTGTTGAGCTTCCAG | 137 995 857 | 230 |
| Primer E FOR | AGGTAGAGTGTCCCTGGAAATAG | 138 001 351 | 150 |
| Primer E REV | CTGAGGACGCTCTGAAACATATC | 138 001 501 | 150 |
| Primer K FOR | GCAAAAGAAGGCTCAATGTGAAG | 138 006 859 | 228 |
| Primer K REV | TTTCCAAGAAGTGAGAGGAGACA | 138 007 087 | 228 |
| Primer L FOR | GAGCGTTCCCATGAAGAAAGTAA | 138 008 103 | 191 |
| Primer L REV | TTCAAATCCCAACCTCTGAGAGA | 138 008 294 | 191 |

# 3 Results Chapter One

## 3.1 MAIT cell development & miR-181a/b-1

### 3.1.1 Thymic MAIT cell development critically depends on miR-181a/b-1

MAIT cells are exceptionally rare in commonly used BL6 strains of mice. Enriching for MAIT cell populations using magnetic beads and MR1-tetramer technology is standard for identification but this method is counterintuitive when determining absolute cell counts. To accurately quantify MAIT cell numbers in mice, we required ample controls to distinguish genuine signals from background noise. All samples were stained with a cocktail of lineage markers to exclude granulocytes, monocytes/macrophages, dendritic cells, NK cells and B cells. Following exclusion of non-MAIT cell lineage markers, we adopted a dual-colour tetramer staining procedure. In doing so, we were able to compare true MAIT cells stained simultaneously with MR1-5-OP-RU in two different fluorophores (APC and PE) as well as MAIT cells stained with MR1-5-OP-RU alongside MR1-Ac-6-FP, a currently accepted negative control. Bona fide MAIT cells were double positive for both MR1-5-OP-RU tetramers and negative for MR1-Ac-6-FP (Figure 3.1a). This allowed us to determine MAIT cell numbers and frequencies in thymi of miR-181a/b-1$^{-/-}$ mice compared to WT controls.

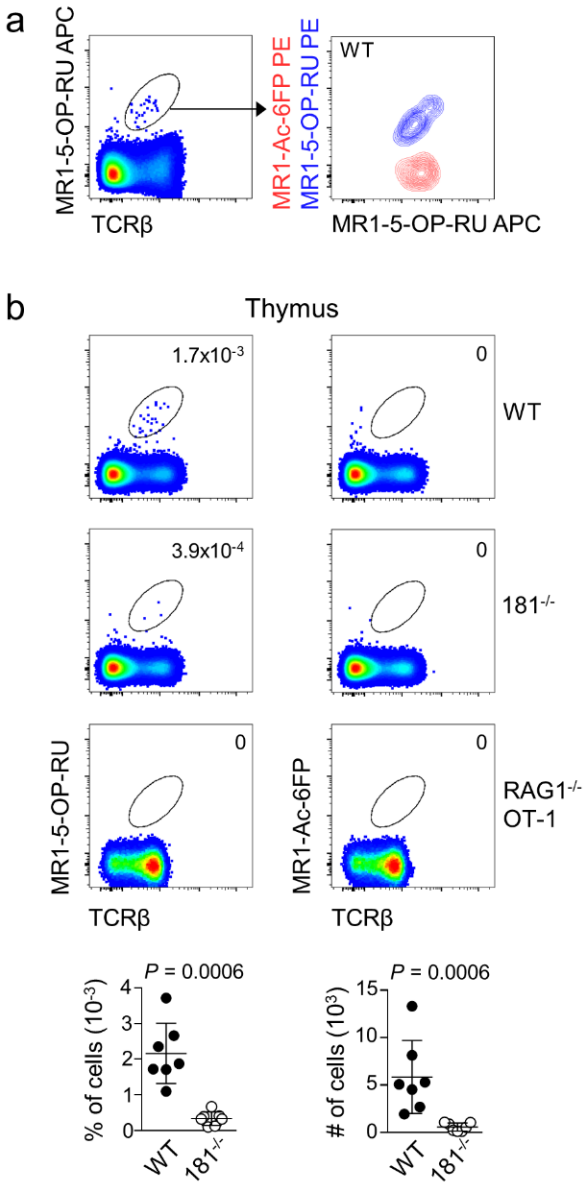


**Figure 3.1 miR-181a/b-1 controls MAIT cell development in the thymus. (a)** Dual-colour tetramer staining to accurately quantify MAIT cells. To control for tetramer staining specificity, dual-tetramer staining was performed in WT mice using MR1-5-OP-RU-APC alongside either MR1-5-OP-RU-PE (blue) or MR1-Ac-6FP-PE (red). **(b)** Identification of MAIT cells in thymus of C57BL/6N (WT), miR-181a/b-1$^{-/-}$ (181$^{-/-}$) and *Rag1*$^{-/-}$OT-I mice. The left column shows thymocytes stained with the MR1-5-OP-RU tetramer (MAIT cells) and the right column shows thymocytes stained with the MR1-Ac-6-FP tetramer (negative control). Percentages of MAIT cells within total thymocytes are indicated in the top right corner of each plot. Frequency and absolute numbers of MR1-5-OP-RU$^{+}$ TCRβ$^{+}$ MAIT cells in total thymocytes of WT and 181$^{-/-}$ mice are graphed (bottom). Data are representative of two independent experiments, with a total of n=7 for each genotype. Each symbol represents an individual mouse and error bars indicate SD. Statistical analyses were performed using Mann–Whitney rank-sum *U*-tests.

Consistent with previous reports, control thymi from C57BL/6 mice contained approximately 6,000 MAIT cells, corresponding to a frequency of 0.002% (Figure 3.1b)[146]. Thymi from miR-181a/b-1$^{-/-}$ mice on the same genetic background contained 10-fold lower numbers and frequencies of MAIT cells (Figure 3.1b). Negative control MR1-Ac-6-FP tetramers did not stain thymic MAIT cells from miR-181a/b-1$^{-/-}$ mice or controls (Figure 3.1b). *Rag1*$^{-/-}$ OT-I-TCR mice have a fixed transgenic OT-I Vα chain whilst synchronously lacking RAG1, an essential protein for V(D)J recombination in non-transgenic cells[332–334]. These mice are therefore not able to undergo Vα19Jα33 TCRα chain rearrangement and thus develop no MAIT cells. To further confirm MR1-5-OP-RU tetramer staining specificity, MAIT cells were not detected in thymi from *Rag1*$^{-/-}$ OT-I-TCR transgenic mice (Figure 3.1b). Together, these controls indicate that despite their scarcity it was possible to accurately determine MAIT cell numbers in thymi of miR-181a/b-1$^{-/-}$ mice. Our data show that intrathymic development of MAIT cells is dependent upon miR-181a/b-1.

### 3.1.2 Reduced peripheral MAIT cell pool in miR-181a/b-1$^{-/-}$ mice

Next, we tested whether loss of central MAIT cells in miR-181a/b-1$^{-/-}$ thymi extended to peripheral organs. To facilitate detection of MAIT cells, MR1-5-OP-RU tetramer$^{+}$ cells were enriched using magnetic microbeads. Enrichment did not massively alter the relative difference in frequencies of MAIT cells in thymi from miR-181a/b-1$^{-/-}$ mice compared to controls and if anything, would understate the reduction observed in miR-181a/b-1$^{-/-}$ mice (Figure 3.2a). Similar effects of enrichment processes

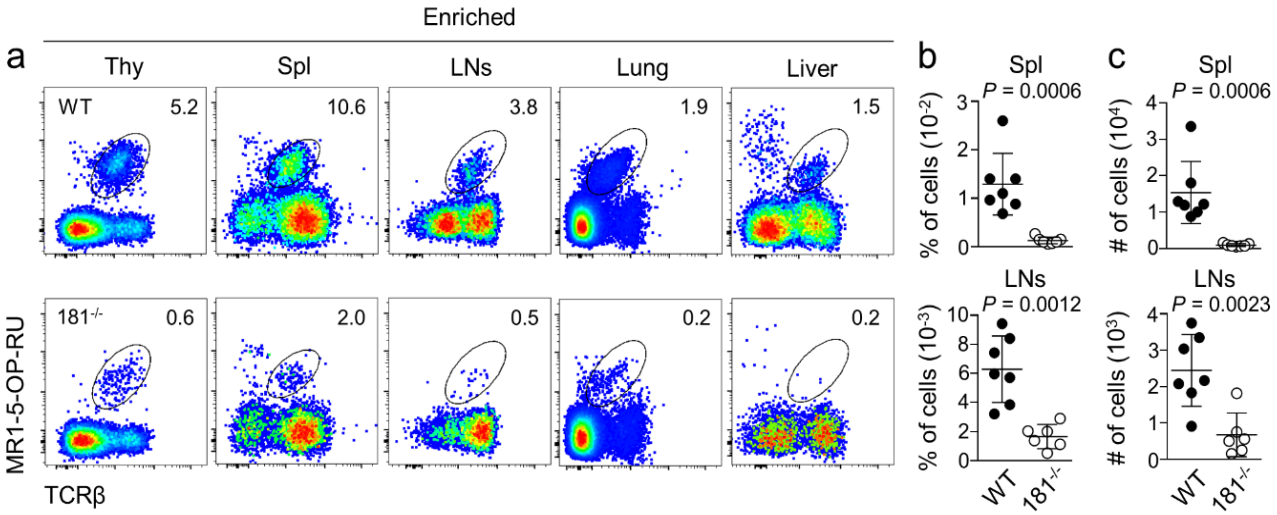


**Figure 3.2 Reduced frequencies of MAIT cells in peripheral organs of miR-181a/b-1$^{-/-}$ mice. (a)** Enriched MAIT cells isolated from thymus (Thy), spleen (Spl), lymph nodes (LNs), lung and liver of WT (top row) and 181$^{-/-}$ mice (bottom row). Numbers in the top right corner of each plot are percentages of MAIT cells in B cell depleted, MR1-5-OP-RU$^{+}$ enriched samples. **(b)** Frequency of MR1-5-OP-RU$^{+}$ TCRβ$^{+}$ MAIT cells in total splenocytes (top) and lymph nodes (bottom) of WT and 181$^{-/-}$ mice. **(c)** Absolute number of MR1-5-OP-RU$^{+}$ TCRβ$^{+}$ MAIT cells in total splenocytes (top) and lymph nodes (bottom) of WT and 181$^{-/-}$ mice. **(a)** Each plot is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. **(b, c)** Each symbol represents MAIT cells (without enrichment) from an individual mouse and error bars indicate SD. Statistical analyses were performed using Mann–Whitney rank-

retaining original differences in cellular frequencies were also observed by us and others for both CD1d and MR1 tetramers[146,2,335,336]. Loss of miR-181a/b-1 resulted in reduced frequencies and numbers of MAIT cells in peripheral lymphoid organs, such as lymph nodes and spleen as well as in liver and lung (Figure 3.2a). These findings were confirmed by analysis of MAIT cells in spleen and lymph nodes without prior enrichment using dual-tetramer staining described in Figure 3.1a (Figure 3.2b-c, 3.3). To further clarify that MAIT cells identified in WT or miR-181a/b-1$^{-/-}$ mice were not rare

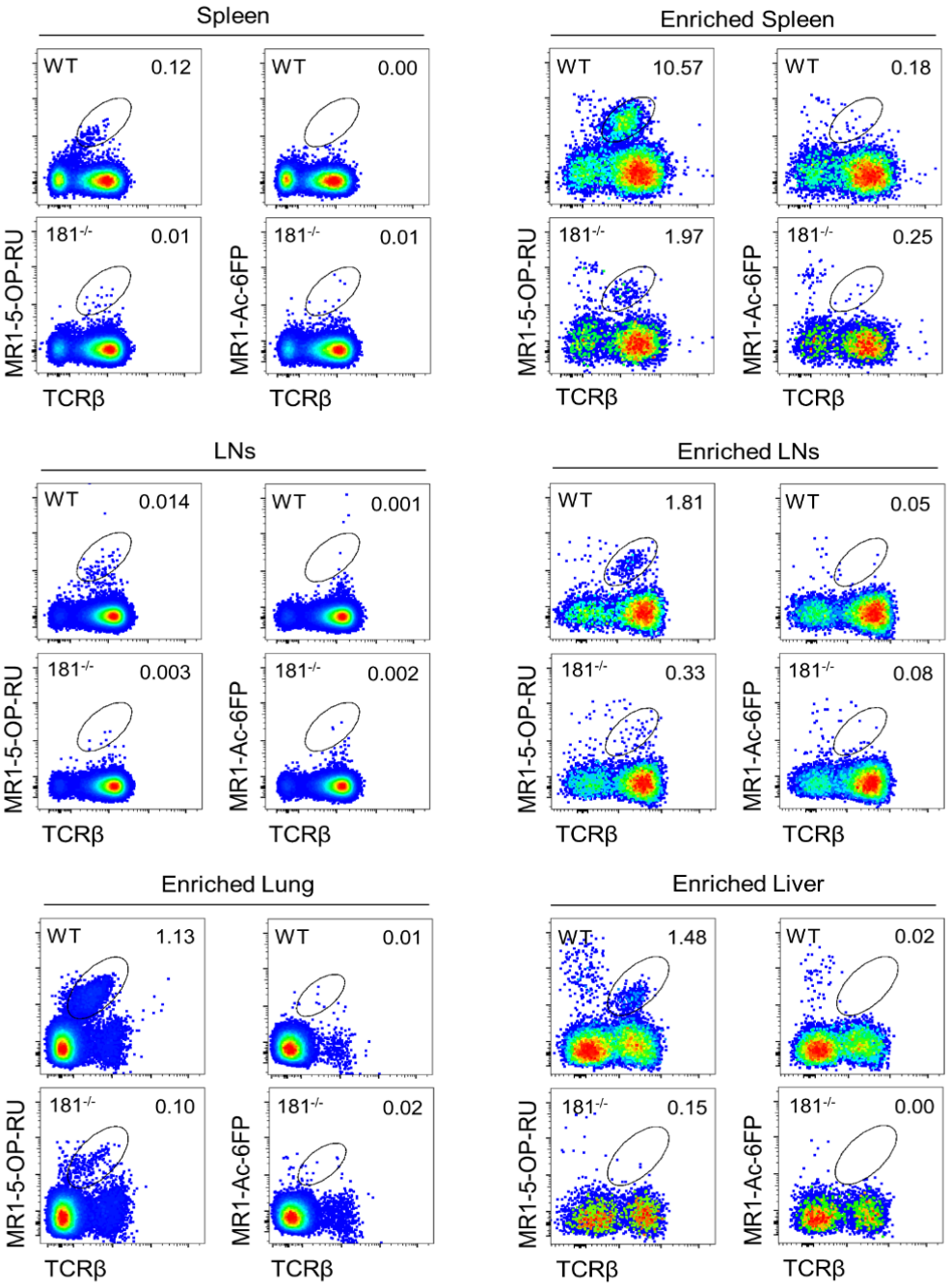


**Figure 3.3 Analysis of MAIT cell staining specificity in various organs.** To show that 181$^{-/-}$ MAIT cells are not rare contaminants, MR1-5-OP-RU was stained alongside MR1-Ac-6FP tetramer (negative control) in both WT and 181$^{-/-}$ samples. Samples shown were either stained directly (spleen, lymph nodes) or following MR1-5-OP-RU enrichment (spleen, lymph nodes, lung and liver).

contaminants, we analysed staining specificity in various organs alongside MR1-Ac-6-FP (Figure 3.3). These data indicate that in the absence of miR-181a/b-1, MAIT cells do not undergo homeostatic expansion in the periphery.

### 3.1.3 miR-181a/b-1 promotes the transition between stage 1 and 2

To pinpoint the stage of MAIT cell development controlled by miR-181a/b-1, we assessed expression of CD24, CD44, CD4 and CD8α on the surface of MR1-5-OP-RU$^{+}$ MAIT cells. The majority of MAIT cells in control thymi were at stage 3 (CD24$^{-}$CD44$^{+}$) and approximately 20% and 15% were at stage 1 (CD24$^{+}$CD44$^{-}$) and 2 (CD24$^{-}$CD44$^{-}$), respectively (Figure 3.4a, b). In contrast, close to 80% of miR-181a/b-1$^{-/-}$ MAIT cells were at stage 1. Given the reduced frequencies of MAIT cells among miR-181a/b-1$^{-/-}$ thymocytes, the overall frequencies of stage 1 cells remained the same, whereas frequencies of stage 2 and stage 3 MAIT cells were reduced by a factor of 8 and 62, respectively (Figure 3.4c). Thus, these data indicate that loss of miR-181a/b-1 leads to a developmental arrest in MAIT cells at stage 1. Stage 1 MAIT cells exhibit heterogeneous CD4 and CD8α co-receptor expression, with the majority of cells being DP or single-positive (SP) for CD4. Analysis of co-receptor expression on stage 1 MAIT cells from miR-181a/b-1$^{-/-}$ mice revealed an increased frequency of DP cells and reduced frequencies of CD4 and CD8α SP cells when compared to controls (Figure 3.4d). Given the developmental origin of MAIT cells it appears likely that DP stage 1 cells are more immature than their SP counterparts and might contain pre-selection MR1-5-OP-RU-tetramer-reactive cells. Our data therefore suggests that miR-181a/b-1 controls the earliest defined stage of MAIT cell development.

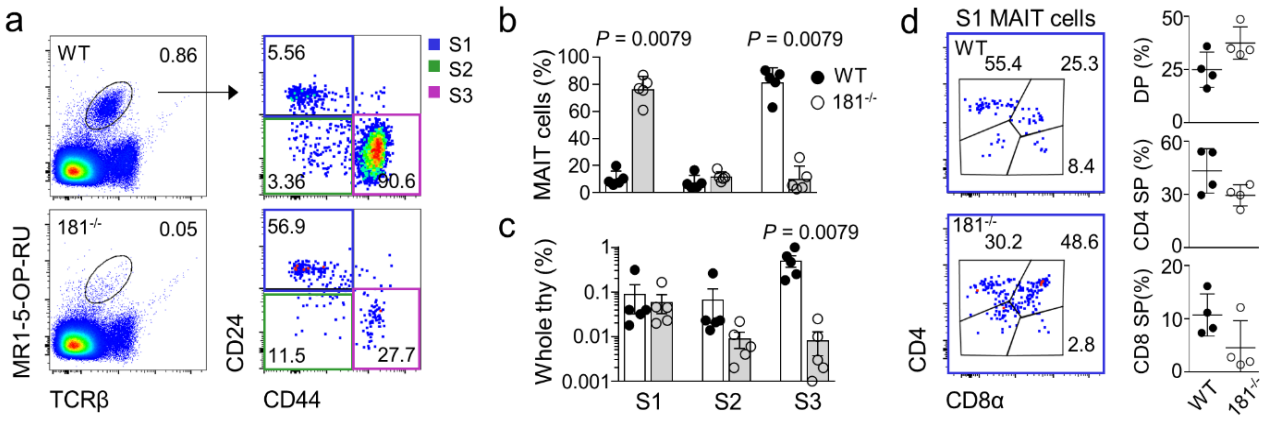


**Figure 3.4 miR-181a/b-1 promotes transition of MAIT cells from developmental stage 1 to stage 2. (a)** Developmental stages of thymic MAIT cells (after magnetic bead enrichment) from WT and 181$^{-/-}$ mice. The three MAIT cell stages are defined as: stage 1 CD24$^{+}$CD44$^{-}$ (S1; blue), stage 2 CD24$^{-}$CD44$^{-}$ (S2; green) and stage 3 CD24$^{-}$CD44$^{+}$ (S3; purple). **(b)** Frequency of enriched thymic MAIT cells at each stage of development in WT and 181$^{-/-}$ mice. **(c)** Frequency of each developmental stage of enriched thymic MAIT cells in whole thymocytes in WT and 181$^{-/-}$ mice. **(d)** Analysis of CD4 and CD8α expression on S1 thymic MAIT cells in WT and 181$^{-/-}$ mice. Percentages of S1 DP, CD4 SP and CD8 SP MAIT cells are shown on the left. Data are representative of 4-5 independent experiments. Each plot is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using Mann–Whitney rank-sum *U*-tests. When no *P*-value is indicated, comparisons revealed no statistical significance.

### 3.1.4 Analysis of developing thymocytes using a *Rag1*$^{GFP}$ molecular timer

To analyse the temporal order of phenotypic changes during intrathymic and peripheral MAIT cell development *in vivo*, we took advantage of a *Rag1*-dependent molecular timer. RAG gene transcription is high in pre-selection DP thymocytes, but is rapidly terminated following positive selection. Mice with GFP under the control of *Rag1* regulatory elements, produce high levels of GFP until *Rag1* transcription is acutely terminated following positive selection. A cells relative expression of GFP therefore acts as a measure of 'pseudo-time' in thymic development that has passed since positive selection. GFP presence in peripheral T cells is also a useful measure of recent emigration from the thymus[9,337] (Figure 3.5a). GFP protein concentration in a cell depends on both its half-life and its division-dependent dilution during proliferation. Essentially, GFP$^{hi}$ cells are developmentally more proximal to the DP stage than GFP$^{lo}$ or GFP$^{-}$ cells. We therefore crossed miR-181a/b-1$^{-/-}$ mice with *Rag1*$^{GFP}$-knock-in mice, and measured GFP expression in developing thymocyte subsets[327] (Figure 3.5b).

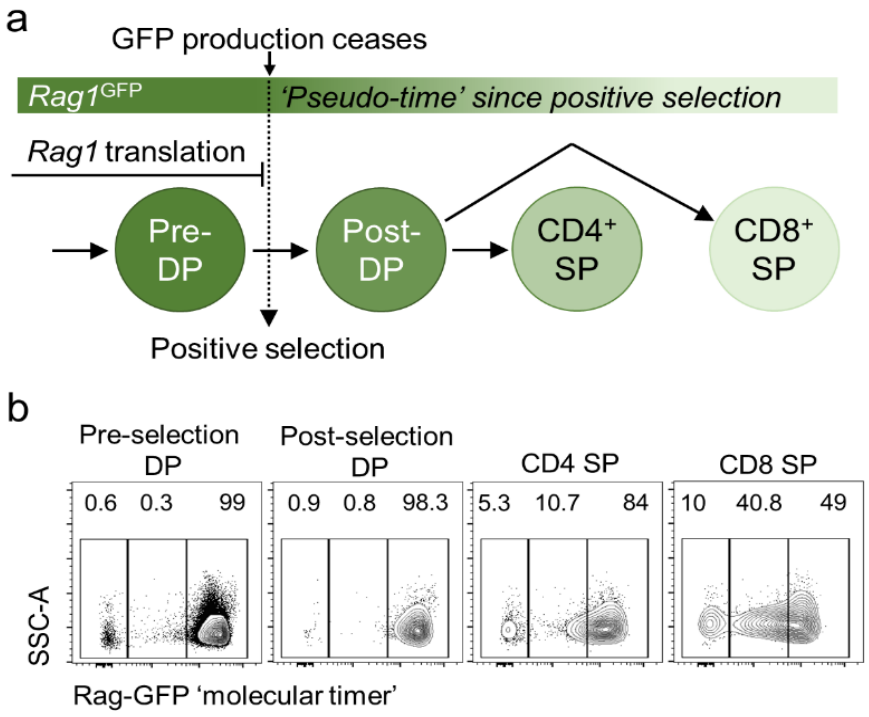


**Figure 3.5 Analysis of developing thymocytes using a *Rag1*$^{GFP}$ molecular timer. (a)** Schematic representation of mice expressing a *Rag1*-GFP knock-in. RAG1 gene transcription is high in pre-selection DP thymocytes (Pre-DP), but is rapidly terminated following positive selection. The degradation of GFP therefore acts as a measure of 'pseudo-time' in thymic development since positive selection. **(b)** *Rag1*$^{GFP}$ expression in pre-selection (TCRb$^{lo}$CD69$^{-}$) and post-selection (TCRb$^{+}$CD69$^{+}$) DP thymocytes, followed by CD4 and CD8 SP thymocytes. Numbers adjacent to outlined areas indicate percentages of each gated subset.

### 3.1.5 miR-181a/b-1 promotes thymic MAIT cell residency

The presence of Rag1-GFP was evaluated in thymic MAIT cells. Analysis of control mice revealed a largely bi-modal distribution of GFP expression (Figure 3.6a). A substantial proportion were GFP$^{hi}$, indicating recent descent from DP thymocytes. A comparatively minor subset was GFP$^{int}$, indicating recent history of proliferation or intermediate residency time in the thymus. A second major population comprised GFP$^{-}$ cells. Thymic cells that have lost GFP expression suggest either a history of massive

proliferation in the thymus, thymic residency or cells that have left and recirculated back to the thymus. In MAIT cells from miR-181a/b-1$^{-/-}$ mice, the proportion of GFP$^{hi}$ cells was substantially increased at the expense of GFP$^{-}$ cells (Figure 3.6a). To delve further into the temporal analysis of MAIT cell biology, we then assessed GFP levels at different stages of MAIT cell development (Figure 3.6b). Consistent with developmental progression from stage 1 through to stage 3, stage 1 cells were uniformly GFP$^{hi}$. Stage 2 cells showed a continuum of peaks of GFP expression ranging from slightly lower levels than stage 1 to GFP$^{int}$ to GFP$^{-}$. These data suggest that proliferative expansion of developing MAIT cells occurs predominantly at stage 2. Stage 3 cells were mostly GFP$^{-}$, consistent with extended residency in the thymus. Parabiosis and transcriptomic experiments reveal that MAIT cells possess tissue-resident phenotypes, reinforcing the notion that stage 3 GFP$^{-}$ cells are long-term inhabitants of the thymus[160,161]. In a miR-181a/b-1-deficient setting, the observed distribution of GFP expression remained essentially unaltered in stage 1 and stage 3 MAIT cells. However, at stage 2,

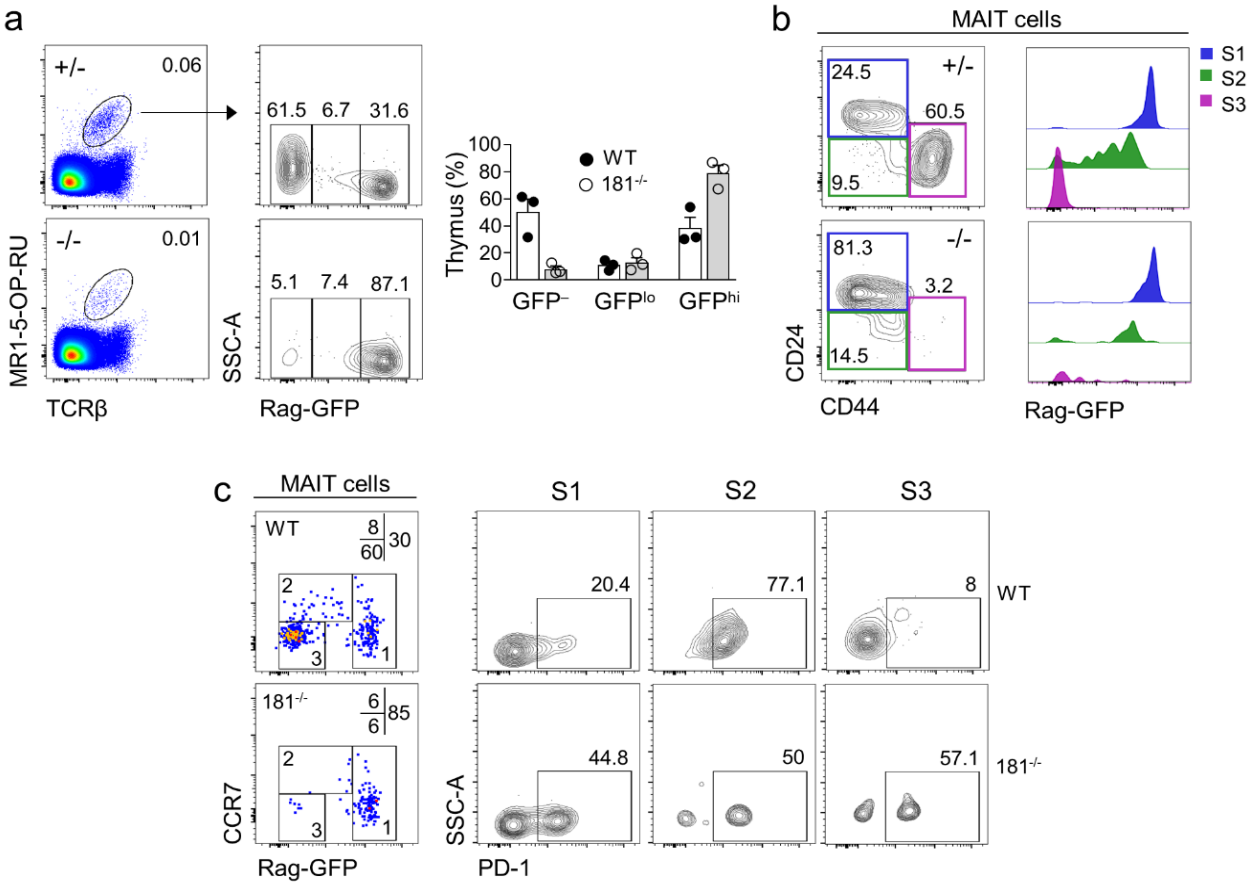


**Figure 3.6 miR-181a/b-1 promotes thymic MAIT cell residency. (a)** Identification of thymic MAIT cells in *Rag1*$^{GFP}$ x 181$^{+/-}$ (+/–) and *Rag1*$^{GFP}$ x 181$^{-/-}$ mice (–/–). Right column shows *Rag1*$^{GFP}$ expression on thymic MAIT cells. Percentages of *Rag1*$^{GFP}$ negative (–), low (lo) and high (hi) MAIT cells depicted (far right). **(b)** Separation of *Rag1*$^{GFP}$ expression into the three stages of MAIT cell development. **(c)** Expression of CCR7 and *Rag1*$^{GFP}$ in thymic MAIT cells. Cells are arbitrarily gated into stage 1-3 MAIT cells and PD-1 expression is plotted for each stage (right). Data is representative of 3 **(a, b)** independent experiments. Each plot and symbol is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using unpaired nonparametric Mann–Whitney rank-sum *U*-tests. When no *P*-value is indicated, comparisons revealed no statistical significance.

fewer miR-181a/b-1$^{-/-}$ cells possessed intermediate levels of GFP, suggesting a proliferative defect extending beyond the developmental block imposed in the absence of miR-181a/b-1 (Figure 3.6b).

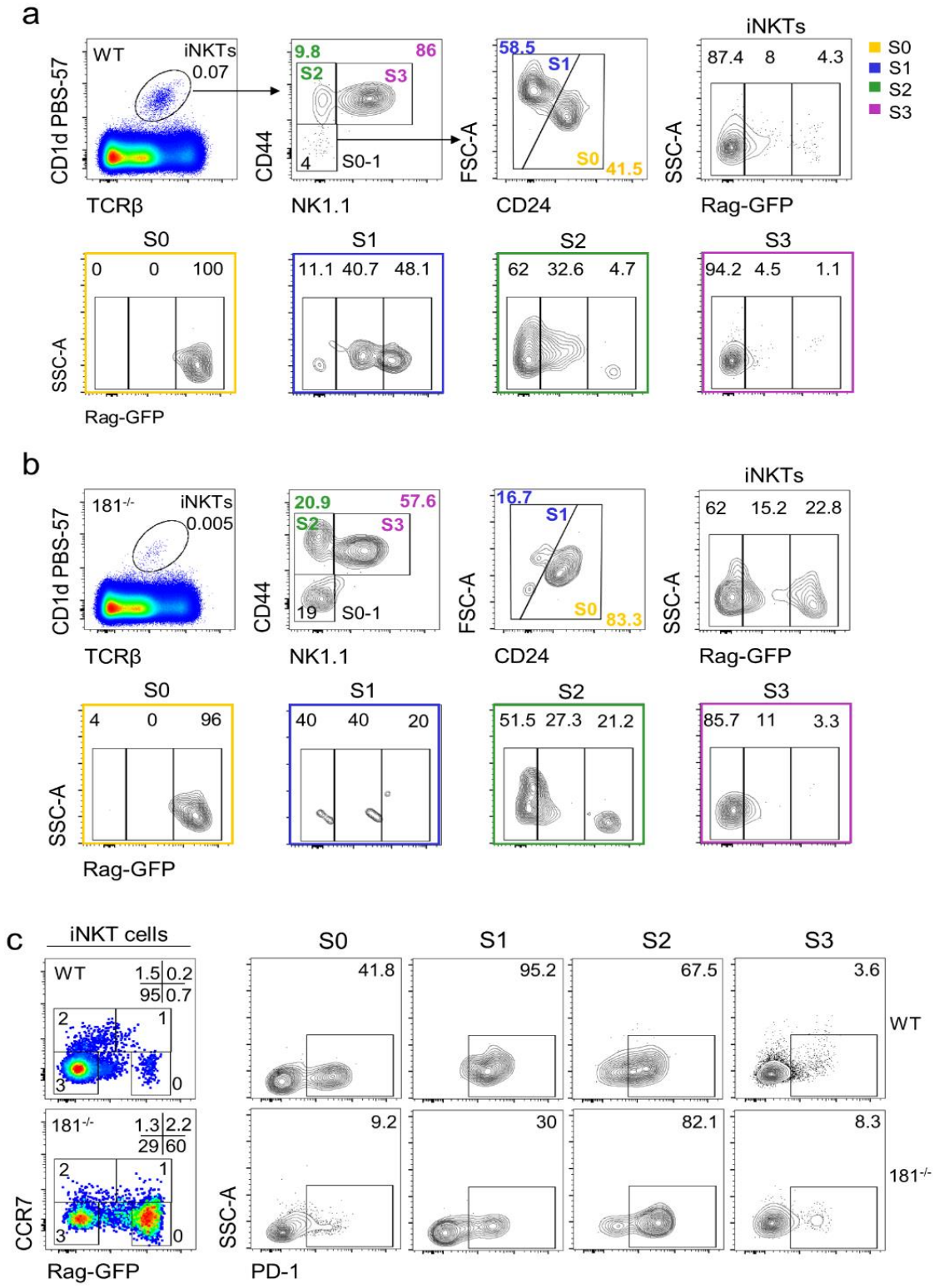


**Figure 3.7 Analysis of thymic iNKT cells using a *Rag1*$^{GFP}$ molecular timer. (a)** Flow cytometric analysis of iNKT cell developmental dynamics in WT thymus using a *Rag1*$^{GFP}$ molecular timer. iNKT cells were defined as TCRβ$^{+}$ and CD1d-PBS-57 tetramer$^{+}$. Developmental stages were discriminated based on expression of CD24, CD44, NK1.1 and size (FCS-A). **(b)** Analysis of iNKT cell developmental dynamics in miR-181a/b-1$^{-/-}$ thymus using a *Rag1*$^{GFP}$ molecular timer. **(c)** Expression of CCR7 and *Rag1*$^{GFP}$ in thymic iNKT cells. Cells are arbitrarily gated into stage 0-3 MAIT cells and PD-1 expression is plotted for each stage (right). Numbers adjacent to outlined areas indicate percentages of each gated subset.

CCR7 has recently been identified to define a precursor population of MAIT cells, before effector differentiation[161]. In conjunction with data from Figure 3.6b, we were able to arbitrarily separate stage 1-3 MAIT cells via CCR7 and Rag-GFP expression (Figure 3.6c). In WT mice newly generated cells (stage 1) upregulate CCR7 (stage 2) and are thought to migrate to the periphery in a similar fashion to iNKT cells[161], before undergoing effector differentiation and returning back to the thymus as Rag-GFP$^{-}$ CCR7$^{-}$ (stage 3) cells (Figure 3.6c). In the absence of miR-181a/b-1, temporal gating of MAIT cells further confirms that differentiation is blocked between stage 1 and 2 and that stage 3 cells fail to recirculate back to the thymus. Furthermore, agonist selected T cells upregulate PD-1 shortly after selection to inhibit negative selection inducing co-stimulatory signals. PD-1 upregulation appeared normal in both WT and miR-181a/b-1-deficient mice suggesting that loss of miR-181a/b-1 did not result in increased co-stimulatory signals through PD-1 dysregulation (Figure 3.6c). Therefore, in the absence of miR-181a/b-1, MAIT cells fail to differentiate beyond stage 1 and therefore fail to exit the thymus and recirculate back as mature effector tissue-resident MAIT cells.

### 3.1.6 Temporal analysis of thymic iNKT cell development

MAIT and iNKT cells share many developmental parallels. Comparison of developmental dynamics of intrathymic MAIT cells with those of iNKT cells in the same experimental model revealed that iNKT cells undergo substantial proliferation at stage 1, resulting in a major population of GFP$^{-}$ stage 2 (Figure 3.7a). Stage 3 iNKT cells were predominantly GFP$^{-}$. Consistent with previous studies, loss of miR-181a/b-1 resulted in an accumulation of GFP$^{hi}$ iNKT cells, although the remaining stage 3 cells were GFP$^{-}$ (Figure 3.7b)[2]. Thus, despite their developmental similarities MAIT and iNKT cells deviate slightly in their proliferative dynamics–with respect to the currently accepted discrimination of developmental stages. As previously reported iNKT cells proceed through development in the thymus by upregulating CCR7 directly after selection (Rag-GFP$^{hi}$) and then emigrating from the thymus. We observe the same pattern in WT mice but CCR7 expression and the presence of CCR7$^{-}$Rag-GFP$^{-}$ recirculating cells is diminished in miR-181a/b-1-deficient mice (Figure 3.7c). Furthermore, PD-1 is upregulated early in development in control cells but is delayed in miR-181a/b-1-deficient mice, suggesting another small uncoupling between MAIT and iNKT cell development (Figure 3.7c).

### 3.1.7 miR-181a/b-1 promotes thymic MAIT cell proliferation

As loss of GFP in this model reflects a combination of half-life and proliferation-dependent dilution we complemented this analysis with Ki67 staining, marking actively proliferating cells. The majority of thymic MAIT cells were actively proliferating, whereas only a minor fraction were Ki67$^{+}$ in miR-181a/b-1$^{-/-}$ mice (Figure 3.8a). Consistent with loss of *Rag1*$^{GFP}$ expression, stage 1 MAIT cells displayed very limited active proliferation, whereas a considerable proportion of stage 2 cells and

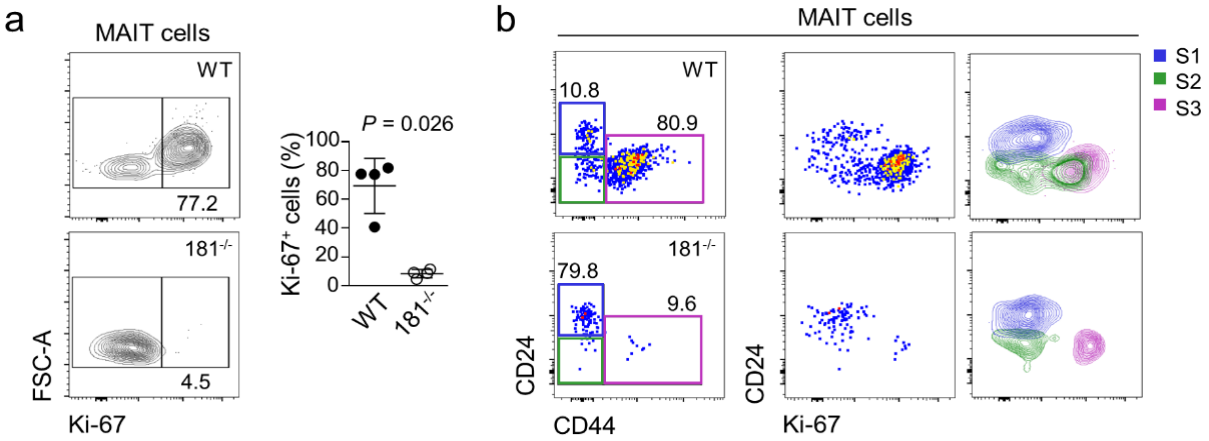


**Figure 3.8 miR-181a/b-1 promotes thymic MAIT cell proliferation at stage 2 but not stage 3. (a)** Frequency of proliferating Ki67$^{+}$ MAIT cells in the thymus of WT and 181$^{-/-}$ mice. **(b)** Identification of the three stages of thymic MAIT cell development (left-most column) in WT and 181$^{-/-}$ mice. Middle column shows CD24 and Ki67 expression of total thymic MAIT cells and the right-most plots depict CD24 and Ki67 expression with the three developmental stages overlaid to show distribution of proliferating cells. Data are representative of 4 independent experiments. Each plot and symbol is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using unpaired nonparametric Mann–Whitney rank-sum *U*-tests.

virtually all stage 3 were actively proliferating, therefore contributing to their loss of GFP (Figure 3.8b). Conversely, in miR-181a/b-1-deficient mice, actively proliferating cells were almost absent at developmental stage 1 and 2. At stage 3 however, proliferation appeared to be independent of miR-181a/b-1. In conclusion, our data have revealed novel developmental dynamics of MAIT cells, showing that in WT thymi proliferation commences at stage 2 and progresses continually at stage 3. Additionally, loss of miR-181a/b-1 precludes proliferation at the former stage, but not the latter.

### 3.1.8 Increased thymic emigration and reduced peripheral proliferation in miR-181a/b-1$^{-/-}$ mice

As previously mentioned, presence of peripheral *Rag1*$^{GFP}$ is an effective method to identify RTEs. Low to intermediate levels of GFP were detectable in approximately 5% of MAIT cells from spleen (Figure 3.9a) and lymph nodes (data not shown). This was attributed to a sizeable proportion of developmentally immature stage 2 MAIT cells that are exported from the thymus (Figure 3.9b). Frequencies of GFP$^{int/lo}$ MAIT cells in peripheral lymphoid organs from miR-181a/b-1$^{-/-}$ mice were substantially increased, comprising 20-25% of all MAIT cells (Figure 3.9a). Detection of increased proportions of peripheral GFP$^{int/lo}$ MAIT cells are attributable to either increased efficiency of thymic export of young MAIT cells or constraints in peripheral proliferation in the absence of miR-181a/b-1. To discriminate between these scenarios, we also analysed peripheral MAIT cells for the expression of Ki67. In WT spleen and lymph nodes approximately 60% of MAIT cells were Ki67$^{+}$ (Figure 3.9c). A lower frequency of peripheral MAIT cells from miR-181a/b-1$^{-/-}$ mice expressed Ki67, demonstrating

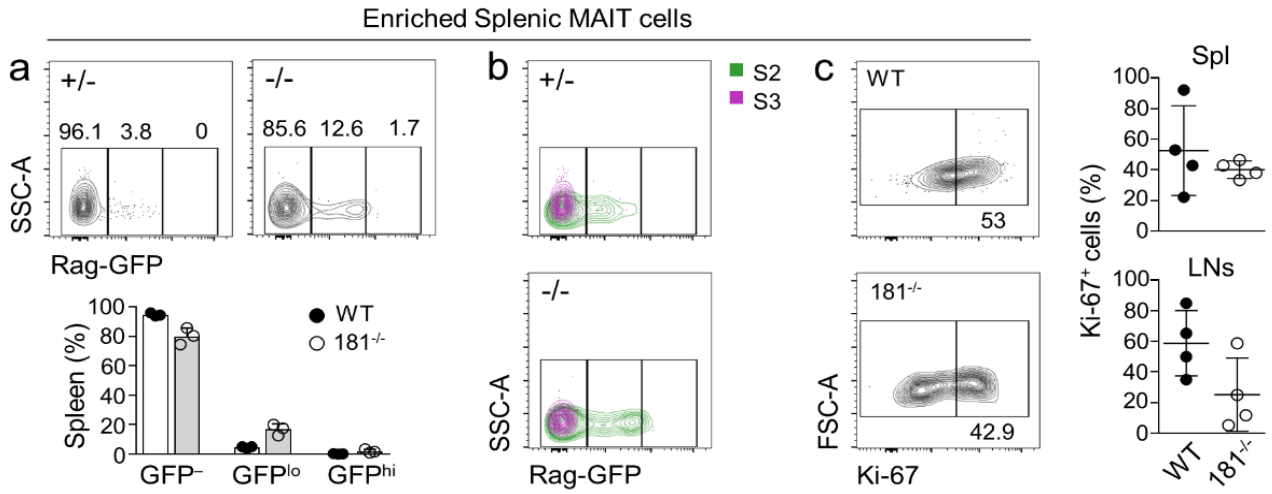


**Figure 3.9 Absence of miR-181a/b-1 results in reduced peripheral MAIT cell proliferation. (a)** Frequencies of *Rag1*$^{GFP}$ negative (–), low (lo) and high (hi) MAIT cells from spleens of both *Rag1*$^{GFP}$ x 181$^{+/–}$ (+/–) and *Rag1*$^{GFP}$ x 181$^{–/–}$ mice (–/–). **(b)** Stage 2 MAIT cells account for GFP$^{lo}$ recent thymic emigrants in both *Rag1*$^{GFP}$ x 181$^{+/–}$ (+/–) and *Rag1*$^{GFP}$ x 181$^{–/–}$ mice (–/–). **(c)** Frequencies of proliferating Ki67$^{+}$ MAIT cells in spleens of WT and 181$^{–/–}$ mice. Graphs on the right depict frequency of proliferating Ki67$^{+}$ cells in spleen (top) and lymph nodes (bottom). Data are representative of 3 **(a, b)** and 4 **(c)** independent experiments. Each plot and symbol is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using unpaired nonparametric Mann–Whitney rank-sum *U*-tests. When no *P*-value is indicated, comparisons revealed no statistical significance.

that proliferative capacity remains impaired beyond thymic export (Figure 3.9c). These data suggest that the apparent increase in frequencies of MAIT RTEs in the absence of miR-181a/b-1 is due to impaired peripheral proliferation, which in turn limits dilution of remaining GFP protein marking those cells. Analysis of *Rag1*$^{GFP}$ mice in conjunction with proliferation indicated that thymic export of MAIT can occur prior to complete functional maturation at stage 2. Furthermore, loss of miR-181a/b-1 results in a profound block in proliferation both within the thymus as well as in peripheral lymphoid organs, thus explaining low numbers of MAIT cells in miR-181a/b-1$^{–/–}$ mice.

### 3.1.9 PLZF acquisition is impaired in miR-181a/b-1$^{-/-}$ MAIT cells

Functional MAIT cell maturity is defined by the ability of a cell to express effector cytokines as well as effector-associated transcription factors PLZF, RORγt and T-bet. Intrathymic MAIT cells do not reach functional maturity before stage 3. Using intracellular staining for these transcription factors, we evaluated the ability of MAIT cells generated in the absence of miR-181a/b-1 to differentiate into mature cells capable of effector functions. PLZF expression precludes functional maturation and is required for MAIT cell transition between stage 2 and 3[146]. Within MAIT cells isolated from control thymi approximately 70% expressed the upstream transcription factor PLZF, which is consistent with the overall frequency of stage 3 MAIT cells (Figure 3.10a, c-d). Analysis of stage 3 MAIT cells revealed that they were uniformly positive for PLZF (Figure 3.10b). In contrast, despite the clear presence,

albeit at low numbers of stage 3 cells, intrathymic miR-181a/b-1$^{-/-}$ MAIT cells failed to express substantial levels of PLZF (Figure 3.10a, b-c). Analysis of residual stage 3 cells revealed that only some of these expressed PLZF (Figure 3.10b). Thus, loss of miR-181a/b-1 precludes functional maturation of MAIT cells in the thymus. We also asked the question of whether peripheral maturation of MAIT cells was possible in the absence of miR-181a/b-1. In controls, the vast majority of MAIT cells in spleen and lymph nodes expressed PLZF (Figure 3.10a, c-d). We detected lower, but sizeable, proportions of PLZF$^{+}$ MAIT cells in peripheral lymphoid organs of miR-181a/b-1$^{-/-}$ mice, which in spleen resembled the frequency of PLZF$^{+}$ cells in intrathymic stage 3 cells (Figure 3.10a, c-d). In lymph nodes however, the difference in PLZF$^{+}$ cells among WT and miR-181a/b-1$^{-/-}$ MAIT cells was smaller, suggesting that peripheral maturation of MAIT cells is not fully precluded by the absence of miR-181a/b-1 (Figure 3.10a-d).

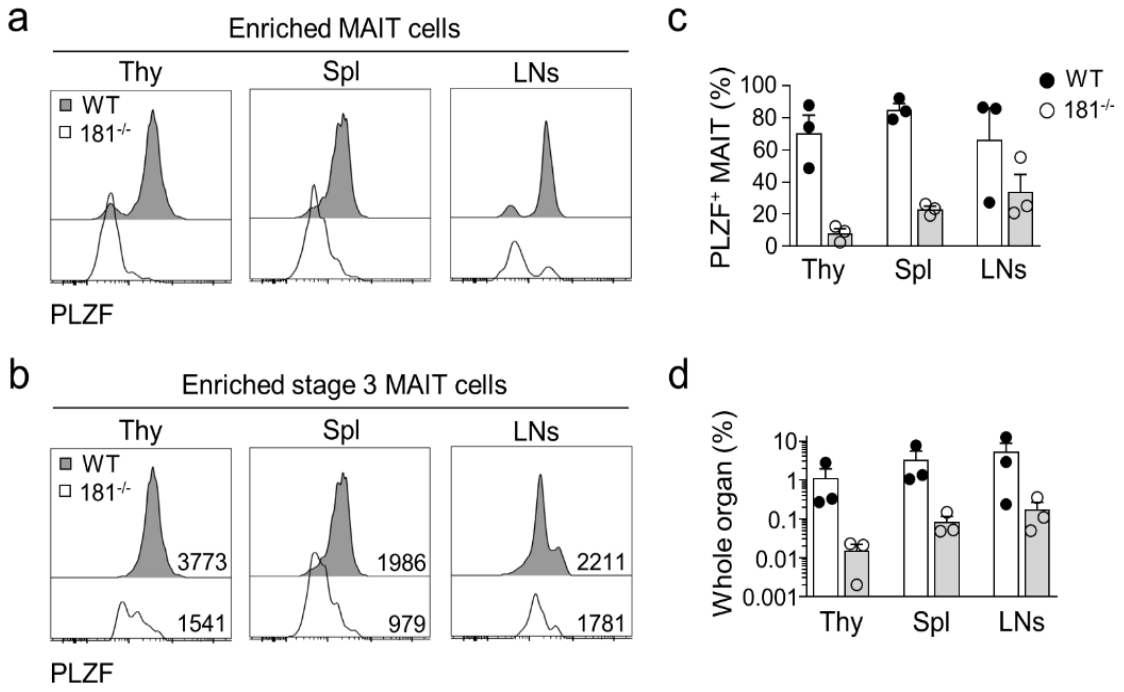


**Figure 3.10 PLZF acquisition is impaired in MAIT cells upon miR-181a/b-1 deletion. (a)** Intracellular PLZF expression in enriched thymus, spleen and lymph nodes of WT and 181$^{-/-}$ mice. **(b)** Intracellular PLZF expression in stage 3 (CD24$^{-}$ CD44$^{+}$) MAIT cells from enriched thymus, spleen and lymph nodes of WT and 181$^{-/-}$ mice. The mean fluorescence intensity (MFI) of PLZF is displayed as the numbers adjacent to histograms. **(c)** Percentage of total PLZF$^{+}$ MAIT cells in each organ. **(d)** Frequency of enriched PLZF$^{+}$ MAIT cells from thymus, spleen and lymph nodes in their respective organ. Data are representative of 3 independent experiments, with a total of n=3. Each plot and symbol is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Error bars indicate SD. Statistical analyses were performed using unpaired nonparametric Mann–Whitney rank-sum *U*-tests and no *P*-value is indicated, comparisons revealed no statistical significance.

### 3.1.10 Effector subsets differentiation is impaired in miR-181a/b-1$^{-/-}$ mice

Consistent with the absence of the upstream transcription factor PLZF, thymic miR-181a/b-1$^{-/-}$ MAIT cells also lacked expression of RORγt and T-bet, whereas in controls between 10% and 20% of all intrathymic MAIT cells expressed T-bet, and 60% to 80% of MAIT cells expressed RORγt (Figure 3.11a-c). Similarly, in controls the majority of lymph node and splenic MAIT cells expressed RORγt, whereas a smaller subset was positive for T-bet in spleen and frequencies of T-bet$^+$ cells were very low in lymph nodes (Figure 3.11a-c). Peripheral T-bet$^+$ cells were less affected by loss of miR-181a/b-1 when compared to RORγt$^+$ cells, resulting in similar overall frequencies of both MAIT cell subsets (Figure 3.11a-c). Despite complete absence in the thymus, small presence of RORγt and T-bet expressing cells in miR-181a/b-1-deficient mice hints towards miR-181a/b-1-independent peripheral maturation. Therefore miR-181a/b-1 is dispensable for peripheral, but not intrathymic, functional maturation of MAIT cells.

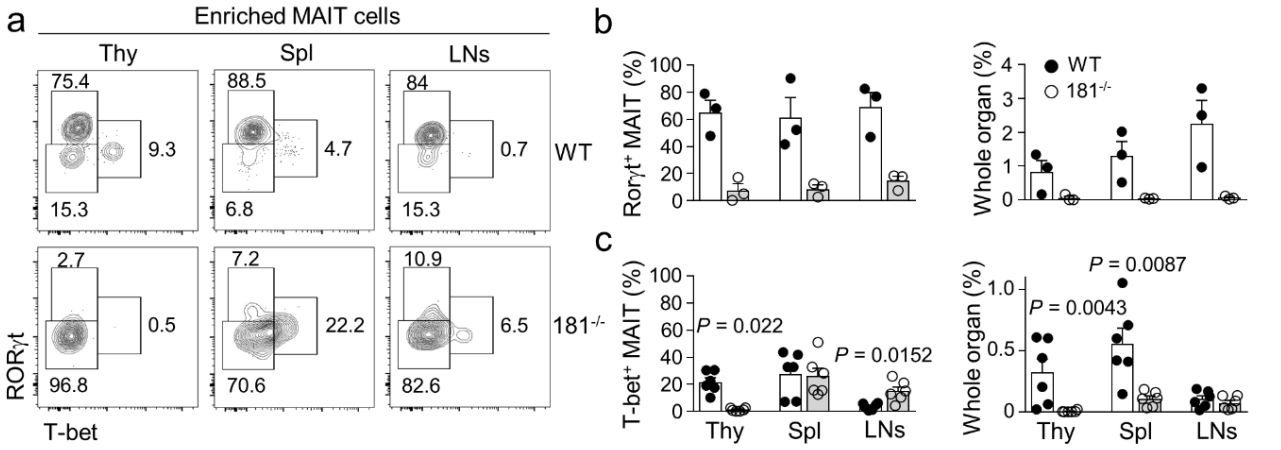


**Figure 3.11 Differentiation into MAIT cell effector subsets is impaired upon miR-181a/b-1 deletion. (a)** Intracellular RORγt and T-bet expression in MAIT cells from thymus, spleen and lymph nodes in WT and 181$^{-/-}$ mice. **(b)** Left graph indicates the percentage of RORγt$^+$ MAIT cells in each organ and the right-most graph indicates the frequency of enriched RORγt$^+$ MAIT cells from thymus, spleen and lymph nodes in their respective organ. **(c)** Left graph indicates the percentage of T-bet$^+$ MAIT cells in each organ and the right-most graph indicates the frequency of enriched T-bet$^+$ MAIT cells from thymus, spleen and lymph nodes in their respective organ. Data are representative of 3 independent experiments, with a total of n=3 (RORγt$^+$) and n=6 (T-bet$^+$) samples. Each plot and symbol is representative of magnetically enriched MAIT cells extracted from 3 pooled mice. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using unpaired nonparametric Mann–Whitney rank-sum *U*-tests. When no *P*-value is indicated, comparisons revealed no statistical significance.

### 3.1.11 Expression of an invariant Vα19Jα33 TCRα chain restores development of MR1-5OP-RU-tetramer-reactive cells in the absence of miR-181a/b-1

To test whether miR-181a/b-1 promoted the development of MAIT cells via regulation of TCR signalling we generated BM chimeric mice, in which BM from control or miR-181a/b-1$^{-/-}$ mice was

transduced with retroviruses encoding the invariant Vα19Jα33 TCRα chain and GFP as a reporter for successful transduction (Figure 3.12a). Eight weeks after reconstitution, MR1-5-OP-RU-tetramer-reactive cells were analysed from the thymus and spleen following magnetic enrichment. For internal controls, we compared both Vα19Jα33-expressing cells with cells that did not express Vα19Jα33 compared both Vα19Jα33-expressing cells with cells that did not express Vα19Jα33 TCRα as well as both empty vector-expressing (encoding only GFP) and non-expressing cells for external controls. In all controls MAIT cell development was impaired in the absence of miR-181a/b-1, showing that this miRNA is required cell-intrinsically (Figure 3.12b, c). Upon overexpression of a pre-arranged Vα19Jα33 TCRα chain the majority of thymocytes recovered after magnetic enrichment constituted of MR1-5-OP-RU-tetramer-reactive cells in donor-derived thymocytes both from controls and miR-181a/b-1$^{-/-}$ mice. The efficiency of rescue in this experimental system was variable and did not fully reach levels of induced MAIT cell development upon expression of the invariant TCRα chain in WT mice. We also analysed these data by creating ratios of GFP$^{+}$ MR1-5-OP-RU-tetramer-reactive cells to GFP$^{+}$ splenic B cells as indicator of reconstitution of transduced cells (Figure 3.12d). Splenic B cells are unaffected by the absence of miR-181a/b-1 and therefore normalization indicated that ectopic expression of Vα19Jα33 TCRα resulted in generation of a larger proportion of MR1-5-OP-RU-tetramer-reactive cells with transduced cells irrespective of transduction rates. The phenotype of peripheral MR1-5-OP-RU-tetramer-reactive cells detected in the spleen mirrored that of the thymus in WT and miR-181a/b-1-deficient mice upon overexpression of a pre-arranged Vα19Jα33 TCRα chain (Figure 3.12e, f). Ratios of GFP$^{+}$ MR1-5-OP-RU-tetramer-reactive cells to GFP$^{+}$ splenic B cells also indicated a competitive advantage for Vα19Jα33 transduced cells. Therefore, expression of an invariant Vα19Jα33 TCRα chain appears to restore development of MR1-5-OP-RU-tetramer-reactive cells in the absence of miR-181a/b-1.

Upon ectopic expression of Vα19Jα33 TCRα chain some MR1-5-OP-RU-tetramer-reactive cells arise that are MR1-independent[201,338]. In order to test whether rescued MR1-5-OP-RU-tetramer-reactive cells displayed characteristics of innate-like T cells, we first determined their distribution within the three developmental stages. The developmental block observed at steady-state was confirmed in GFP$^{-}$ miR-181a/b-1$^{-/-}$ donor cells showing increased frequencies of S1 cells at the expense of S3 cells (Figure 3.13a, b). This distribution was not markedly altered upon ectopic expression of the invariant Vα19Jα33 TCRα chain.

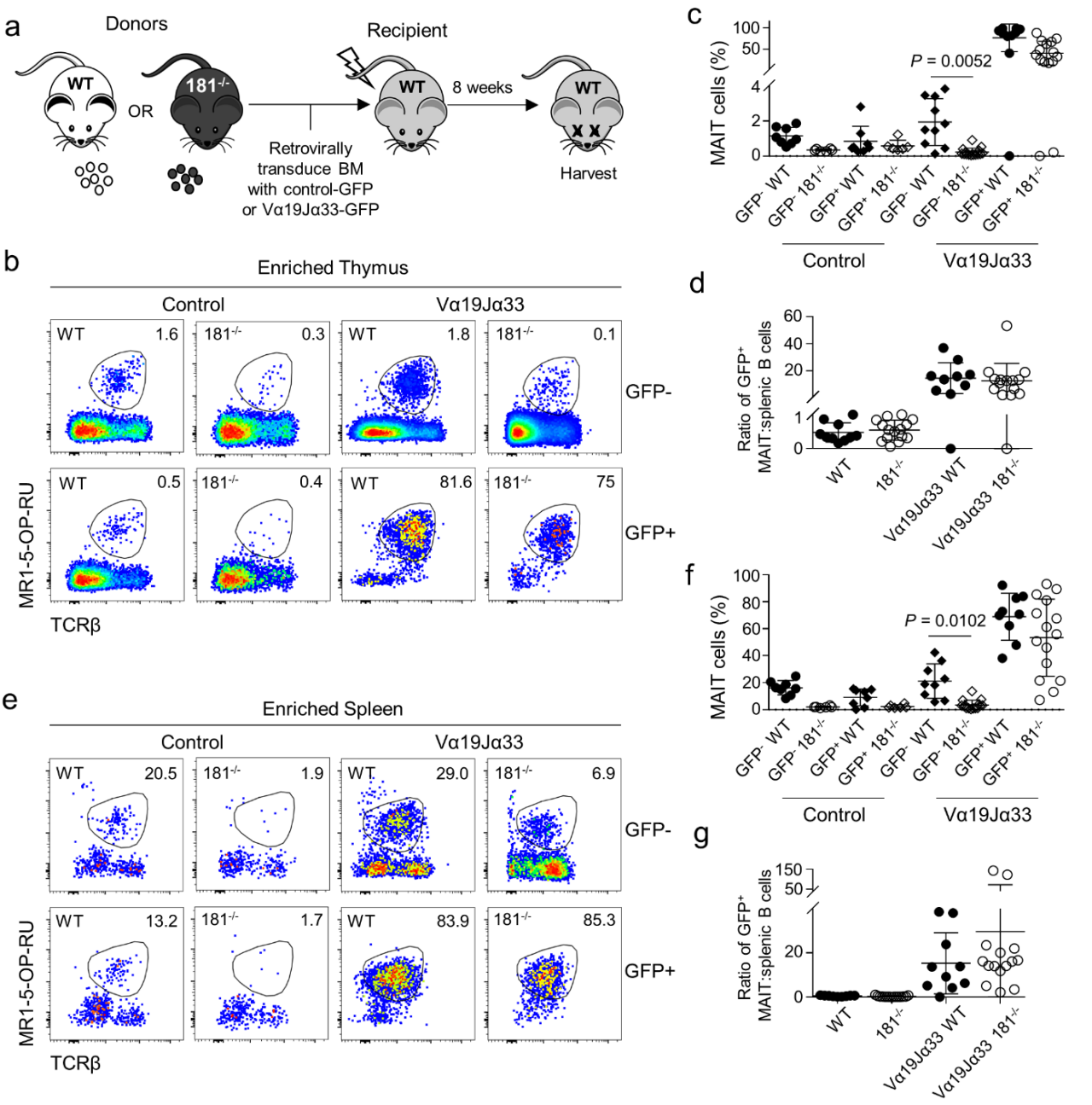


**Figure 3.12 Expression of an invariant Vα19Jα33 TCRα chain restores development MR1-5-OP-RU -tetramer-reactive thymocytes in the absence of miR-181a/b-1. (a)** Schematic representation of BM chimera generation. Lineage-negative BM from WT and 181$^{-/-}$ mice was transduced with control-GFP or Vα19Jα33-GFP-expressing retrovirus. Cells were then injected into lethally irradiated WT recipients and thymi and spleens were harvested after 8 weeks. **(b)** Expression of untransduced (GFP$^{-}$) and transduced (GFP$^{+}$) MR1-5-OP-RU-tetramer-reactive cells in the thymus following MAIT cell magnetic bead enrichment. **(c)** Frequencies represented in **(b)** are plotted in **(c)** as the percentage of enriched MR1-5-OP-RU -tetramer-reactive cells in the thymus. **(d)** Ratio of Vα19Jα33 transduced thymic MAIT cells to Vα19Jα33 transduced splenic B cells to demonstrate competitive advantage of Vα19Jα33 transduced cells. **(e)** As described in **(b)**, MAIT cells in the spleen were analysed following MAIT cell magnetic bead enrichment. **(f)** Frequencies represented in **(e)** are plotted in **(f)** as the percentage of enriched MAIT cells in the spleen. **(g)** Ratio of Vα19Jα33 transduced splenic MAIT cells to Vα19Jα33 transduced splenic B cells to demonstrate competitive advantage of Vα19Jα33 transduced cells. Data are representative of 2 independent experiments, with a total of n=15 (Vα19Jα33 181$^{-/-}$), n=9 (Vα19Jα33 WT), n=8 (empty vector WT) and n=6 (empty vector 181$^{-/-}$). Each plot and symbol are representative of MR1-5-OP-RU -tetramer-reactive cells enriched from one mouse and error bars indicate SD. Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's post-test for pairwise comparisons. Pairwise comparisons were performed between WT and 181$^{-/-}$ for each condition. Numbers adjacent to outlined areas indicate percentages of each gated subset.

However, phenotypic differences based on surface markers were also observed in iNKT cells from Vα14 transgenic mice[339,3]. Therefore, we also analysed expression of PLZF. We observed a marked increase in the fraction of PLZF+ cells within the MR1-5-OP-RU-tetramer-reactive population upon ectopic expression of Vα19Jα33 TCRα chain when compared to MR1-5-OP-RU-tetramer-reactive GFP$^{-}$ miR-181a/b-1$^{-/-}$ controls (Figure 3.13c). This indicates that miR-181a/b-1 promotes development of MR1-5-OP-RU-tetramer-reactive innate-like T cells at the level of the TCR. Finally, we tested whether ectopic expression of TCRα was able to restore the balance between effector subsets by staining for transcription factors RORγt and T-bet. Interestingly, we did not observe restoration of effector phenotypes in miR-181a/b-1$^{-/-}$ cells (Figure 3.13d). This finding suggests that miR-181a/b-1 acts via different mechanisms during early MAIT cell development and acquisition of effector function.

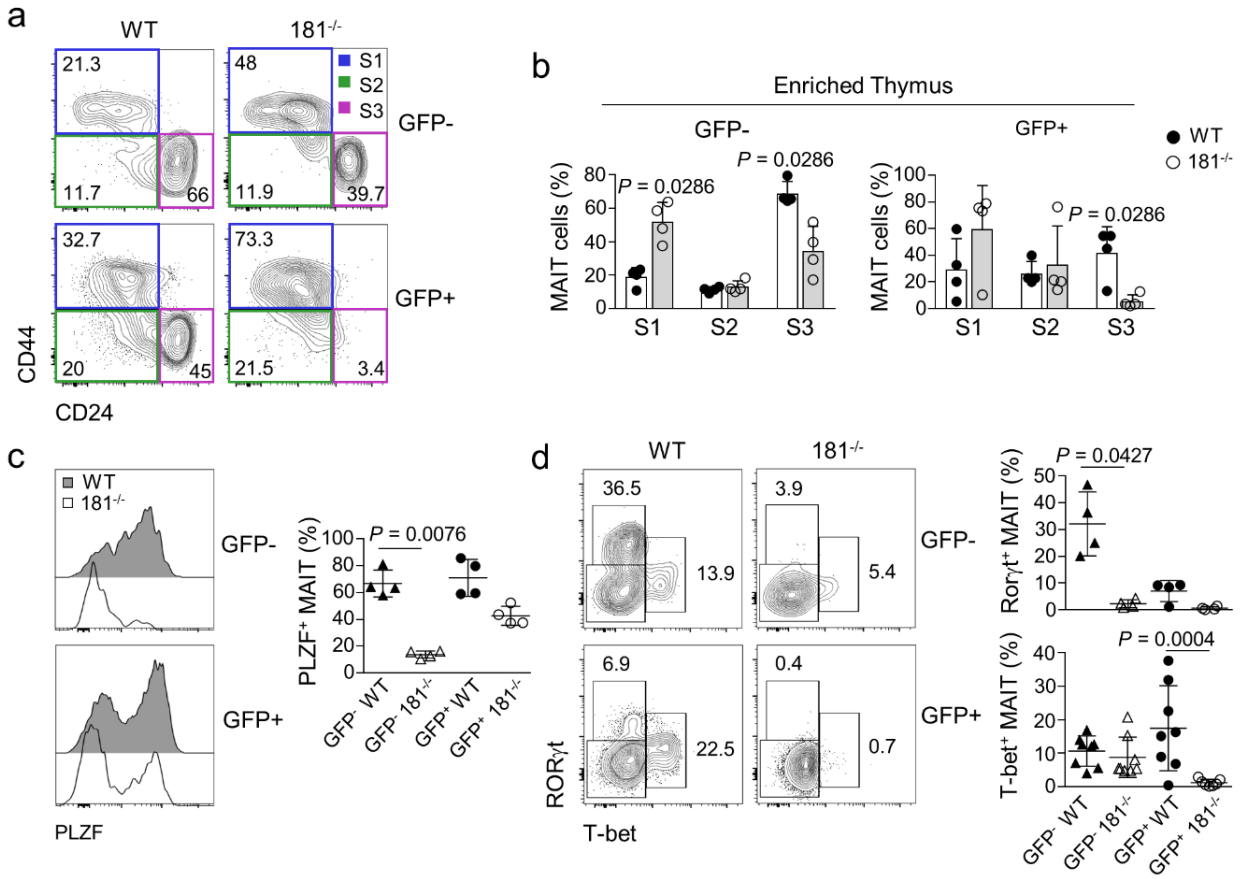


**Figure 3.13 Expression of an invariant Vα19Jα33 TCRα chain restores MR1-5-OP-RU PLZF expression, but not functional maturity. (a)** Thymocytes described in Figure 12b, were additionally stained with CD24 and CD44 to identify MAIT cell developmental stages following reconstitution of Vα19Jα33-transduced WT or 181$^{-/-}$ BM. **(b)** Graphs show percentages within untransduced (GFP$^{-}$) and Vα19Jα33-transduced (GFP$^{+}$) MR1-5-OP-RU-tetramer-reactive cells. **(c)** Histograms depict thymic PLZF expression in both WT and 181$^{-/-}$, untransduced (GFP$^{-}$) and Vα19Jα33-transduced (GFP$^{+}$) MR1-5-OP-RU -reactive cells. Percentages of PLZF$^{+}$ MR1-5-OP-RU-tetramer-reactive cells are shown right. **(d)** Thymic RORγt and T-bet expression from WT and 181$^{-/-}$, untransduced (GFP$^{-}$) and Vα19Jα33-transduced (GFP$^{+}$) MR1-5-OP-RU -reactive cells. Percentages are shown right. Data are representative of 2 independent experiments, with a total of n=4 (Vα19Jα33 181$^{-/-}$) and n=4 (Vα19Jα33 WT). Each plot and symbol is representative of MR1-5-OP-RU-tetramer-reactive cells enriched from one mouse and error bars indicate SD. Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's post-test for the pairwise comparisons. Pairwise comparisons were performed between WT and 181$^{-/-}$ for each condition.

# 4 Results Chapter Two

## 4.1 Treg development & miR-181a/b-1

### 4.1.1 miR-181a/b-1 controls the intrathymic development of MAIT cells

With experimental evidence presented for miR181a/b-1's role in fine-tuning the TCR signalling thresholds governing agonist selection[340,2,3], this begged the question – what about Tregs? Tregs are a subset of $CD4^+$ T cells expressing the lineage-defining transcription factor Foxp3. They develop from agonist signalling interactions in the thymus causing them to fall under the category of cell populations undergoing 'agonist selection'. To distinguish between young thymically-generated tTregs and either mature thymus-resident tTreg or recirculating peripheral-induced iTregs we again utilized mice harbouring a *Rag1*$^{GFP}$ knock-in allele described in Figure 3.5a. We found that frequencies and absolute numbers of *de novo* generated tTregs were decreased by approximately 2-fold in the thymus of miR-181a/b-1-deficient mice in comparison to controls (Figure 4.1a). Furthermore, when analysing the Rag-GFP negative portion of Tregs in the thymus, which accounts for mature thymus-resident tTregs and recirculating iTregs, we saw no change in frequency between controls and miR-181a/b-1-deficient mice (Figure 4.1a). Consistent with this finding, total splenic Tregs were also present at normal absolute numbers and frequencies in miR-181a/b-1-deficient mice (Figure 4.1b). Homeostatic proliferation is a normal physiological process that occurs to maintain a constant frequency of T cells. Upon discrimination of Rag-GFP+ RTEs in spleen, it was apparent that the export of tTregs from the thymus was indeed impaired in miR-181a/b-1-deficient mice (Figure 4.1b). It therefore possible that the rescue of overall Treg numbers in the periphery of miR-181a/b-1-deficient mice is due to homeostatic expansion of Tregs to fill the available niche.

### 4.1.2 Treg developmental dynamics are slower in the absence of miR-181a/b-1

Reduced numbers of freshly generated tTregs is suggestive of a disruption in normal Treg development. Tregs are known to develop via two distinct precursors stages, either CD4SP $CD25^-$ $Foxp3^+$ ($Foxp3^+$ precursor 1a)[237] or CD4SP $CD25^+Foxp3^-$ ($CD25^+$ precursor 1b)[236]. Temporal assessment of both maturation pathways was achieved using the aforementioned *Rag1*$^{GFP}$ mice. Thymic Rag-$GFP^+$ cells were arbitrarily gated into 5 populations based on progressive loss of GFP, reflecting the time/distance since positive selection and the cessation of *Rag1* gene expression

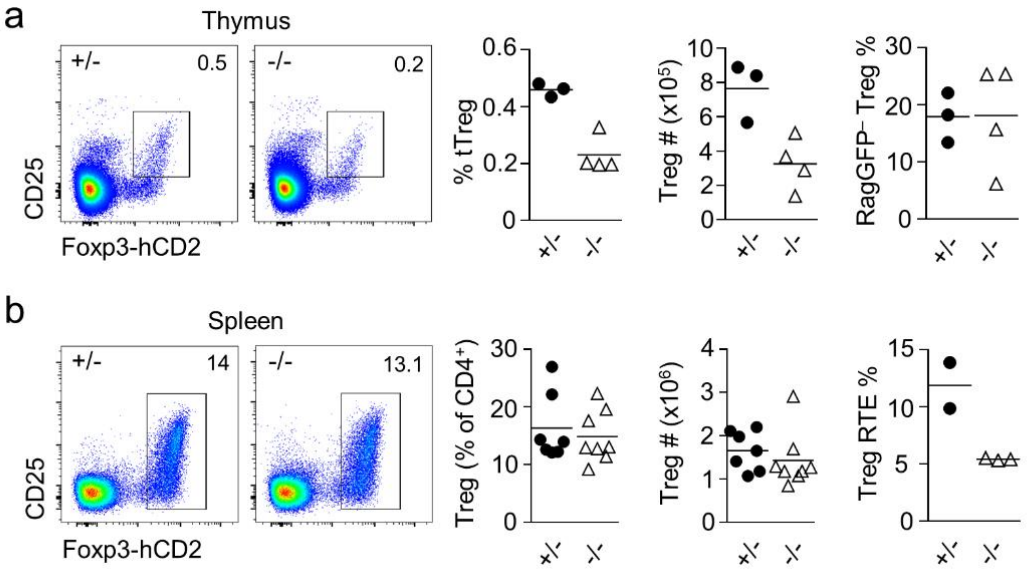


**Figure 4.1 Thymic Treg development is impaired in the absence of miR-181a/b-1 despite peripheral Treg recovery.** **(a)** Representative plots, frequencies and absolute numbers of tTreg cells ($CD4SP^+Rag\text{-}GFP^+CD25^+Foxp3^+$) in the thymus of heterozygous (+/–) and homozygous (–/–) miR-181a/b-1 knock-out mice. Right most graph indicates the frequency of $Rag\text{-}GFP^-$ thymic Treg cells. **(b)** Representative plots, frequencies and absolute numbers of Treg cells ($CD4^+Foxp3^+$) in spleens of heterozygous (+/–) and homozygous (–/–) miR-181a/b-1 knock-out mice. Right-most graph indicates the frequency of $Rag\text{-}GFP^+$ recent thymic emigrants (RTE). Data are representative of 2-3 independent experiments with **(a)** n=3/4, **(b)** n=7/8, where each data point represents one mouse. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using unpaired non-parametric Mann–Whitney rank-sum *U*-tests. When no *P*-value is indicated, comparisons revealed no statistical significance.

(Figure 4.2a). Consistent with previous reports[226,238,239], $CD25^+$ precursor 1b cells were the first to be generated, followed by the slower induction of $Foxp3^+$ precursor 1a cells, which were closely follwed by mature $CD25^+Foxp3^+$ Treg cells (Figure 4.2a). However in the absence of miR-181a/b-1, developmental dynamics were altered. The development of $Foxp3^+$ precursor 1a cells was slower and their numbers did not reach WT frequencies (Figure 4.2a). Consistent with this observation, mature Treg induction was slower and in reduced frequencies when compared to WT Tregs. Interestingly, $CD25^+$ precursor 1b cells behaved in a different manner. These cells were substantially elevated in young $GFP^{hi}$ cells from miR-181a/b-1-deficient mice (Figure 4.2a). To understand what may be causing such alterations in Treg dynamics we looked towards TCR signalling in developing thymocytes. In line with this, precursor 1b cells have been reported to receive stronger TCR signals during development[238,239,236], which may have elevated sensitivity to loss of miR-181a/b-1. To analyse TCR signalling we stained for Nur77, a surrogate marker for TCR signalling[341]. Nur77 detection using commercially available antibodies is weak and only reveals slight differernces in staining intensities regardless of the actual physiological shift in Nur77 expression. To combat this we labelled and then

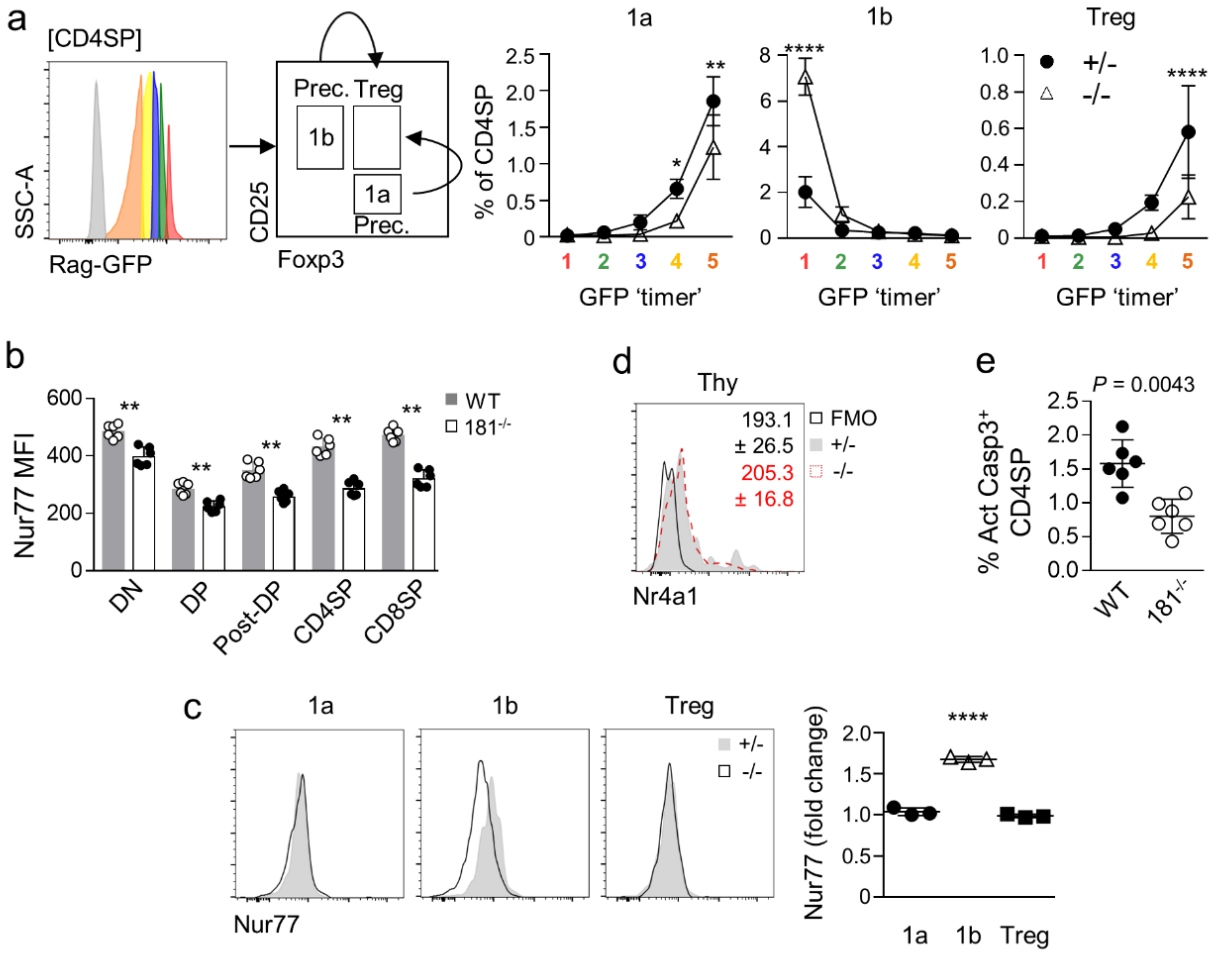


**Figure 4.2 . Treg development dynamics are slower in the absence of miR-181a/b-1. (a)** A molecular-timer based analysis of miR-181a/b-1-dependent tTreg development. CD4SP cells are divided into 5 groups (labelled by colour) depending on Rag-GFP expression to create a measure of 'pseudo-time'. Representative plot and gating strategy (left) and quantification (right). Graphs to the right display the percentage of each stage over 'pseudo-time'. **(b)** Nur77 intracellular protein expression in developing thymocyte subsets from WT or $181^{-/-}$ mice. **(c)** Nur77 intracellular protein expression in precursor 1a, 1b and Treg cells in the thymus. Fold increase of Nur77 expression in the heterozygous knock-out controls (+/–) compared to miR-181a/b-$1^{-/-}$ (–/–) mice. A fold change higher than 1 represents a decrease in total Nur77 expression in miR-181a/b-1-deficient mice. **(d)** RNA flow-cytometry analysis of the expression of Nr4a1 (encoding Nur77) by TCRβ+CD4+Foxp3+ thymic Tregs. Numbers indicate average MFI ±SD. **(e)** Percentage of activated caspase-$3^{+}$ CD4SP cells in WT and $181^{-/-}$ thymocytes. Data are representative of 1-2 independent experiments with **(a)** n=3/4, **(b)** n=3, **(c)** n=3, **(d)** n=6 and **(e)** n=6. Error bars indicate SD. Statistical analyses were performed using **(a)** two-way Anova, **(b)** multiple unpaired *t*-test and **(c)** paired *t*-test (* p=0.0011** p=0.0021, *** p=0.0002 and **** p<0.0001). When no *P*-value is indicated, comparisons revealed no statistical significance.

mixed both WT and miR-181a/b-1 samples, so that Nur77 staining could be carried out in the same tube, thereby controlling for small differences in fluorescences resulting from slight alterations in staining intensities. Analysis of Nur77 protein levels in developing thymocytes showed that in the absence of miR-181a/b-1, all major thymic cell populations dsiplayed a dampening of TCR signalling (Figure 4.2b). Further analysis of developing Treg subsets showed that both $Foxp3^{+}$ precursor 1a and mature Treg populations had normal levels of Nur77, whilst $CD25^{+}$ precusor 1b cells had consistenly

reduced Nur77 and therefore TCR signalling in the absence of miR-181a/b-1 (Figure 4.2c). Due to poor intracellular Nur77 antibody staining we optimised a new tool to stain and visualise mRNA transcripts via flow cytometry. Utilising this, we show that mature thymic Treg cells from either genotype expressed similar levels of Nur77 transcripts, *Nr4a1* (Figure 4.2d). Therefore, it is apparent that miR-181-a/b-1 limits TCR signal strength prior to the emergence of mature Treg cells. Cells undergoing negative selection-mediated apoptosis express activated caspase-3[35]. A significant reduction in apoptotic cells is seen in miR-181a/b-1-deficient CD4SP cells (Figure 4.2e). We therefore conclude that due to $CD25^+$ precursor 1b cells developing from strong TCR signalling interactions that are normally conductive to clonal deletion, the absence of miR-181a/b-1 dampens TCR signals and therefore negative selection of these cells, increasing their abundance. Moreover, loss of miR-181a/b-1 results in an overall reduction and delay of $Foxp3^+$ precursor 1a and subsequently mature Treg-cell formation.

To understand the altered kinetics and frequencies of miR-181a/b-1-deficent Tregs and their precursors, we assessed the status of immature developing thymocytes in the absence of miR-181a/b-1. On visual examination, slight differences exist in the presence of DN thymocytes (Figure 4.3a). Firstly, there appears to be a significant decrease in the frequencies of DN1 thymocytes,

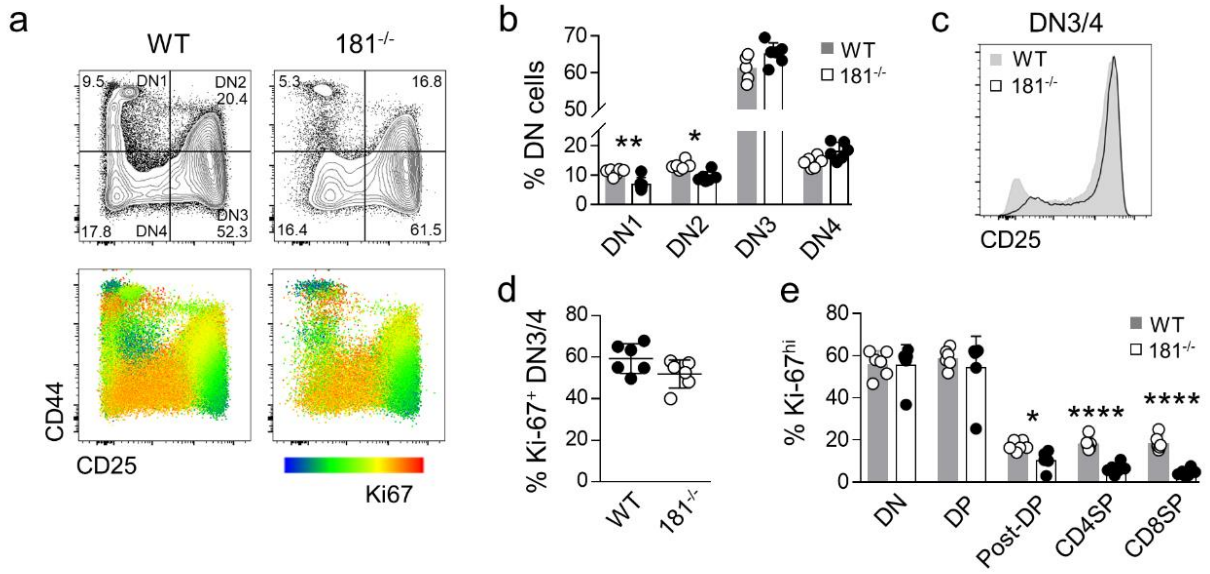


**Figure 4.3 Slower developmental kinetics in developing miR-181a/b-1$^{-/-}$ thymocytes. (a)** Representative plot of the distribution of thymic DN subsets in WT and 181$^{-/-}$ mice (top row). Heat map of the proliferation marker Ki67 overlayed onto thymic DN subsets (bottom row), with blue/green indicating no to low expression and orange/red indicating high Ki67 expression. **(b)** Frequencies of DN subsets in WT and 181$^{-/-}$ mice. **(c)** Representative histogram of CD25 expression in DN3 and DN4 cells ($TCR\beta^-CD4^-CD8\alpha^-CD44^-$). **(d)** Frequencies of combined DN3 and DN4 (DN3/4) $Ki67^+$ proliferating cells. **(e)** Frequencies of proliferating $Ki67^+$ total DNs, DPs, post-selection ($CD69^+TCR\beta^+$) DPs, CD4SPs and CD8SP cells in WT and 181$^{-/-}$ mice. **(a-e)** Data are representative of 1 experiment with n=6. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using Multiple unpaired non-parametric *t*–test (* p=0.0332, ** p=0.0021, *** p=0.0002 and **** p<0.0001).

which continues as cells progress to the DN2 stage (Figure 4.3a). This reduction in DN1/2 frequencies coincided with an increase in the later DN3 and DN4 stages, albeit insignificantly (Figure 4.3a). Loss of CD25 expression on $CD44^-$ cells correlates with the maturation of DN3 cells to the DN4 stage which then progress into DP thymocytes. When assessing CD25 expression on combined DN3 and DN4 cells, CD25 is lost from the surface of DN4 cells in more gradual fashion, likely resulting in DN4 cells which proceed to the DP stage with higher surface expression of CD25 (Figure 4.3b). Developing cells with increased CD25 expression initiated prior to the emergence of defined Treg-cell precursors could also explain the presence of increased $CD25^+$ precursor 1b cells in miR-181a/b-1-deficient mice.

The observed reduction in CD25 degradation could be a consequence of reduced proliferation. Ki67 accumulation occurs during S, G2, and M phases of the cell-cycle, following which it is degraded in G1 and G0. Ki67 can therefore be used as a graded marker of cell proliferation[342]. Utilising *ex vivo* Ki67 staining, we characterised the proliferation status of immature DN thymocytes. Cells pertaining to the DN1 phenotype ($CD44^+CD25^-$), had a surprisingly altered make-up in terms of proliferating populations (Figure 4.3c). These arbitrarily defined 'DN1' cells consist of a mixed bag of thymic precursors and haematopoietic cell populations, so further characterisation would require the addition of c-kit antibodies to accurately identify true DN1 cells and therefore make robust conclusions. Cells transitioning from the DN3 to DN4 stage have slightly reduced expression of Ki67 (Figure 4.3d), which could account for the retention of CD25 seen in Figure 4.3b. Upon wider examination of thymocyte subsets, no change in proliferation was detected in total DP cells, but following selection, DP and downstream SP cells showed a significantly reduced capacity to proliferate in comparison to controls (Figure 4.3e). It is therefore possible that slower developmental kinetics before the emergence of defined Treg cell precursors could account for the impaired development of $Foxp3^+$ precursor 1a cells. Additionally, sluggish kinetics at the late DN stages could result in higher retention of CD25, which could contribute to the increased presence of $CD25^+$ precursor 1b cells in the absence of miR-181a/b-1.

### 4.1.3 CTLA-4 is intrinsically upregulated in miR-181a/b-1-deficient mice

To identify whether the altered Treg developmental dynamics observed in miR-181a/b-1-deficient mice are a result of cell-internal or external factors, we generated competitive bone marrow chimeras (Figure 4.4a). Competitive BM chimeras with 1:1 mixtures of donor cells from WT and miR-181a/b-$1^{-/-}$ mice revealed that miR-181a/b-1-deficient Tregs were disadvantageous in thymic Treg-cell generation which extended to a competitive disadvantage in the periphery (Figure 4.4b). miR-181a/b-1 therefore acts in a cell-intrinsic manner during Treg cell development. We then assessed whether impaired generation in the thymus led to an altered Treg phenotype in the absence

of miR-181a/b-1. Notably, CTLA-4 – a protein involved in the suppressive function of Tregs[250] – was markedly increased in miR-181a/b-1-deficient Tregs (Figure 4.4c). To ascertain whether CTLA-4 dysregulation is a result of peripheral expansion, we also assessed intracellular CTLA-4 levels in miR-181a/b-1$^{-/-}$ and control Treg cells isolated from the same mixed BM chimeric mice. CTLA-4 levels were consistently higher in miR-181a/b-1-deficient Treg cells despite competition by their WT counterparts, indicating that CTLA-4 levels are regulated cell-intrinsically and do not depend on peripheral expansion (Figure 4.4d). We also tested whether alterations in tonic TCR signalling result in elevated expression of CTLA-4. Nur77 is a reliable read-out for tonic signalling[101,102]and Treg cells from miR-181a/b-1$^{-/-}$ and control mice expressed comparable levels of Nur77 transcripts (*Nr4a1*), suggesting that tonic signalling through the TCR is similar (Figure 4.4e). Overall, these findings further support the hypothesis that miR-181a/b-1-dependent control of CTLA-4 expression is elicited in the thymus and subsequently sustained in the periphery.

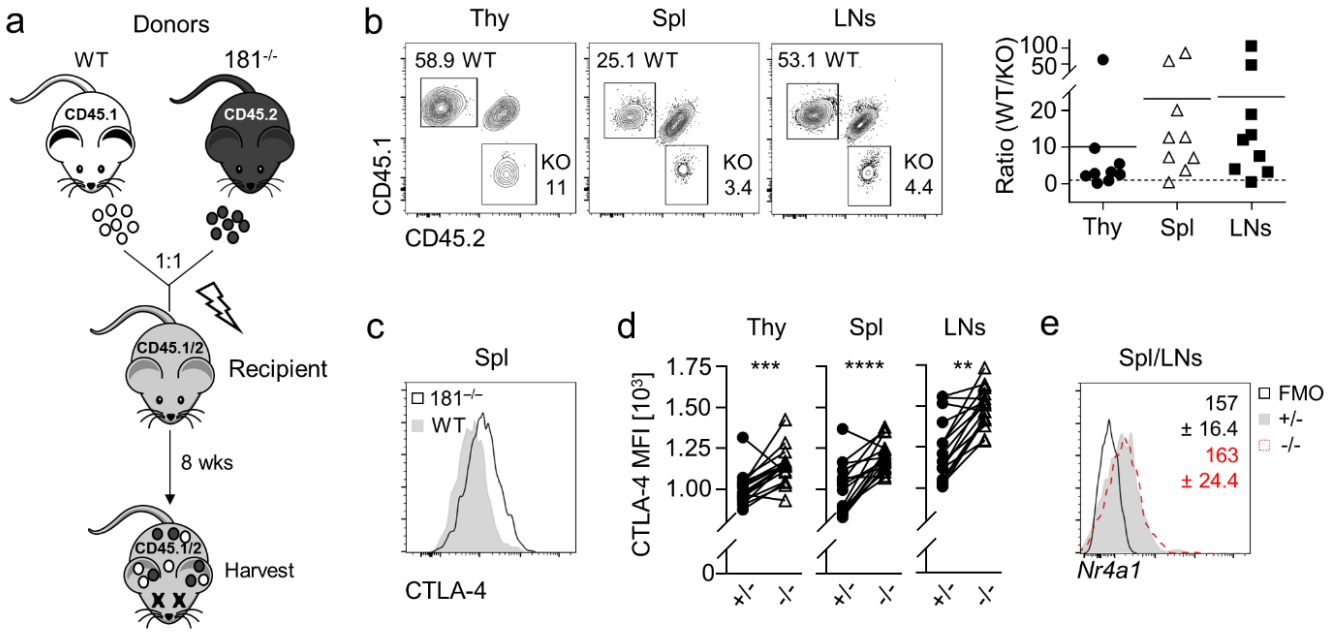


**Figure 4.4 CTLA-4 is cell-intrinsically upregulated in miR-181a/b-1-deficient mice. (a)** Schematic representation of mixed BM chimera generation. WT (CD45.1) and 181$^{-/-}$ (CD45.2) BM was isolated and mixed in an equal 1:1 ration before being transferred into lethally irradiates WT recipients (CD45.1$^{+}$CD45.2$^{+}$). **(b)** Identification of WT (CD45.1) and 181$^{-/-}$ (CD45.2) Tregs in thymus, spleen and lymph nodes of 1:1 mixed BM chimeras. Graph to the right represents the ratio of WT:181$^{-/-}$ Tregs. Ratios above 1 represent a competitive disadvantage for 181$^{-/-}$ Tregs. **(c)** Representative flow cytometric analysis of increased intracellular CTLA-4 expression in peripheral 181$^{-/-}$ Tregs in comparison to WT controls. **(d)** Cell-intrinsic upregulation of CTLA-4 in the absence of miR-181a/b-1. Lymphoid organs from mixed BM chimeras described in **(a)** were analysed for intracellular CTLA-4 expression in Tregs. Graphical representation of paired CTLA-4 MFI values are depicted. **(e)** RNA flow-cytometry analysis of the expression of Nr4a1 by TCRβ+CD4+Foxp3+ Tregs from pooled spleen and lymph nodes. Numbers indicate average MFI±SD. Data are representative of 2 individual experiments with **(b)** n=9, **(d)** n=18 and **(e)** n=6. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using paired non-parametric *t*–test (** p=0.0021, *** p=0.0002 and **** p<0.0001) **(d)**.

### 4.1.4 CTLA-4 is post-transcriptionally upregulated in miR-181a/b-1$^{-/-}$ mice

CTLA-4 protein increase was confirmed via independent methods (flow cytometry and immunohistochemistry), albeit transcript levels of CTLA-4 were unaltered[343]. Therefore, thymic generation in the absence of miR-181a/b-1 results in post-transcriptionally controlled up-regulation of CTLA-4 protein in Treg cells. Under *in vitro* stimulatory conditions, isolated miR-181a/b-1-deficient Tregs accumulated higher levels of CTLA-4 protein when compared to controls (Figure 4.5a). To ascertain whether increased CTLA-4 is due to altered protein degradation we stimulated Tregs in the presence of absence of the translation inhibitor cycloheximide (CHX). Inhibition of translation for 2 hours reduced CTLA-4 protein to similar levels in miR-181a/b-1$^{-/-}$ Treg cells and controls (Figure 4.5b). Given the higher protein levels when translation is active, degradation rates of CTLA-4 are higher in the absence of miR-181a/b-1. Furthermore, this data predicts that if protein degradation is intact, elevated levels of CTLA-4 protein arise as a result of increased rates of protein translation. To test this prediction, we assessed accumulation of CTLA-4 protein in Treg cells in the presence of bafilomycin, an inhibitor of lysosomal protein degradation, *in vitro*. Over the course of 3 hours, miR-181a/b-1$^{-/-}$ Treg cells accumulated significantly more CTLA-4 protein when compared to their WT counterparts (Figure 4.5c). Elevated levels of CTLA-4 in peripheral Treg cells in the absence of miR-181a/b-1 may therefore occur due to increased rates of translation.

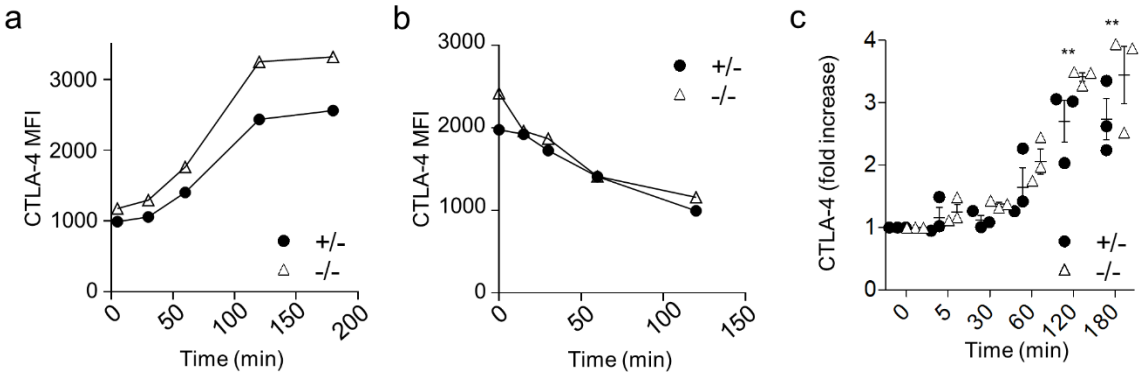


**Figure 4.5 Increased CTLA-4 as a result of increased translation in miR-181a/b-1-deficient mice. (a)** Increased accumulation of intracellular CTLA-4 after *in vitro* αCD3/CD28 stimulation of isolated Tregs **(b)** Enhanced degradation of CTLA-4 in the absence of miR-181a/b-1. Isolated heterozygous (+/–) and homozygous (–/–) miR-181a/b-1 knock-out Tregs from pooled spleen and LNs were incubated with Cycloheximide (CHX) and intracellular CTLA-4 expression was analysed after several time points to assess protein degradation. **(c)** Lysosome degradation inhibitor assay to assess protein translation. Isolated heterozygous (+/–) and homozygous (–/–) miR-181a/b-1 knock-out Tregs from pooled spleen and LNs were incubated with Bafilomycin and intracellular CTLA-4 expression was analysed after several time points. Data are representative of 2-3 independent experiments with **(a)** n=2, **(b)** n=2 and **(c)** n=3. Statistical analyses were performed using two-way Anova (p-values for effect of genotype, ** p=0.0021). When no *P*-value is indicated, comparisons revealed no statistical significance.

### 4.1.5 Changes in miRNA expression upon loss of miR-181a/b-1 do not directly contribute to CTLA-4 dysregulation.

CTLA-4 dysregulation due to an increase in translation in miR-181a/b-1-deficient thymocytes occurred despite CTLA-4 not being a direct target of miR-181a/b-1 miRNAs. We hypothesised that a reduction in TCR signal strength might result in failure of TCR-dependent miRNAs to reach physiological levels and that such miRNAs might directly target CTLA-4 mRNA. To assess this, we magnetically enriched $CD4^+CD25^+$ thymic Tregs from both miR-181a/b-1-sufficeint and -deficient mice and sorted mature $CD25^+Foxp3^+$ cells for small RNA-seq (Figure 4.5a). Consistent with the overall small changes in the transcriptome, we identified 4 miRNAs (miR-15b, miR-150, miR-342, and let-7g) that were moderately downregulated in Treg cells from miR-181a/b-$1^{-/-}$ mice (Figure 4.5b). However, *in silico* analysis of *Ctla4* mRNA using Targetscan7 and RNA22 provided no evidence for the existence of either canonical or non-canonical binding sites for any of these miRNAs, suggesting that elevated protein levels of CTLA-4 are not caused by reduced miRNA expression.

### 4.1.6 Increased suppressive capacity of miR-181a/b-$1^{-/-}$ Tregs is not mediated through proliferation

CTLA-4 has been shown to regulate the suppressive capacity of Tregs[344,345]. *In vivo* suppression assays demonstrated that due to increased CTLA-4, miR-181a/b-1-deficient Tregs have a stronger capacity to suppress lymphopenia-driven expansion of conventional T cells (Tconv) cells[343].

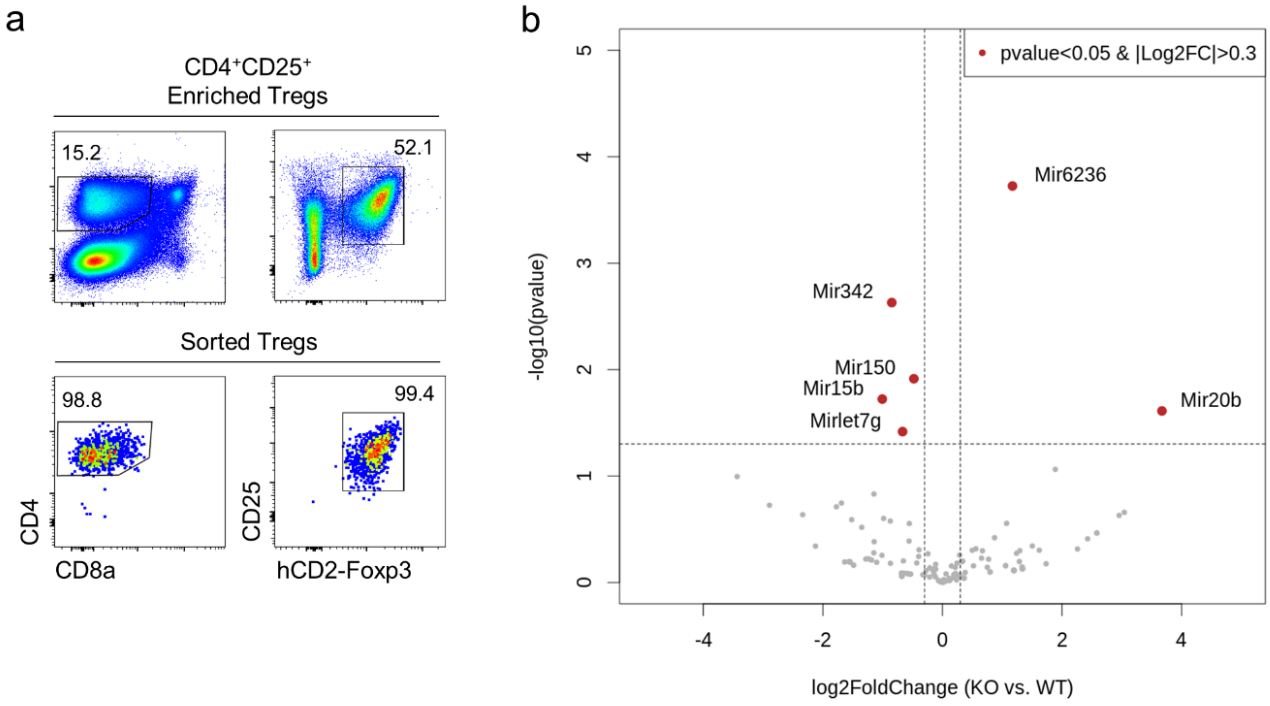


**Figure 4.6 Changes in miRNA expression upon loss of miR-181a/b-1 do not directly contribute to CTLA-4 dysregulation. (a)** Sorting strategy to isolated $CD4^+CD25^+Foxp3^+$ thymic Tregs from WT and miR-181a/b-1-deficient mice. **(b)** Small-RNAseq volcano plot of differentially regulated miRNAs in miR-181a/b-$1^{-/-}$ compared to WT thymic Tregs. Data from three independent replicates, with n=2 (pool) for each genotype. *Bioinformatics data was analysed by Nikita Verheyden.*

Suppressive capacity of Tregs over CD4 Tconv cells can be elicited via regulating their proliferation[346,347]. Therefore, we assessed the *in vivo* suppressive capacity of Treg cells by transfer of congenically labelled 1:1 mixtures of miR-181a/b-1-sufficient or miR-181a/b-1-deficient Treg cells (CD45.2) and CellTrace Violet™ labelled conventional naïve CD4 T cells (CD45.1) into *Rag1*$^{-/-}$ recipients. 5 days after transfer, proliferation of CD4 Tconv cells was assessed (Figure 4.6a). We observed no difference in proliferation of CD4 Tconv cells in the presence of either control or miR-181a/b-1-deficient Tregs (Figure 4.6b). Enhanced Treg suppressive capacity by increased CTLA-4 on miR-181a/b-1-deficient Tregs is either not a result of reduced proliferative capacity of CD4 Tconv cells or perhaps differences in proliferation cannot be visualised as early as 5 days – the upper limit to which CTV labelling can still be observed.

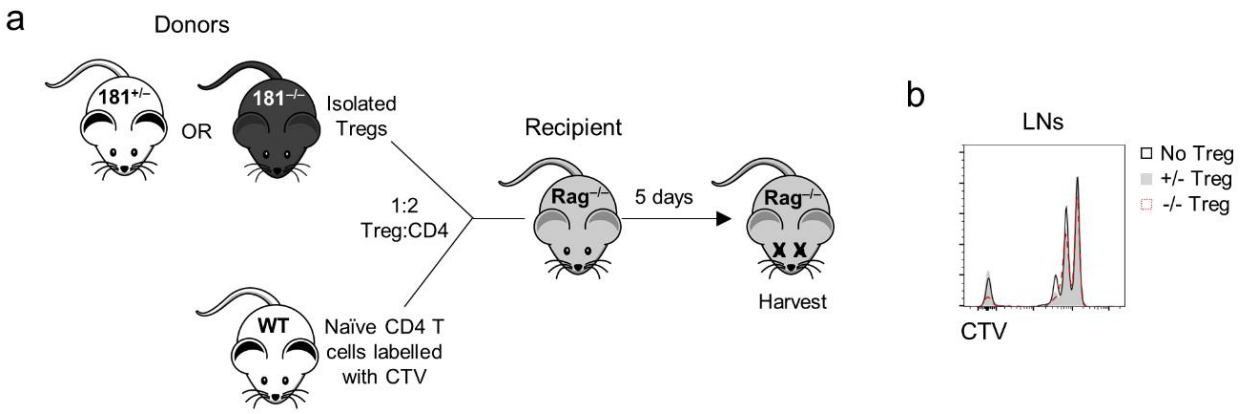


**Figure 4.7 CTLA-4-mediated increased suppressive capacity has no effect on naïve CD4 proliferation. (a)** Schematic overview of *in vivo* Treg suppression assay. Tregs were isolated from either heterozygous (+/–) and homozygous (–/–) miR-181a/b-1 knock-out mice and mixed in a 1:2 (Treg:CD4) ratio with isolated WT naïve CD4 T cells labelled with Cell-Trace Violet (CTV). Cell mixture is then intravenously transferred into Rag$^{-/-}$ recipient mice. Spleens and lymph nodes were harvested after 5 days. **(b)** Proliferation of CTV labelled donor CD4 T cells from mice receiving no Tregs, +/– Tregs and –/– Tregs. Data are representative of 2 independent experiments with n=5 per genotype.

# 5 Results Chapter Three

## 5.1 Conventional T cell selection & miR-181a/b-1

### 5.1.1 miR-181a/b-1 sets a threshold for conventional selection

Unlike agonist-selected populations with limited TCR repertoires, alterations in conventional selection are generally more complicated to assess. Populations containing a small range of TCRs respond to changes in selection with a discrete loss of cells, as seen in the case of iNKT[2] and MAIT cells in miR-181a/b-1-deficient mice[340]. Conventional cells with an expansive repertoire of TCRs most likely respond to changes in selection thresholds with simple shifts in their TCR repertoires, leading to no obvious differences in selection output. Transgenic TCR-specific immunogenic peptides that are modified to create APLs have been used to define an incredibly narrow window between positive and negative selection[115]. To utilise this APL system to visualise the selection thresholds in the absence of miR-181a/b-1, we began by generating transgenic OT-I x miR-181$^{-/-}$ mice. The OT-I transgene encodes a pre-arranged TCR specific for the chicken ovalbumin peptide, SIINFEKL (OVA), presented by MHC class-I and these mice possess an abundance of CD8$^{+}$ OVA-specific T cells[348]. APLs have varied affinities for the OT-I TCR due to single amino acid substitutions, which trigger known selection outcomes. The three APLs used here are SIINFEKL (N4), SIITFEKL (T4) and SIIVFEKL (V4), which

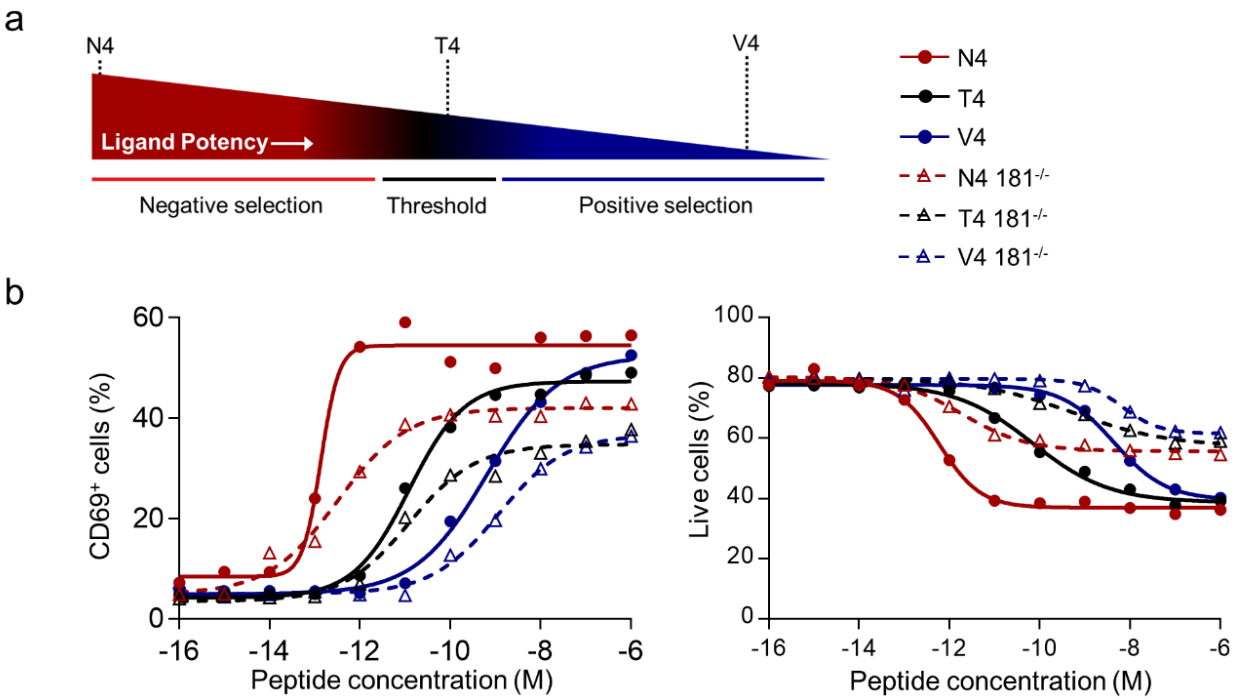


**Figure 5.1 Altered conventional selection in response to OVA-APLs. (a)** Schematic representation of selection outcomes in defined OVA-APLs. N4 (SIINFEKL) is a negatively selection peptide, while T4 (SIITFEKL) lies on the border between positive and negative selection and V4 (SIIVFEKL) induces positive selection. **(b)** OT-I pre-selection DP thymocytes were stimulated with peptide pulsed T2Kb APCs at various concentrations and then analysed for CD69 expression. **(c)** In the same assay system detailed in (b) the percentage of live cells was calculated using Zombie Aqua live/dead staining. Dotted lines represent thymocytes obtained from 181$^{-/-}$ OT-I mice.

trigger negative selection, lie on the border or result in positive selection respectively (Figure 5.1a)[115]. By incubating these peptides at various concentrations with pre-selection OT-I DP cells we assessed the strength of the TCR pool to the respective peptides. Pre-selection DP cells that have received a signal through their TCR upregulate CD69, which is used as a read-out. Using this method, we show that the absence of miR-181a/b-1 in pre-selection DP thymocytes upon OVA-APL stimulation results in a reduced maximal signalling capacity (Figure 5.1b). On inspection of cellular death in the same assay system using intracellular live/dead discrimination, it is apparent that miR-181a/b-1-deficient OT-I transgenic cells are remarkably less prone to cell death, despite the stimuli they recieve (Figure 5.1c). Ligand potencies were calculated using a non-linear regression to establish the peptide concentration that induces half the maximal CD69 expression (EC50). This value was then normalised to WT N4 values (Figure 5.2). Ligand potencies between WT and miR-181a/b-1-deficeint OT-I transgenic were similar for the negative selection N4 and borderline T4 peptides. On the other end of the spectrum, the weak positively selecting peptide V4 required double the ligand potency to activate miR-181a/b-1-deficient thymocytes. This signifies that double the amount of peptide was required to induce half the maximal response in comparison to controls, which is reflective of their reduced capacity to signal through the TCR. The presence of reduced maximal signalling, despite relatively normal EC50 values in the N4 and T4 peptides, is suggestive of a shift in the negative selection border with no or only minor alterations at the lower positive selection border. Furthermore, a shift in the negative selection border is indicated due to a dramatic reduction in cell death. We therefore propose that miR-181a/b-1 sets a threshold for negative selection.

### 5.1.2 Decreased negative selection in miR-181a/b-1$^{-/-}$ mice

*In vitro* studies using the FTOC experimental system concluded that miR-181a/b-1 modulated negative selection[1]. More recent reports using H-Y transgenic system showed no alteration in negative selection in male H-Y antigen mice in the absence of miR-181a/b-1, conflicting with previous reports[4]. It is important to note that the H-Y system as a transgenic TCR model is not without its

| | | | Maximal | CD69 | | |
|---|---|---|---|---|---|---|
| **WT** | Name | Sequence | response | EC 50 | 1/potency | Selection |
| | N4 (OVA) | SIINFEKL | 54.48 | 1,368E-13 | 1.0 | Negative |
| | T4 | SIITFEKL | 47.30 | 1,227E-11 | 89.7 | Border |
| | V4 | SIIVFEKL | 52.47 | 6,458E-10 | 4720.8 | Positive |
| **181$^{-/-}$** | | | | | | |
| | N4 (OVA) | SIINFEKL | 42.02 | 3,197E-13 | 2.3 | Negative |
| | T4 | SIITFEKL | 34.75 | 1,23E-11 | 89.9 | Border |
| | V4 | SIIVFEKL | 36.70 | 1,154E-09 | 8435.7 | Positive |

**Figure 5.2 Ligand potencies in the absence of miR-181a/b-1.** Data from figure 5.1b were analysed using non-linear regression to establish the peptide concentration needed to induce CD69 expression in 50% (EC50) of the pre-selection DP thymocytes from WT and 181$^{-/-}$ OT-I transgenic mice. 1/potency was normalised to WT N4 (OVA).

caveats. To address this open question, we looked to further characterising the thymic subsets in miR-181a/b-1-deficient mice (Figure 5.3a). Previous reports suggested no significant differences in the frequencies of thymic subsets of 8-week-old miR-181a/b-1-deficient mice[2]. Here we observed a shift towards post-selection populations in 16-week-old miR-181a/b-1-deficient mice (Figure 5.3a, b). It appears the effects of miR-181a/b-1 are more pronounced in aged mice, with significant increases in the DN cell pool, as well as post-selection DP cells and CD4SP cells (Figure 5.3b). This increase in CD4SP cells could represent both an increase in positive selection or a decrease in negative selection, leading to a larger number of cells passing through the DP stage and maturing into SP thymocytes. We further assessed thymic development using an *in situ* organotypic thymic culture system[136]. The principle of the thymic slice system involves extracting thymi from mice, cutting them into thin sections and the culturing these slices. The cortical and medullary regions of the thymus are maintained supporting thymocyte migration and selection. Specific peptides can be overlaid onto thymic slices, which are consequently endocytosed and presented on the surface of thymic DCs to developing thymocytes. TCR transgenic thymocyte subsets can then be overlaid onto slices, following which they naturally migrate into their appropriate microenvironmental niche and proceed through development. Overlaid cells can be distinguished using fluorescent labelling or using congenic markers. Here we incubated WT thymic slices with the negative selection-inducing OVA peptide, SIINFEKL (N4). miR-181a/b-1-deficient or -sufficient OT-I transgenic preselection DP thymocytes, were then sorted and overlaid onto slices and successful migration into the slice was assessed using congenic markers. After 24 hours, the selection status of overlaid thymocytes was assessed using antibody staining for the CD4 and CD8α co-receptors. OT-I transgenic thymocytes in the absence of OVA-peptide (N4) were mostly still blocked at the DP stage in both the presence and absence of miR-181a/b-1. Addition of OVA-peptide (N4) to control OT-I thymocytes induced a loss in DP thymocytes, signified by the downregulation of the CD4 and CD8α coreceptors (Figure 5.3c), consistent with previous reports in FTOC experiments[115]. miR-181a/b-1-deficient thymocytes displayed the same phenotype, albeit to a lesser extent, as much fewer cells presented as co-receptor deficient, suggesting that less cells underwent negative selection (Figure 5.3c).

One of the mechanisms behind negative selection is the activation of the caspase-3 apoptotic cascade[349]. We therefore stained for activated caspase-3 in developing thymocytes to visualise the number of cells undergoing clonal deletion. High expression was seen at the DP stage in control thymi, which is expected since the majority of negative selection occurs at this stage (Figure 5.3d). Consistent with our results suggesting that loss of miR-181a/b-1 causes a defect in negative selection, we see a dramatic reduction in the processing of caspase-3 (Figure 5.3d). Cells that have received strong TCR signals and are likely to undergo negative selection are high for CD5 and TCRβ[350]. By first isolating these cells with autoreactive potential and then assessing activation of caspase-3, we

see a clear reduction in caspase-3 processing to levels seen in pre-selection control cells in miR-181a/b-1-1deficient mice (Figure 5.3e). Consistently low activation of caspase-3 in control cells is likely due to cells that are newly entering the apoptotic cascade. In summary, the increase in SP thymocytes coupled with the reduction in apoptosis in miR-181a/b-1-deficient mice signifies that miR-181a/b-1 sets a threshold for negative selection.

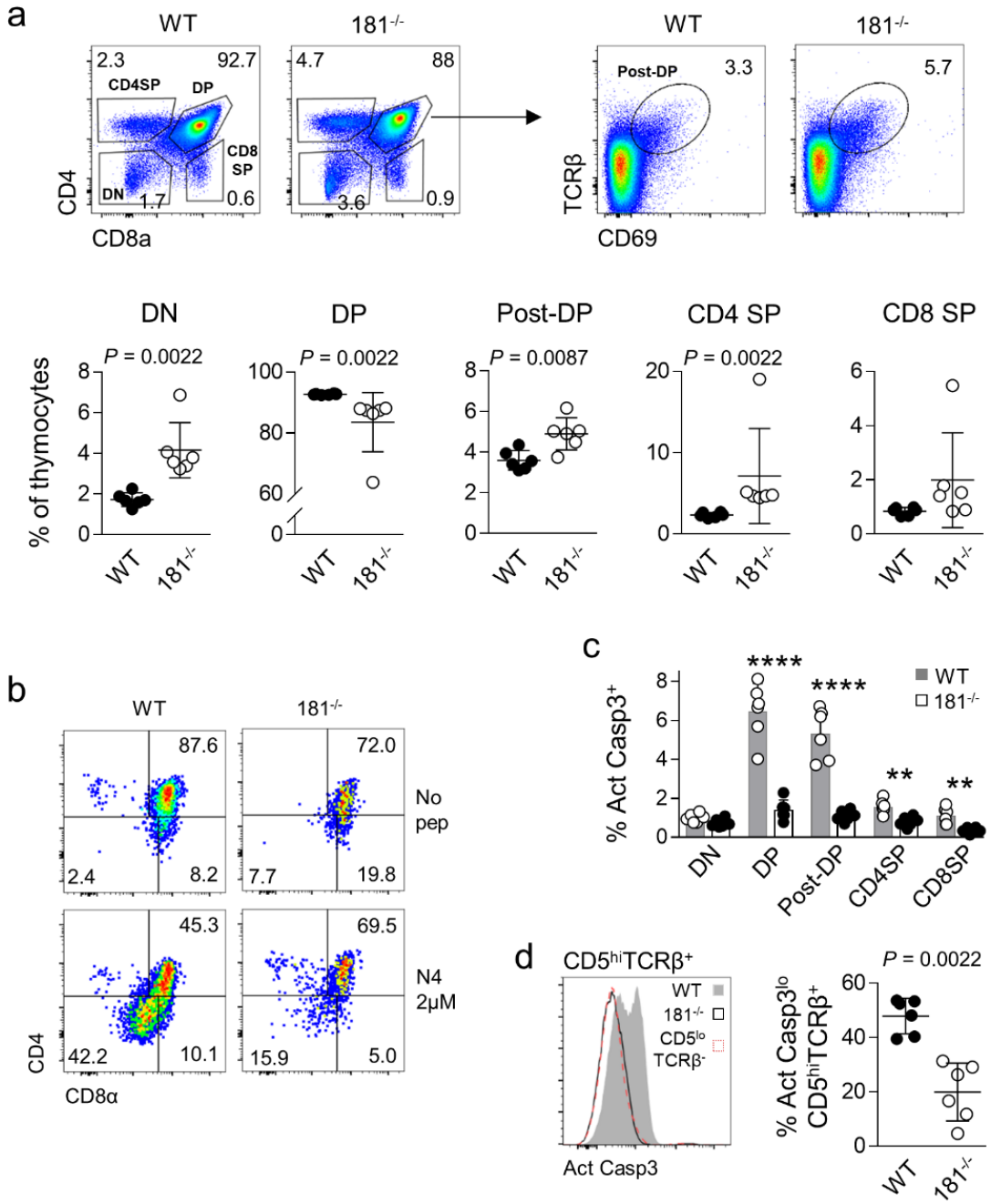


**Figure 5.3 miR-181a/b-1 potentiates negative selection. (a)** Gating strategy and frequencies of thymic subsets in WT and 181$^{-/-}$ mice. Mice were 16 weeks old. **(b)** Sorted pre-selection DPs from OT-I WT or 181$^{-/-}$ mice were overlaid on WT thymic slices loaded with N4 (OVA) peptide. After 24 hours the developmental progression was analysed. **(c)** The percentage of cleaved caspase-3 in developing thymocyte subsets. **(d)** Cleaved caspase-3 expression in CD5$^{hi}$TCRβ$^{+}$ cells undergoing negative selection. **(a-d)** Data are representative of 1 experiment with n=6 **(a, c, d**) and n=1 **(b)**. Numbers adjacent to outlined areas indicate percentages of each gated subset. Error bars indicate SD. Statistical analyses were performed using **(a, c)** Mann–Whitney rank-sum *U*-tests and **(d)** Multiple unpaired non-parametric *t*–test (* p=0.0332, ** p=0.0021, *** p=0.0002 and **** p<0.0001).

# 6 Results Chapter Four

## 6.1 The pri-miR-181a/b-1 transcript

### 6.1.1 Mapping the murine pri-miR-181a/b-1transcript

By this point, we have a decent understanding of the function of mature miR-181a/b-1 in the thymus, but research into what regulates the transcription of miR-181a/b-1 itself is lacking. Before we can understand the regulatory network that modulates the dynamic expression of miR-181a/b-1 during thymocyte development we must look to the primary miR-181a/b-1 transcript. To date, the miR-181a-1 and miR-181b-1 transcriptional unit and their promoter has not been established. DIANA miRGen v3 predicts the mir181a-1 transcription start site (TSS) to be approximately 70kb upstream of the mature miRNA sequences in a transcript region with abundant histone methylation as indicated using Ensembl software. This region also overlapped with an annotated lncRNA Gm4258. In an attempt to characterise this transcriptional unit, we performed RT-qPCR with several homologous primers at multiple sites upstream and downstream of the mature miR-181a/b-1 sequences, a method termed

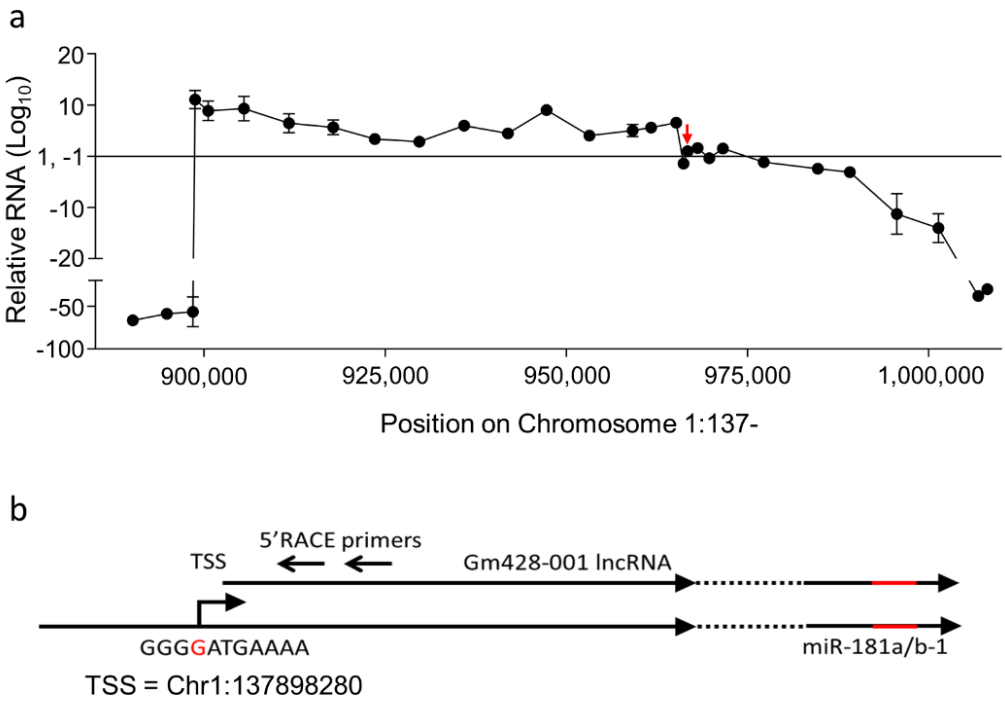


**Figure 6.1 Identification of the miR-181a/b-1 primary transcript. (a)** Mapping of pri-miR-181a/b-1. Total RNA was extracted from WT thymi and reverse transcribed. Following reverse transcription, multiple qPCRs were performed using primers homologous to mRNA up and downstream of the mature miR-181a-1 and b-1 (represented by each data point), in a method known as chromosome walking. Relative RNA levels were determined by normalising to the 'middle primers' located in between the two mature transcripts (indicated by the red arrow). Fold difference = $2^{(\text{Ct middle primer} - \text{Ct specific primer})}$. The numbers on the x-axis represent the genomic position, for example '900,000' indicates chromosome 1:137,900,000. **(b)** Schematic representation of TSS identification through 5'RACE. RNA extracted from WT thymi was used for 5'RACE and TSS was identified to correspond with the TSS for the lncRNA Gm4258-001.

'chromosome walking' (Figure 6.1a). A reference or 'middle' primer that amplified an area between the two miR-181a-1 and miR-181b-1 transcripts was used to determine the relative RNA levels. RT-qPCR signals were relatively constant for several kB upstream and downstream of the mature miR-181a/b-1 sequences in WT mouse thymocytes (Figure 6.1a). Variations in signal strength could be attributed to unequal RNA degradation rates, differences in primer efficiency or RNA secondary structure formation affecting reverse transcription efficiency. Relative constancy of RNA levels across the transcriptional unit suggests that the major form is an unspliced transcript. As we 'walked' upstream of the mature miR-181a/b-1 sites, RNA levels were relatively constant until they rapidly decline approximately 70kB upstream, signifying a dominant TSS (Figure 6.1a). Walking downstream of the mature miR-181a/b-1 transcripts revealed a gradual decline in RNA levels, suggesting an absence of a single dominant RNA polymerase II termination site (Figure 6.1a). At the estimated TSS, Ensembl data reveal abundant histone methylation, consistent with a TSS. To confirm and more precisely map the TSS we performed 5'RACE on total RNA isolated from WT mouse thymocytes, using PCR primers homologous to a region just downstream of the putative TSS. 5'RACE data confirmed the existence of this dominant TSS at position 137,898,280 on mouse chromosome 1 (Figure 6.1b).

### 6.1.2 Pattern of pri-miR-181a/b-1 correlates with mature levels

The relative abundance of mature miR-181a during thymic development shows a spike in miR-181a between the DN and DP stages followed by a rapid decrease in the CD4SP and CD8SP populations[295] (Figure 1.10). To assess whether levels of pri-miR-181a/b-1 corresponded with mature miRNA abundance we utilised fluorescent probes that bound to our putative pri-miR-181a/b-1 transcript and visualised fluorescence intensity using RNA flow cytometry (Figure 6.2). Here we see a similar pattern, whereby pri-miR-181a/b-1 levels are low in DN thymocytes and increase upon transitioning to the DP stages, which is followed by a rapid drop in expression in the CD4SP and CD8SP populations (Figure 6.2). Therefore, expression patterns of pri-miR-181a/b-1 correlate with the

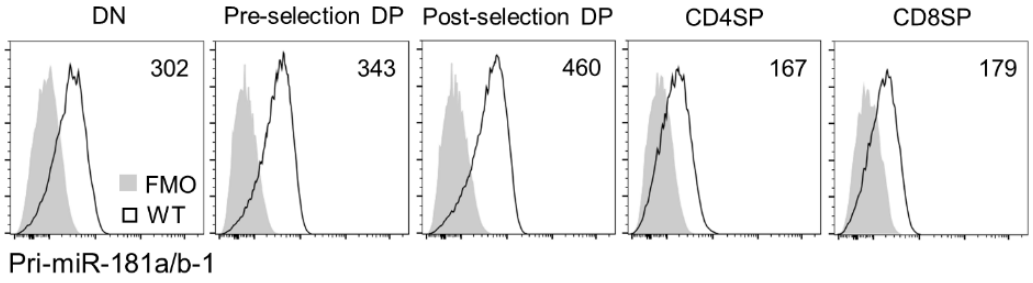


**Figure 6.2 Pri-miR-181a/b-1 expression corresponds with mature miR-181a/b-1 during thymic development.** RNA flow cytometry was performed on WT thymi with probes targeting the putative pri-miR-181a/b-1 transcript. Analysis of pri-miR-181a/b-1 in thymocyte subsets was performed and compared to FMO controls. Values on plots represent the median fluorescence intensity (MFI) of WT samples in their corresponding thymocyte subset.

abundance of mature miR-181a/b-1 supporting the idea that this long transcript is the host gene for pri-miR-181a/b-1.

### 6.1.3 Putative pri-miR-181a/b-1 transcript levels accumulate upon deletion of miR-181a/b-1

Our miR-181a/b-1 deficient mouse model was created by generating a germline deletion of approximately 250bp surrounding both mature miR-181 a-1 and b-1 sequences. We asked the question of whether lack of mature miR-181a/b-1 expression in thymocytes altered the abundance of pri-mi-181a/b-1. To do so, we took thymi from either WT of miR-181a/b-1-deficient mice and repeated the chromosome walking experiment established in Figure 6.1a. Fold increase was calculated by normalising to the housekeeping gene TBP. Here we saw that upon deletion of miR-181a/b-1 overall primary transcript levels increased (Figure 6.3). In addition, primary transcript levels after the excised mature miRNA site appeared to be in even higher abundance. We hypothesise that the accumulation of pri-miR-181a/b-1 in the absence of its mature miRNA transcripts it due to the lack of processing events initiated by the DROSHA/DGCR8 microprocessor complex.

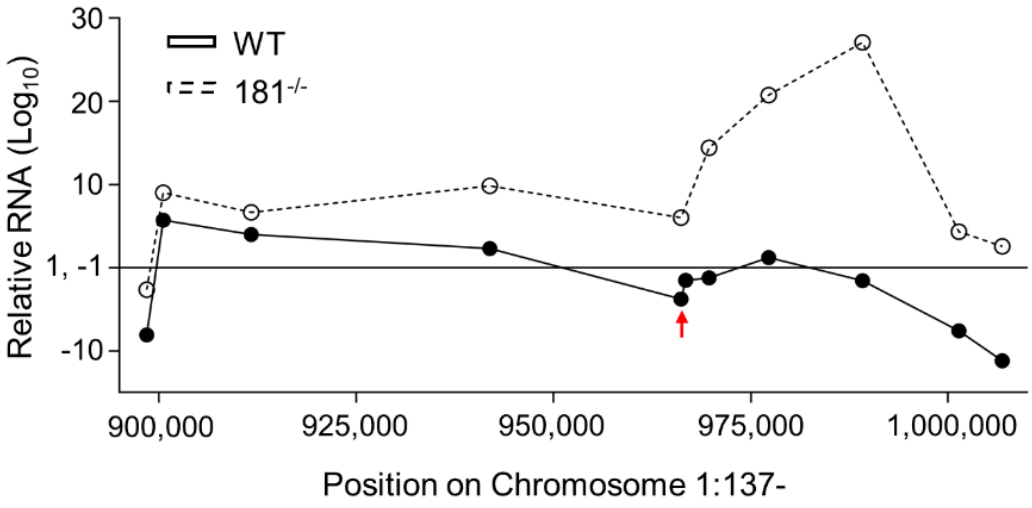


**Figure 6.3 Accumulation of pri-miR-181a/b-1 in the absence of miRNA processing.** Chromosome walk of RNA extracted and reverse transcribed from WT and $181^{-/-}$ thymi. Relative RNA is calculated by normalising to the housekeeping gene TBP. Red arrow indicates middle primer, which is only present in WT sample due to transcript knock-out in $181^{-/-}$ thymi.

# 7 Discussion

## 7.1 miR-181a/b-1 controls MAIT cell development

Low cell frequencies and lack of available antibodies has impeded the field of murine MAIT cell biology. Recent advances in MR1-tetramer technology have allowed us to reliably identify MAIT cell populations in humans and mice alike[201]. By using a combination of MR1-tetramer-based enrichment and genetic miR-181a/b-1-knock-out mouse models we were able to demonstrate that miR-181a/b-1 is required to drive the development of MAIT cells in the thymus at an early stage. Additionally, analysis of MAIT cells in miR-181a/b-1$^{-/-}$ mice co-expressing a *Rag1*$^{GFP}$ molecular timer provided new insight into the developmental dynamics of MAIT cells as well as their functional maturation.

Through interference with miRNA processing machinery, mouse models lacking canonically processed miRNAs can give us an insight into cellular functions and developmental processes that are heavily dependent on miRNA-mediated control. Comparing total miRNA-deficient models with individual miRNA knock-outs allows us to assess the overall contribution of that individual miRNA in said cellular or developmental processes[351]. In MAIT cells, the loss of miR-181a/b-1 mirrors the loss of all miRNA in T cell-specific Drosha-deficient mice[146]. It is therefore plausible to conclude that miR181a/b-1 constitutes a major player of the miRNA network controlling MAIT cell development.

Due to the paucity of MAIT cells in common strains of laboratory mice, there is a high risk that some cells captured in the MR1-5-OP-RU$^{+}$ tetramer gate are rare contaminants. This is even more problematic when detecting MAIT cells in miR-181a/b-1-deficient mice containing a 10-fold reduction in an already rare subset. Our experiments are complemented with dual-tetramer staining and parallel MR1-Ac-6-FP staining to support the analysis of *bona fide* miR-181a/b-1-deficient MAIT cells. Parallel studies using *Rag1*-deficient OT-I-transgenic mice which are bereft of MAIT cells, further complement the staining specificity of our MR1-5-OP-RU tetramers. We therefore were able to sufficiently analyse miR-181a/b-1 deficiency on remaining MAIT cell populations. Future MAIT cell research may benefit from utilising B6-MAIT$^{CAST}$ mice, which due to a genetic trait have higher Vα19 rearrangement and therefore approximately 20 times more MAIT cells than normal BL6 strains[209].

A major caveat of murine MAIT cell research is that it is very difficult to accurately identify MAIT cells without prior enrichment in a WT setting. Therefore tetramer-based MAIT cell enrichment was crucial for adequate characterisation of rare miR-181a/b-1-deficient MAIT cells. It is worth noting that via using this method, we are selectively enriching our population of interest which may lead to an underestimation of the effect of miR-181a/b-1 loss on MAIT cells. The same method has similarly been used when comparing the differences between both PLZF and Drosha-deficiency to WT mice[146].

Despite the potential drawbacks of enrichment, we reliably saw a ~10-fold reduction in MAIT cell frequencies in miR-181a/b-1-deficient mice, which corresponds to absolute numbers and frequencies obtained from unenriched lymphoid organs.

A three-stage model of MAIT cell development was recently proposed[146]. Using a *Rag1*$^{GFP}$ knock-in model as molecular timer to capture the kinetics of MAIT cell development *in vivo*, we were able to confirm the sequence of MAIT cell developmental stages previously established using the *in vitro* OP9-DL1 system. Of note, stage 1 MAIT cells contained uniformly high GFP expression, indicative of a sharp transition into stage 2. Stage 2 MAIT cells exhibited a broad distribution of GFP spanning more than 3 orders of magnitude. GFP half-life is approximately 56 hours and such a wide spread of GFP distribution is probably due to a highly proliferative state[9]. The appearance of GFP 'peaks' in stage 2 MAIT cells is also similar to what's seen in proliferation studies, such as CFSE labelling. In these experiments, cells tagged with a fluorescent dye halve their fluorescence intensity with every cell division forming a fluorescent readout that translates to a succession of spikes or peaks[352]. Here we speculate that the peaks observed in stage 2 MAIT cells may indicate that these cells are undergoing several rounds of division, thus 'diluting' their GFP. Lastly, stage 3 MAIT cells were homogeneously negative for GFP expression most likely representing tissue-resident cells within the thymus. iNKT cells were recently shown to exit the thymus at stage 2 (CCR7$^{+}$Rag-GFP$^{lo}$) then undergo effector maturation and colonise tissues[161]. Therefore stage 3 iNKTs are recirculating iNKT cells that reside for prolonged periods of time in the thymus[161,160]. Due to their similarities and tissue-resident transcriptional programs it is likely that stage 3 MAIT cells are also members of a recirculating thymic-resident pool.

Surprisingly, proliferation dynamics during MAIT cell development differed from those observed in developing iNKT cells. Stage 0 iNKT cells homogeneously undergo a large proliferative burst, which slows as the cells progress through development until late stage effector iNKTs predominantly cease proliferation[175,353–355]. In stark contrast, we show that MAIT cell development proceeds in an opposing manner. MAIT cells show little signs of proliferation until they reach stage 2, in which cells are heterogeneously positive for proliferation markers and *Rag1*-GFP. By the final stage of MAIT cell development, most cells are proliferating and have lost GFP expression. Due to the competitive nature of the MAIT and iNKT cell pools[146], perhaps the lower proliferation rate during early MAIT cell development can partially account for the low frequencies of MAIT cells in mice.

MAIT cell proliferation was selectively dwarfed at stage 2 but managed to recover later during development in miR-181a/b-1-deficient mice. This observation suggests that miR-181a/b-1 does not control proliferative processes in general, but rather that stage-specific loss of proliferation is a consequence of perturbed TCR signalling. This interpretation is consistent with the hypothesis that

miR-181a serves as a rheostat for TCR signalling during thymic selection[1]. Additionally, this finding parallels the consequences of miR-181a/b-1-deficiency on development of iNKT cells[2,3]. Furthermore, these data show that late stage proliferation in the thymus is unable to compensate for ineffective generation of both MAIT and iNKT cells.

Our observation of stage 2 MAIT cells retaining low GFP levels in peripheral organs indicates that some MAIT cells undergo early thymic export consistent with recent reports for iNKT cells[161,175]. Furthermore, evidence of transcription factor expression in peripheral MAITs in the absence of miR-181a/b-1, despite no expression in thymic MAIT cells, is an indicator of extra-thymic maturation. Complete functional immaturity of thymic MAIT cells despite the presence of at least some mature cells in the periphery suggests that stage 3 MAIT cells may have failed to undergo thymic export and be long-term resident cells rather than cells that have recirculated from the periphery. Additionally, MAIT and iNKT cells have been shown to have tissue-resident transcriptomic profiles as well as strong residency phenotypes in parabiosis experiments[160,356]. It was also shown that specific subsets of MAIT and iNKT cells, preferentially reside in certain tissues[160]. Developmentally immature cells would therefore benefit from peripheral plasticity, homing to tissues first and then undergoing further maturation with respect to the needs of their surrounding microenvironment. Therefore, we provide further evidence that early thymic export plays a role in the efficient maintenance of the peripheral MAIT cell pool.

In conventional thymocytes, the absence of PD-1 has been shown to lead to greater levels of negative selection[357]. PD-1 expression is seen in early IELp cells in the thymus which is suggested to inhibit costimulatory signals in order to avoid negative selection[350,358]. In the absence of miR-181a/b-1, MAIT cells appear to upregulate PD-1 at the early stages which could suggest that the developmental block lies after cells upregulate PD-1 on their surface. PD-1 expression is also sustained in later developmental subsets as well as in iNKT cells regardless of miR-181a/b-1 presence. Due to this is appears that the defect seen in miR-181a/b-1-deficient mice most likely doesn't pertain to costimulatory induced negative selection, which is seen in the absence of PD-1.

Innate-like lymphocytes have the capacity to share niches and compensate for each other. For instance, in the absence of CD1d, the selecting ligand of iNKT cells, MAIT cells are expanded in thymus and periphery[146]. Furthermore, we observed that NKT cells carrying a $\gamma\delta$ TCR are enriched in mice lacking miR-181a/b-1[326]. Our study suggests that iNKT cells and MAIT share a similar miR-181a/b-1-dependent developmental program precluding compensatory replacement of iNKT cells by MAIT cells in the absence of miR-181a/b-1. Consistently and similar to MAIT cells, iNKT cells fail to develop in the absence of all miRNAs[144,145,359].

In contrast to iNKT cells, MAIT cells fail to upregulate PLZF in the thymus in the absence of miR-181a/b-1[2]. The timing of PLZF expression during MAIT cell and iNKT cell development differs markedly. iNKT cells already express substantial amounts of PLZF at the earliest stage of development, whereas in MAIT cells, expression of PLZF rather coincides with functional maturation[2,184,146]. Of note, some peripheral miR-181a/b-1-deficient MAIT cells expressed PLZF, indicating that PLZF expression is independent of miR-181a/b-1 in MAIT cells. Engagement of the homophilic SLAM family receptor Ly108 amplifies TCR signalling in early iNKT cell precursors and results in PLZF expression[360,361]. During later stages of iNKT cell maturation, Jarid2 is induced by similar TCR stimulation and acts as a repressor of PLZF[362]. TCR signalling therefore plays a role in the transcriptional regulation of PLZF during iNKT cell development. In accordance, despite a lack of PLZF expression in miR-181a/b-1-deficient thymic MAIT cells, overexpression of the rearranged Vα19Jα33 TCRα chain was able to rescue PLZF expression in MR1-5-OP-RU tetramer-reactive cells. miR-181a/b-1-independent rescue of PLZF via modulating TCR signalling further suggests a potential uncoupling of PLZF and miR-181a/b-1 and an involvement of TCR signalling in the transcriptional regulation of PLZF during MAIT cell development. Nevertheless, expression of PLZF alone in the absence of miR-181a/b-1 in MAIT cells was insufficient for the differentiation of MAIT effector phenotypes.

Innate-like T cells are segregated into various subsets based on effector functions. γδ T cells develop into either $CD27^+$ IFNγ-producing or $CD27^-$ IL-17A-producing γδ T cells[267], whilst iNKT cells can develop into either iNKT1, iNKT2 or iNKT17 populations. Studies have shown that both γδ T and iNKT cell subsets develop from varying strengths of TCR signals during selection in the thymus[259,264,363,364]. Weak signals promote IL17-producing γδ T cells and iNKT1 cells whereas progressively stronger signals promote IFNγ-producing γδ T lineages and iNKT2 and iNKT17[264,363,364]. Corresponding with this, it is possible that based on their transcription factor expression, differing MAIT cell subsets may also develop from various intensities of TCR signalling. In mild support of this notion, we witnessed a slight difference in the effect of miR-81a/b-1 deficiency on the different subsets. T-$bet^+$ MAIT cell numbers are low in comparison to RORγt cells in controls, but they appeared to be less effected by the loss of miR-181a/b-1, allowing us to speculate that this subset may require weaker TCR signals during development, similar to their iNKT1 counterparts.

A surprising study demonstrated that a small fraction of iNKT thymocytes could originate from DN thymocytes and pass the DP stage in Rag-deficient mice[183]. Cells that develop through this pathway favoured the IFNγ-producing NKT1 lineage and showed patterns of preferential homing, such as high frequencies in the liver. In the case that a small population of both MAIT and iNKT cells follow this alterative 'DN pathway' of development, this may account for the remaining cells observed in miR-181a/b-1-deficient mice[2]. Our data suggest that most remaining miR-181a/b-1-deficient MAIT

cells that are able to completely differentiate favour the T-bet$^+$ MAIT1 population. If MAIT cells were able to differentiate through the DN pathway, this may account for the remaining population of cells in miR-181a/b-1-deficient mice. γδ T cells that also bypass the DP stage and differentiate from DN cells, also remain unaffected by loss of miR-181a/b-1, showing that it's robust effects do not begin until the DP stage of development[326].

Although our study establishes an essential role of miR-181a/b-1 in driving the differentiation of MAIT cells, the precise mechanistic details of miR-181a/b-1's regulatory footprint during the thymic development of innate-like T cells needs further investigation. Previously, it has been shown that miR-181a/b-1 interacts with negative regulators of TCR signalling, setting thresholds during T-cell development and selection[1,320,4,2]. With experiments supporting this[2,3,340] we therefore suspect a similar regulatory landscape for miR-181a/b-1 in both MAIT and iNKT cell development. Furthermore, it is possible that differential mechanisms of miR-181a/b-1 regulation occurs at multiple stages through development. Through TCRα overexpression we were able to overcome the TCR signalling and early expansion defect, rescuing MR1-5-OP-RU-tetramer$^+$ cell numbers and PLZF expression.

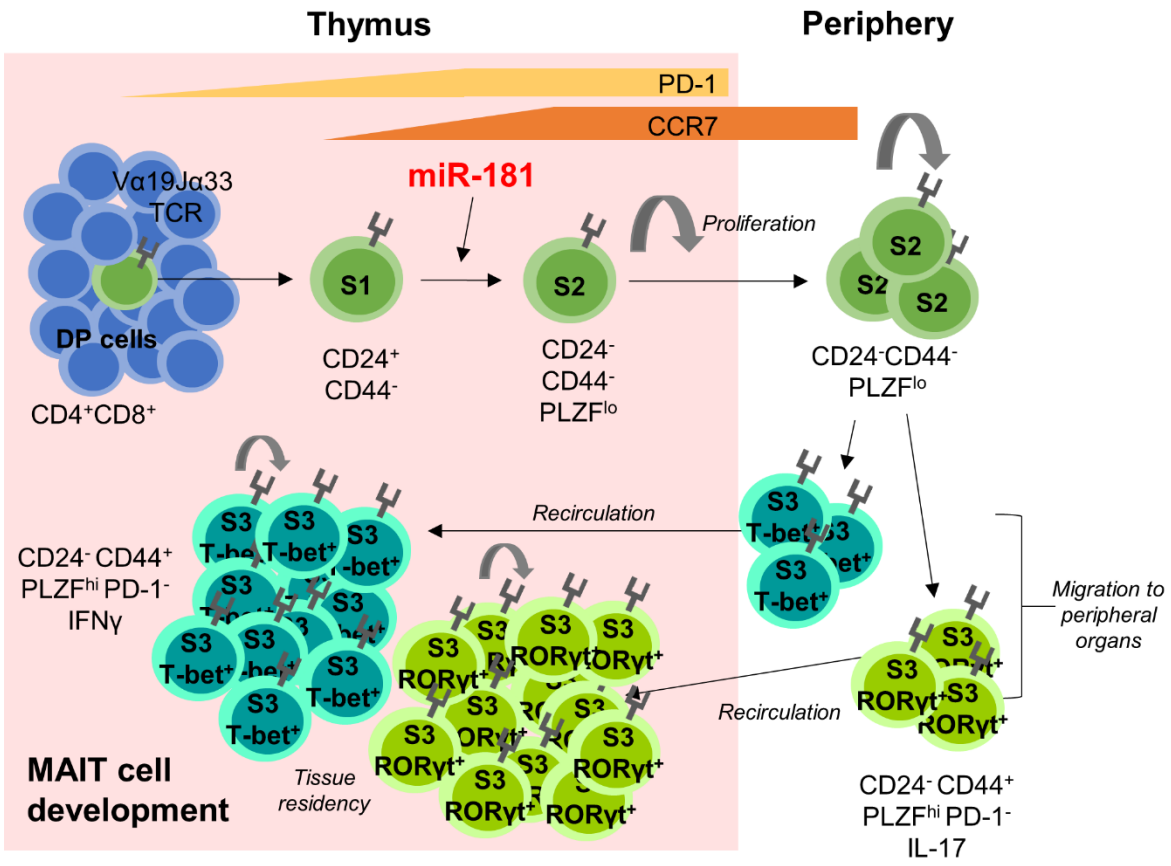


**Figure 7.1 Newly defined characteristics of MAIT cell development.** We have shown that the transition between stage 1 (S1) and stage 2 (S2) critically requires miR-181a/b-1 in a TCR-dependent manner. Additionally, S1 MAIT cells do not proliferate. Once they upregulate CCR7 and downregulate CD24, they begin to proliferate, and it is likely that they migrate out of the thymus. In the periphery, MAIT cells undergo effector differentiation and recirculate back into the thymus where they are highly proliferative and remain resident for long periods of time.

This method was unable to rescue effector subset differentiation, which could indicate a second stage of miR-181a/b-1 requirement but through a TCR signalling-independent mechanism. It is also possible that as a caveat of retroviral overexpression of the TCRα chain, the development of MAIT cells is altered. In support of this, MAIT cells that develop in Vα19Jα33 Cα$^{-/-}$ transgenic mice, exhibited a naïve phenotype and lacked expression of CD44 which coincides with effector differentiation[194,203]. This should be kept in mind when interpreting results from the MAIT TCR overexpression studies.

The mechanism behind the rescue of MAIT cell expansion through retroviral overexpression of the invariant MAIT cell TCRα chain is rather speculative at this stage. We predict that by increasing the level of transgenic TCR precursors, the spatial relationship between the MAIT cell TCRs and self-ligand-MR1-presenting DP cells improved. In a normal WT setting, DP thymocytes are exquisitely sensitive to antigen. Unlike peripheral T cells which require interactions with at least an estimated 100 pMHC complexes to undergo activation, developing thymocytes can require as little as one or two interactions with pMHC complexes to undergo clonal deletion[365,366]. Therefore, by increasing the levels of TCRs on the cell surface of DP thymocytes, we may also increase the likelihood interaction with a ligand-MR1 complex with high enough affinity to overcome the agonist selection threshold in miR-181a/b-1-deficient mice. This may also account for the increased numbers (compared to steady-state), whilst simultaneously recognising that the miR-181a/b-1-deficient TCR numbers were approximately half that of the WT. In contrast, the ratio of WT and miR-181a/b-1-deficient TCR expressing MAIT cells in spleens was closer to equal, which could be due to some form of peripheral homeostatic expansion. Further investigation is required to pinpoint the mechanism behind the rescue of MAIT cell numbers through retroviral TCR overexpression.

It is becoming more and more accepted that MAIT and iNKT cells share a very similar developmental process[160]. iNKT cell research is markedly more advanced than the adolescent MAIT cell field, providing a suitable framework to make educated hypotheses on MAIT cell biological processes. Like MAIT cells, iNKT cells are under strict miRNA control during their early stages of development as demonstrated in total miRNA-deficient mice[145,144,146]. Aside from miR-181a/b-1, studies have revealed multiple individual miRNAs that contribute to this elaborate web of regulation, including Let-7, miR-155, miR-150 and miR-17~92[304,302,305,306,308,309]. Let-7 family members were shown to uniquely target the transcription factor PLZF and be important in the development of iNKT cells[304]. Considering the involvement of PLZF in late MAIT cell differentiation, we predict that Let-7 regulation also plays a role in modulating the development of MAIT cells. TGFβR2 was shown to be a target of miR-17~92, perturbing iNKT cell development at stage 2[302]. The involvement of TGFβ signalling in MAIT cell development is currently undefined, leaving a suitable open question regarding its involvement and furthermore its regulation by miR-17~92. Overall, the field of miRNA regulation in MAIT cell biology is

in its earliest stages. We predict that in the upcoming years the complex network governing MAIT cell developmental biology will be unravelled.

In conclusion, our study shows that one individual pair of miRNAs, miR-181a/b-1 controls MAIT cell development in a TCR-dependent manner and highlights both parallels and key differences between MAIT cell and iNKT cell development.

## 7.2 miR-181a/b-1 modulates Treg development

In Results Chapter Two, we addressed the role of miR-181a/b-1 in Treg cell development and function. Here we demonstrated that intrathymic generation of Treg cells requires sufficient expression of miR-181a/b-1 to transduce the strong TCR signals required for Treg development. A reduced thymic Treg pool was reflected in peripheral RTEs in miR-181a/b-1-deficeint mice, despite homeostatic expansion recovering peripheral Treg numbers. The developmental legacy inscribed into miR-181a/b-1-deficient Tregs resulted in an intrinsic post-translational upregulation of CTLA-4 via an unknown mechanism. miR-181a/b-1-deficient Tregs displayed increased suppressive abilities *in vivo* but efforts to characterise the suppressive mode-of-action were insufficient. Overall, miR-181a/b-1 tunes TCR signalling during early Treg cell development leading to post-translational control of aberrant CTLA-4 expression.

Unlike MAIT or iNKT cells, the Treg pool possesses a large polyclonal TCR repertoire, with an array of affinities for self-peptides overlapping with both positive and negative conventional selection thresholds[234,228,229,367,230–233]. Due to this we predicted that clear observations of selection defects in the absence of miR-181a/b-1 might have been mild or completely confounded by small shifts in selection thresholds. To our surprise, when assessing Treg cell numbers in the thymus we saw a significant reduction in freshly generated thymic Tregs, which extended to Tregs that had recently exited the thymus. When observing Treg numbers both in the periphery and those that had recirculated back to the thymus we saw that upon emigration, the Treg pool was able to equilibrate in numbers. $CD4^+CD25^-$ RTEs from miR-181a/b-$1^{-/-}$ mice did not produce more iTreg cells upon transfer into lymphopenic hosts when compared to controls, suggesting that Treg-cell induction is not the primary mechanism to equilibrate peripheral Treg numbers in miR-181a/b-$1^{-/-}$ mice[343]. Additionally, TCR repertoire sequencing carried out by a collaborator showed that TCR clonality in the thymus of WT and miR-181a/b-1-deficient mice was similar, but peripheral Tregs had reduced TCR diversity. This suggested that the thymically generated pool of Tregs emigrating to the periphery was responsible for the equilibrated Treg numbers through homeostatic expansion.

Treg development is a two-step process that occurs via two separate pathways. When observing the temporal dynamics of these pathways we noticed some clear differences. Firstly, the $CD25^+$ precursor 1b pathway appeared to accumulate at a much higher level early after selection in miR-181a/b-1-deficient mice. This precursor population has been shown to span the border of negative selection in TCR transgenic mice in the presence of cognate antigen[368] and expresses very high levels of TCR signalling markers[239,238,236]. Negative selection also appears to constrain overall Treg development, leading to a bias toward selection of low-abundance self-peptides[234] and recent studies suggest that $CD25^+$ precursors 1b cells have a higher expression of apoptotic proteins that are triggered during clonal deletion[239]. Collectively, it can be interpreted that the $CD25^+$ precursor 1b population develops from stronger interactions though the TCR which are normally conductive for negative selection in conventional T cells. Additionally, this population appears to be subjected to negative selection. Our data show a large and rapid increase in this precursor pool in the absence of miR-181a/b-1, but there was no evidence of increased onward development to the mature Treg phenotype, which were in fact decreased in overall number. Reduced overall apoptosis in the CD4SP compartment as well as reduced levels of TCR signalling markers exclusively in the $CD25^+$ precursor 1b population in the absence of miR-181a/b-1 suggests an impairment of negative selection-induced apoptosis which could be a downstream effect of dampening TCR signals. It is possible that in the absence of miR-181a/b-1, Treg TCR clones with high self-reactivity that would normally undergo negative selection, have reduced TCR signal transduction and therefore do not trigger negative selection and the consequential apoptotic cascade. This may then explain the witnessed accumulation of $CD25^+$ precursor 1b cells, as cells that would normally be deleted from the repertoire. This is in line with our hypothesis that miR-181a/b-1 sets selection thresholds at steady-state.

Mice lacking the tyrosine kinase ITK have defects in negative selection[369]. The adaptor ADAP is downstream of ITK and is also required for negative selection[370]. Analysis of Treg precursors in both models revealed that inhibition of negative selection increased the frequencies of $CD25^+$ precursor 1b cells. Interestingly, discrepancies lied within the $Foxp3^+$ precursor 1a population[239]. $Adap^{-/-}$ mice showed no difference in frequencies, whilst $Itk^{-/-}$ mice had a substantial increase in these precursors coupled with what appears to be an increase in mature Treg frequencies, although mature Treg frequencies were not statistically analysed. This discrepancy was suggested to occur due to known increases in IL-4 production by iNKT cells in $Ikt^{-/-}$ mice[371,372], which does not occur in $Adap^{-/-}$ mice. Upon crossing $Ikt^{-/-}$ mice with $IL4Ra^{-/-}$ mice, $Foxp3^+$ precursor 1a cell frequency was decreased to WT levels as well as mature Treg frequency[239]. This study mimics what we see in our miR-181a/b-1-deficient mice. Here we have a hypothesised decrease in negative selection pathways as well as a reduction in IL-4-producing iNKT cells. Therefore, our data resembles $Adap^{-/-}$ and $Ikt^{-/-}$ x $IL4Ra^{-/-}$ mice which have an increase in $CD25^+$ precursor 1b cells that does not affect $Foxp3^+$ precursor 1a cells

and does not result in increased conversion into mature Tregs. Furthermore, it is important to note that this study does not completely replicate our findings of a reduced Foxp3$^+$ precursor 1a compartment. This is most likely due to Adap$^{-/-}$ and Ikt$^{-/-}$ mice having a disruption in negative selection alone. miR-181a/b-1 is suggested to set selection thresholds at both ends of the spectrum, therefore affecting both agonist selection and negative selection thresholds which could account for the decrease seen in Foxp3$^+$ precursor 1a frequencies and therefore mature Tregs.

The dampening of negative selection processes in miR-181a/b-1-deficient mice was also observed by artificially increasing TCR signalling in developing thymocytes and in paired transgenic antigen TCR model experiments[343]. Overexpression of the Nur77 family member *Nr4a2* in WT and miR-181a/b-1-deficient mice resulted in two different outcomes. Increased *Nr4a2* and hence TCR signalling in WT mice led to a decrease in mature Treg numbers, whilst the same increase in TCR signalling in miR-181a/b-1-deficient mice led to an increase in Treg numbers that went beyond steady-state WT levels. This indicated that in the WT system where negative selection is intact, increasing the strength of TCR signalling leads to clonal deletion of Tregs, providing further evidence for Treg development being restrained via negative selection. In the absence of miR-181a/b-1, negative selection is dysregulated resulting in an increased number of cells that can overcome the lower agonist selection threshold and therefore be diverted into the Treg lineage, whilst concurrently escaping clonal deletion. Additionally, clonal deletion of miR-181a/b-1$^{-/-}$ OT-II Tregs was also impaired in RIP-mOVA mice, whilst fewer donor-derived miR-181a/b-1$^{-/-}$ OT-II Treg cells developed[343]. Therefore, negative selection was decreased and fewer cells were diverted into the Treg lineage due an inability to overcome the lower agonist selection threshold stemming from dampened TCR signalling capacities.

The second pathway of Treg development was also altered, but in an opposing manner. Unlike their CD25$^+$ counterparts, the Foxp3$^+$ precursor 1a population traversed through development in a slower fashion and in reduced numbers in the absence of miR-181a/b-1. This precursor population characteristically shows much lower TCR triggering than their CD25$^+$ siblings[239,238,236]. Experiments have also shown that total Treg TCR repertoires do overlap with conventional T cell repertoires suggesting that some Treg selection lies below the conventional negative selection boundary[228,229,219,230,367]. It was revealed recently that the TCR repertoires of both precursor populations rarely overlap and Foxp3$^+$ precursor 1a cells were identified in the thymic cortex, a feature shared with conventional T cells undergoing positive selection[239]. This precursor population therefore appears to develop from the other end of the spectrum, requiring weaker interactions through their TCR and potentially undergoing a form of selection in the cortex through interactions with ubiquitous antigens – although this requires further experimental validation. Consistent with this, we observed a reduction in the development of these precursors coupled with reduced proliferation in the post-

selection DP and CD4SP compartments, which could account for their slower developmental kinetics in miR-181a/b-1-deficient mice. An overall reduction in the numbers of Foxp3$^+$ precursor 1a cells was also followed by an overall decrease in mature Treg formation, suggesting that this developmental defect is due to a diminished contribution of mature Tregs from the Foxp3$^+$ precursor 1a pool. We hypothesise, that similar to MAIT and iNKT cell selection, adequate miR-181a/b-1 levels are required to overcome a lower agonist selection boundary. In our knock-out model, TCR signalling is reduced in Treg precursors that would normally reach a threshold that allows them to divert into the Treg lineage, resulting in fewer cells reaching this requirement. Coupled with a reduced proliferation capacity, we see a decrease in these cells that can further differentiate and contribute to the mature Treg pool.

The DN phenotype of miR-181a/b-1-deficient mice revealed that DN4 cells had higher levels of surface CD25 than their WT counterparts. Coupled with a slight reduction in proliferation kinetics it is possible to hypothesise that higher expression of CD25$^+$ precursor 1b cells is a developmental remnant from the DN4 stage, due to inefficient dilution of CD25. Furthermore, post-selection DPs and CD4SP cells had a significantly reduced proliferation capacity. This reduction in proliferation could at least in part be responsible for the slower developmental kinetics and reduced frequencies of the Foxp3$^+$ precursor 1a population. Lastly, analysis of DN subsets revealed a slight alteration in early DN1 and DN2 cells in miR-181a/b-1-deficient mice. miR-181a/b-1 has been reported to target negative regulators of NOTCH signalling, which is required for early thymic progenitors to enter the T cell lineage and sustains early thymocyte differentiation[373]. In light of this, we cannot exclude that loss of miR-181a/b-1 results in a defect in the early DN1 and DN2 stages of development, but this is out of the scope of this thesis.

Onward progression between the early CD25$^+$ 1b or Foxp3$^+$ 1a precursors to the mature CD25$^+$Foxp3$^+$ Treg stage was shown to be independent of TCR signalling[238]. In this particular study, researchers make use of an inducible Zap70 which is required for TCR signal transduction. Zap70-deficient thymocytes are arrested at the DP stage and inducing Zap70 expression for a short 48-hour window allowed the assessment of persistent TCR signalling on Treg development. During this 48-hour window normal development of both Treg precursors occurred and upon cessation of Zap70-induced TCR signalling, onward development of Treg precursors to mature Treg continued normally, despite no new development of CD4SP cells. It therefore appears that TCR signalling is not involved in the progression of Treg precursors to mature Tregs, but is required prior to, during the selection of Treg precursors. This therefore corroborates our data that shows that despite an increase in CD25$^+$ precursor 1b cells, we do not see an additional increase in mature Treg numbers because the progression between these stages is independent of TCR signalling and most likely unaffected by miR-181a/b-1 loss. Progression of Foxp3$^+$ precursor 1a cells to mature Tregs most likely occurs

normally in miR-181a/b-1-deficient mice, albeit is reduced simply due to decreased input numbers. Previous studies have showed that a defect in the development of a single precursor population is not compensated for by the other in the case of IL-15Ra$^{-/-}$ mice[238]. IL-15 is required for Foxp3$^{+}$ precursor 1a cells to differentiate into mature Tregs, but not for CD25$^{+}$ precursor 1b cells. Similar to our study, IL-15Ra$^{-/-}$ mice have reduced numbers of Foxp3$^{+}$ precursor 1a cells and a corresponding reduction in mature Tregs. CD25$^{+}$ precursor 1b frequencies remain unaltered.

The requirement for IL-4 in Treg development was also shown to affect the Treg precursors differently[239]. *In vitro* incubation with IL-4 enhanced the differentiation of Foxp3$^{+}$ precursor 1a cells into mature Tregs but had no effect on CD25$^{+}$ precursor 1b cells. iNKT cells have been reported to be the main IL-4 producers in the thymus[162]. When assessing Treg precursor frequencies in CD1d$^{-/-}$ mice which contain no iNKTs, a reduction in the frequencies of Foxp3$^{+}$ precursor 1a cells was reported[239]. One could perhaps argue that the loss of iNKT cells observed in our miR-181a/b-1-deficient model could in turn lead to a reduction in Foxp3$^{+}$ precursor 1a generation due to reduced IL-4 signalling. We hypothesise that reduced IL-4 signalling in the absence of iNKT cells is not the prime cause of reduced Foxp3$^{+}$ precursor 1a cells observed in out model. Firstly, IL-4 producing iNKT cells are rare in BL6 mice, the genetic background of our knock-out model. These studies of CD1d-deficienecy were carried out on a BALB/c background which is known for having a much higher percentage of IL-4-producing iNKT cells and the same group suggests that the same studies on a BL6 background revealed no differences. Therefore, it appears that a reduction in iNKT-derived IL-4 most likely plays a minor or additional role in our observed phenotype of reduced Foxp3$^{+}$ precursor 1a cells.

To determine whether loss of miR-181a/b-1 and therefore a reduction in the thymically generated Treg pool had any functional consequences, a broad range of typical Treg markers were screened. Only one protein appeared to be significantly and consistently dysregulated in the absence of miR-181a/b-1, CTLA-4. CTLA-4 upregulation was found to occur cell-intrinsically when assessed using mixed BM chimeras and tonic signalling levels between peripheral Tregs were equal between WT and miR-181a/b-1-deficient mice, suggesting that this did not play a role in the upregulation of CTLA-4. This is consistent with a massive reduction in miR-181a/b-1 levels in peripheral Tregs, which should therefore allow WT levels of TCR signalling in the periphery. Unfortunately, we did not combine CTLA-4 protein analysis with our *Rag1*-GFP timer. In doing so, we could answer the question of whether the higher CTLA-4 expression is present in freshly generated Tregs or whether selective homeostatic expansion of thymic derived Tregs with high expression of CTLA-4 occurs. Furthermore, transcriptomic analysis revealed no alterations in any Treg-associated genes or CTLA-4 itself, suggesting that the increase in CTLA-4 is via a post-transcriptional mechanism. miR-181a/b-1 does possess non-canonical target sites in the coding sequence of the *Ctla4* mRNA, but targeted

repression was not observed in luciferase assays[343]. To delve further into what may be causing this increase in CTLA-4 we carried out a number of assays that revealed CTLA-4 upregulation resulted from increased translation rather than an increase in lysosomal degradation.

miRNA regulation occurs in complex networks, with single miRNAs simultaneously regulating hundreds of mRNAs, whilst a single mRNA can be a target of multiple miRNAs. Due to the apparent lack of direct targeting of CTLA-4 mRNA by miR-181a/b-1, we hypothesised that loss of miR-181a/b-1 interfered with a complex miRNA network, which in turn caused the derepression of CTLA-4. Small RNA-seq analysis revealed an alteration in miRNA networks with several miRNAs identified to be up or down regulated. Validation of the 4 downregulated miRNAs was performed via RT-qPCR[343]. Based on Targetscan7 and RNA22, none of these miRNAs carried canonical or non-canonical predicted binding sites in CTLA-4 mRNA. Lack of predicted targeting combined with relatively mild levels of downregulation led us to conclude that these mild adjustments in miRNA expression most likely do not contribute or contribute in a complex combinatorial manner to the post-transcriptional accumulation of CTLA-4 in miR-181a/b-1-deficient mice.

CTLA-4 mediates the suppressive capacity of Tregs[251,250]. *In vivo* suppression assays to test the functionality of Tregs generated in the absence of miR-181a/b-1 were performed[343]. Most likely attributed to their increased levels of CTLA-4, miR-181a/b-1-deficient Tregs had an enhanced suppressive function. Suppression may be elicited by controlling conventional T cell numbers by impacting proliferation or survival. To assess proliferation, *in vivo* suppression assays were set up using CTV to monitor proliferation over time. Unfortunately, preliminary studies showed that proliferation monitoring with CTV had an upper limit of 5-7 days. *In vivo* suppression assays performed for this shortened interval did not show a significant alteration in conventional T cell proliferation in the presence of miR-181a/b-1-deficient or -sufficient Tregs. Proliferation data obtained from longer term suppression assays was inconclusive and are not shown here. The results from these experiments are not fully conclusive and it is therefore still unknown whether Tregs limit proliferation or survival of conventional T cells. It is also possible that the sensitivity of this assay is ultimately too low to discriminate between a factor of 2 difference in Tconv numbers after 12 days of *in vivo* suppression from miR-181a/b-1-sufficient and deficient Treg. Finally, it is possible that due to expansion of both subsets over time, considerable suppression only occurs towards the end of a two-week *in vivo* suppression assay. Regrettably, we could not adequately address this question.

Intrathymic development of Treg cells depends on CD28-mediated co-stimulatory signals[152]. Thus, it might be possible that elevated expression of CTLA-4 by Treg cells in the thymus contributes to impaired development. CD28 is required early during Treg-cell differentiation prior to expression of CTLA-4[152]. Co-stimulation via CD28 is required for efficient generation of $Foxp3^{-}CD25^{+}$ Treg-cell

precursors, but less so during later Treg-cell development, suggesting that CD28 signalling protects thymocytes from clonal deletion[374–376]. Loss of CD28 signalling does not result in export of autoreactive cells into the periphery, indicating that it does not simply act as an amplifier of TCR signal strength[375]. Consistently, loss of CD28-mediated co-stimulation and loss of miR-181a/b-1 generate phenotypically distinct developmental defects, also supporting the notion that elevated levels of CTLA-4 in miR-181a/b-1$^{-/-}$ Treg cells are a consequence rather than cause of inefficient generation of Treg cells in these mice.

One recent study provided brief evidence that Treg cells generated through either precursor pathway had remarkably different suppressive capacities[239]. CD25$^{+}$ precursor 1b cells differentiate into Tregs that have a greater suppressive capacity than Tregs originating from Foxp3$^{+}$ precursor 1a cells in a model of EAE. Immunisation with MOG peptide causes the induction of EAE and most MOG-reactive TCRs were identified in the CD25$^{+}$ precursor 1b population. Altered contribution of these precursors to the mature Treg pool in miR-181a/b-1-deficient mice as hypothesised, would mean that a higher proportion of Tregs exiting the thymus would be from CD25$^{+}$ precursor 1b origin due to a reduction in Foxp3$^{+}$ precursor 1a cells. Homeostatic expansion that then follows thymic export may favour Tregs with CD25$^{+}$ precursor 1b origin. The mechanism behind the variations in suppressive capacity was left unanswered but it would be interesting to see if altered CTLA-4 expression played a role.

Another study which focused on engineering Tregs to express MOG reactive TCRs to combat EAE disease progression showed that the functional avidity of specific Treg TCRs for MOG determines their protective function against EAE[377]. Silencing of CTLA-4 in these Tregs decreased the protection afforded by MOG-reactive Tregs. Upon silencing, these Tregs retained low amounts of CTLA-4 and proliferated less than their MOG-reactive counterparts with normal CTLA-4 levels. From this, it is possible to hypothesise that due to increased input from the CD25$^{+}$ precursor 1b pool in miR-181a/b-1-deficient mice, Tregs have higher TCR avidities for self-peptides which could afford them with higher protective functions. Furthermore, increased CTLA-4 may be a result of increased CD25$^{+}$ precursor 1b progenitors, which could in turn cause increased expansion of these progenitors. To resolve a few of these questions we could assess the input of each of our miR-181a/b-1-deficient precursors to the mature Treg pool by sorting both precursors and injecting them back into thymi of mice on a different congenic background. An appropriate amount of time later, the conversion of these precursors into mature Tregs could be monitored and their levels of CTLA-4 analysed. This could provide an understanding on the relative contribution of each precursor to the mature Treg pool in the absence of miR-181a/b-1, whilst also identifying whether the source of increased CTLA-4 is a developmental legacy imprinted via a single precursor pathway.

Our data suggest that alterations in thymic selection caused by the absence of miR-181a/b-1 have long-term impact and are translated to increased suppressive activity of peripheral Treg cells. We therefore propose that the developmental legacy of TCR signal strength during agonist selection determines Treg-cell function in the periphery. Thus, altered TCR thresholds during selection might affect a Treg-cell's responsiveness to tonic signalling. Similar observations have previously been reported for both CD4 and CD8 Tconv cells[378,379]. The avidity of positively selecting self-peptides and thus strength of the TCR signal during selection determines the outcome of a T-cell immune response even of T cells recognizing the same foreign antigen with an identical avidity[378]. In contrast to Treg cells, differential reactivity to self-peptide by CD8 Tconv cells was accompanied by clear differences in gene expression profiles[379]. Although in Tconv cells the capacity for tonic signalling in the periphery contributes to distinct responsiveness to pathogens, thymically pre-determined levels of the feedback regulator of TCR signalling CD5 are likely to help control tonic signals[378]. Thus, the quality of protective T-cell responses as well as Treg-cell mediated suppression appears to be pre-set during thymic selection.

In summary, we propose that upon loss of miR-181a/b-1 the threshold for both agonist and negative selection are shifted resulting in more $CD25^+$ precursor 1b cells protected from clonal deletion, whilst simultaneously decreasing the amount of progenitors with high enough TCR reactivity to enter the $Foxp3^+$ precursor 1a pathway. Due to this a higher proportion of mature Tregs arise from the $CD25^+$ precursor 1b pathway. Homeostatic expansion of thymically generated Tregs is then responsible for the equilibration of peripheral Treg numbers. A developmental legacy of amplified CTLA-4 through a post-transcriptional increase in translation is present, coupled with heightened suppressive

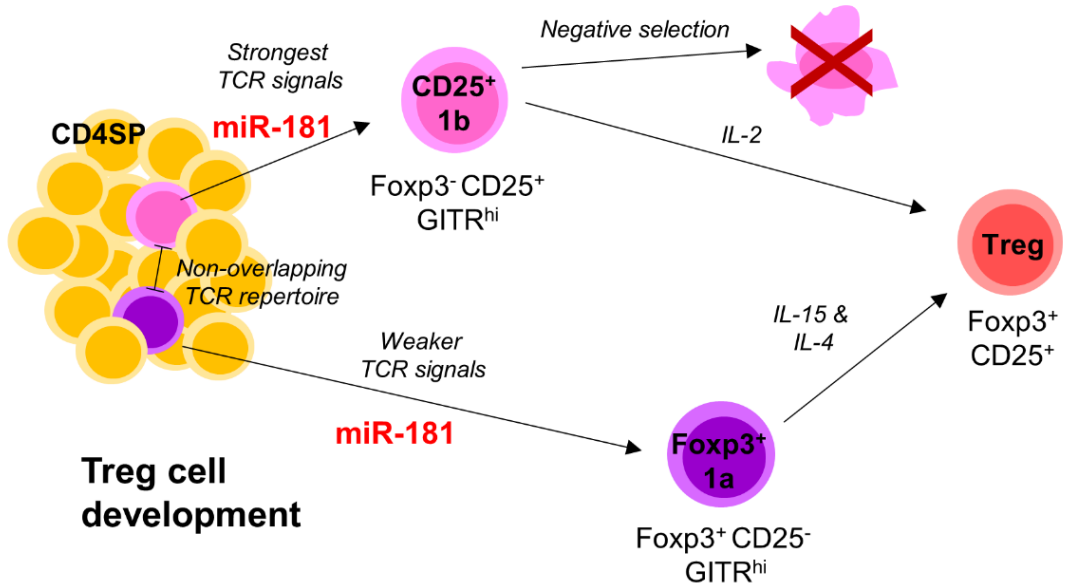


**Figure 7.2 Thymic Treg development. miR**-181a/b-1 modulation of TCR signalling is required for normal development of Treg precursors at stage 1 of their two-step pathway.

capacities. These outcomes could both be a result of increased input from higher self-reactive $CD25^+$ precursor Tregs that escaped negative selection and exited to the periphery in higher proportions than usual. Higher self-reactivity in the thymus may go hand-in-hand with increased CTLA-4 levels and these cells may also have been subjected to increased homeostatic proliferation in the periphery. This supports the notion that miR-181a/-1 is a modulator of TCR signalling thresholds during thymic development and selection.

## 7.3 miR-181a/b-1 modulates conventional selection

The role of miR-181a/b-1 in conventional selection was addressed in Results Chapter Three. Using a transgenic TCR model combined with APLs and assessing for clonal deletion-induced apoptosis, we identified that miR-181a/b-1 is required for thymic negative selection. Loss of miR-181a/b-1 reduced the number of cells able to signal through their TCR upon incubation with optimal (or higher) concentration of selection-inducing peptides. Cells receiving strong signals normally inductive to negative selection also presented with less activation of caspase-3 in the absence of miR-181a/b-1. Overall, it is likely that the alterations in selection from the loss of miR-181a/b-1 lead to an altered peripheral T cell repertoire, skewed towards higher self-reactivity. Therefore miR-181a/b-1 is fundamental in moderating TCR signalling levels during conventional selection and therefore generating a functional, self-tolerant peripheral T cell pool.

Experiments examining the TCR reactivity to peptides with defined selection outcomes and concentrations led us to believe that miR-181a/b-1 modulated the upper negative selection threshold alone. We are aware that miR-181a/b-1-deficient developing thymocytes have a reduced TCR signalling capacity, therefore in a situation where the selection outcomes were not altered, we would see the same pool of developing T cells, albeit with reduced TCR signalling and hence would require more peptide to induce half the maximal signalling capacity (EC50) therefore increasing the ligand potency values. In our case however, we don't see a change in ligand potencies of our samples stimulated with the strong and borderline agonist peptides, N4 and T4. This suggests that the TCR repertoire has in fact been skewed to higher reactivity considering TCR signals are diminished, yet the TCR pool is still able to respond to the same concentrations of agonist peptides. The reduced maximal signalling capacity may indicate that in the developing thymocyte pool of miR-181a/b-1-deficient mice there is a decrease in the frequencies of cells that are able to respond to OVA-APL stimulation. Therefore, if there was no shift in the lower positive selection threshold you would have more cells that are unable to respond to agonist peptide due to their decreased TCR signalling and therefore a reduced maximal signalling threshold. A special case exists for the weakest peptide, V4. In pre-selection DP thymocytes lacking miR-181a/b-1, stimulation with the weakest APL V4 which is

normally inductive for positive selection, resulted in the same reduced maximal signalling capacity but this time with higher EC50 and ligand potency values. Simply, this implies that almost double the concentration of peptide is needed to stimulate half of the thymocyte pool with the weak V4 ligand. Therefore, selection with peptides at the weak end of the spectrum by cells exhibiting a reduced TCR signalling capacity requires increased concentrations to generate a response. From this, it appears the miR-181a/b-1 is responsible for heightening TCR sensitivity in conventional thymocytes to detect for autoreactive cells and therefore eliminate them through negative selection.

In conjunction with OVA-APL stimulation assays, we assessed thymic cell subsets in miR-181a/b-1 mice to see if they contained any signs of altered thymic selection. Increased frequencies of CD4 and CD8 SP thymocytes is likely due to the decrease in negative selection observed in the OVA-APL stimulation assays. The fact that this increase in SP frequencies became more pronounced in older mice may be an indicator that miR-181a/b-1 tuning of TCR signals in the thymus is more important throughout ageing. A further understanding of miR-181a/b-1 and the effects of ageing would benefit from the characterisation of the pri-miR-181a/b-1 to identify whether factors that increased or decreased with age modulated the expression of miR-181a/b-1. Additionally, the levels of caspase-3 processing in thymocyte subsets, including the ones that have received strong signals inductive for negative selection, were massively reduced. Upon clonal deletion the apoptotic cascade involves the activation of caspase-3. Due to this it seems that less miR-181a/b-1-deficeint thymocytes are undergoing apoptosis at the selection stage, which is reflective of reduced negative selection.

The decrease in negative selection observed in miR-181a/b-1-deficient mice pertains to higher emigration of self-reactive T cell clones into the periphery. On first thought it might appear that regardless of the TCR repertoire, miR-181a/b-1-deficient T cells should signal less and therefore be less reactive in the periphery. This is not an accurate assumption as levels of miR-181a/b-1 in peripheral T cells are so low in comparison to developing thymocytes, that the basal levels of miR-181a/b-1 are unlikely to affect TCR signalling[4,343]. Therefore, in the wake of WT levels of TCR signalling, a more autoreactive peripheral T cell pool in theory should be more conductive to spontaneous autoimmunity. Interestingly, we do not observe any signs of autoimmunity in miR-181a/b-1-deficient mice which is discussed below (7.5 Conclusion).

Quantitative understanding of the control elicited by miR-181a/b-1 during conventional selection would give us a deeper insight into its functions and the mechanisms behind them. Downstream applications involving the modification of miR-181a/b-1 to alter TCR sensitivity and therefore peripheral repertoires would also highly benefit from such an understanding. Introduction of defined concentrations of miRNA into respective miR-181a/b-1-deficeint mice through intrathymic injections

of miRNA mimics would add to the discourse. On top of this, the impact of miR-181a/b-1 on positive selection remains unaddressed here. Unlike negatively selected cells which require rapid and robust ERK activation, cells undergoing positive selection require more gradual and sustained ERK activation[67]. Therefore, analysing intracellular ERK activation over time following stimulation with OVA-APLs of defined selection outcomes would be one way to address this question. Peripheral T cell auto-reactivity can also be addressed upon response to microbial challenge, in which genetically modified *Listeria monocytogenes* (Lm) strains express various OVA-APLs[128]. Monitoring the activation and expansion of OVA-specific T cells upon Lm-OVA-APL challenge could be a means for assessing the self-tolerance of the peripheral T cell pool.

When forming conclusions about miR-181a/b-1 and its role in thymic selection, it is important to consider not only the direct, but also the indirect effects of the miRNA's absence. As we have already seen, loss of miR-181a/b-1 leads to loss of iNKT[2,3] and MAIT cells[340], which may influence thymic homeostasis. NKT2 and NKT17 cells have been reported to produce RANKL which regulates the differentiation of mTECs, promoting Aire expression[163,161]. Qa2 expression is upregulated during late T cell maturation in the thymus. NKT1 cells were also implicated in supporting Qa2 expression on CD4 and CD8 SP thymocytes, through their secretion of IFNγ[161]. Moreover, NKT2 cells were recently shown to mediate thymic emigration via the secretion of IL-4 and IL-13[162]. In lieu of such data implicating multiple roles for iNKT cells in thymic development, it is important to consider the absence of iNKT cells in miR-181a/b-1-deficient models that may also influence conventional selection outcomes. Mixed BM chimeras would be a method to control for this. Additionally, the regulatory roles of MAIT cells in T cell development is currently unknown, but due to their strong parallels with iNKT cells, their absence should be taken into account.

## 7.4 The primary miR-181a/b-1 transcript

miRNA research is heavily focused on the regulatory network modulated by individual miRNAs. Here we begin to address the importance of moving the focus upstream and investigating what modulates the miRNA itself. To first understand this, characterisation of the primary miRNA transcript is needed. Previous research has shown that in humans the primary transcript for the miR-181a/b-1 subset is very long and dynamically regulated in NK cells in response to different cytokines[380]. For miR-181a/b-1, we have provided evidence for a long primary transcript which extends approximately 70kB upstream from the site of the mature miR-181a-1 and b-1 transcripts. Pri-miR-181a/b-1 levels appear to accumulate in the absence of mature miRNA processing and expression levels follow the same pattern as mature miR-181a/b-1 levels obtained from published qPCR data in developing

thymocyte subsets. Overall, we have identified the pri-miR-181a/b-1 transcript, opening the doors for future characterisation.

In general, pri-miRNA transcripts give rise to pre-miRNA which are relatively short, approximately 50-70bp[381]. Originally it was believed that mature miRNAs stemmed from short pri-miRNAs only several hundred to a few kilo base pairs long[285,382]. This changed when it was identified that most miRNA genes ranged from tens of kilobases to approximately 170kB long[295]. Therefore ~99.95% of a pri-miRNA is transcribed, processed to give rise to pre-miRNA and then discarded. Due to efficient processing by the microprocessor complex, methods such as northern blot to identify pri-miRNAs have proven difficult. Here we used a 'primer walk' method to roughly visualise transcript expression upstream and downstream of the mature miR-181a-1 and b-1. This indicated a dominant TSS approximately 70kB upstream of the mature miRNAs. Histone modifications surrounding the putative TSS, such as H3K4me3 peaks, corresponded with reported data suggesting that miRNA genes carry chromatin modifications similar to that of protein-coding genes[383]. Our miR-181a/b-1-deficient mice carry an approximate 250bp deletion surrounding both mature transcripts[2]. Due to the absence of microprocessor recognition, the pri-miRNA remains unprocessed in our miR-181a/b-1 knock-out mouse. This resulted in the accumulation of our putative pri-miRNA, corresponding with similar experiments that remove microprocessor intermediates to study pri-miRNAs[295,147,285,384]. Furthermore, total thymic RNA-seq data comparing WT and miR-181a/b-1-deficient mice revealed a similar transcript-wide increase in expression of our putative pri-miRNA in the absence of miR-181a/b-1 processing (data not shown).

It is relatively unclear why pri-miR-181a/b-1 or pri-miRNA transcripts in general are so long. It may be that the remaining transcript functions as a lncRNA or is involved in regulating pri-miRNA processing. *In vitro* data suggests that processing of stem-loop-containing RNAs occurs at different efficiencies which could be due to distal *cis* elements in flanking pri-miRNA transcripts[301,381]. To begin to understand the function of the remaining 70kB of pri-miR-181a/b-1, we performed RNA-FISH experiments to visualise its localisation within the cell. Unfortunately, the mouse fibroblast cell line used (3T3) expressed very low levels of pri-miR-181a/b-1 that localised to one or two focal points within the nucleus (data not shown). Future RNA-FISH experiments would benefit from assessing pri-miR-181a/b-1 in developing thymocytes due to its known high expression and until then we cannot make any strong conclusions. Generating a mouse model that is deficient in the pri-miR-181a/b-1 transcript yet retains mature miR-181a/b-1 expression may also be a way to assess the function of these long flanking regions.

Studies to characterise the promoter region through luciferase assays and identification of transcription factor binding sites would be beneficial to understand what induces a dramatic increase

in transcription of pri-miR-181a/b-1 during T-cell development. Perhaps identification of specific transcription factors the regulate miR-181a/b-1 expression may provide another target to artificially modulate miR-181a/b-1 expression without using antagomirs or miRNA mimics. The understanding of what induces the miRNA itself, seems just as vital as the understanding of what genes miRNAs themselves modulate, to piece together the complex puzzle of gene regulatory networks.

## 7.5 Conclusion

In summary, we present here a single miRNA subset with broad implications in T-cell development. In disagreement with central dogma that individual miRNAs generally provide weak to moderate modulation over cellular pathways, we present the miR-181 family subset, miR-181a/b-1 as an efficient regulator of TCR signalling pathways. Due to the sensitive nature of TCR signalling during thymocytes selection, miR-181a/b-1 elicits gross effects, which are essential for central tolerance and generating a functional self-tolerant peripheral T cell repertoire. From our studies it is clear that miR-181a/b-1 heightens TCR sensitivity in developing thymocytes, allowing certain subsets of autoreactive cells to pass a selection threshold and differentiate into agonist-selected T cells. In doing so, our data also suggests that miR-181a/b-1's effects are not just limited to the agonist selection checkpoint, but also the upper negative selection threshold. By heightening the TCR sensitivity to self-pMHC, miR-181a/b-1 aids in the detection and subsequent elimination of autoreactive thymocytes. We therefore conclude that miR-181a/b-1 is fundamental in T-cell development as a whole.

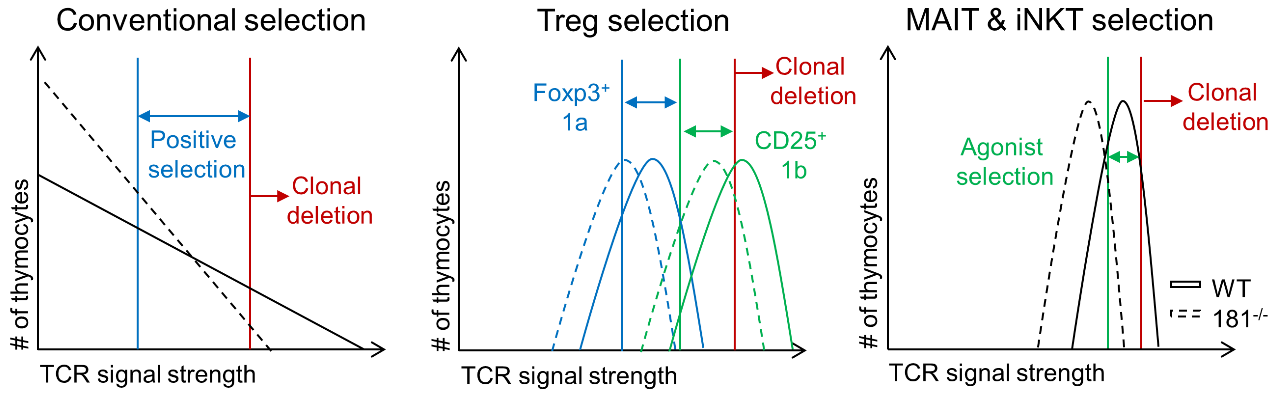


**Figure 7.3 Loss of miR-181a/b-1 dampens TCR signalling and skews developing thymocyte populations in respect to their selection thresholds.** Schematic model representing the shift in TCR sensitivity upon loss of mir-181a/b-1 regulation (dotted lines) in respect to defined selection thresholds.

One interesting aspect that still must be addressed is the apparent discrepancy between our studies and others which point towards the loss of miR-181a/b-1 skewing the conventional T-cell repertoire to be more autoreactive[1,320], whilst *in vivo* models of autoimmunity suggest otherwise[4]. Induction of EAE, a model of multiple sclerosis dependent on MOG-reactive T cells, was observed to be slower and disease severity was weaker in miR-181a/b-1-deficient mice. This occurred despite the detection of increased MOG-reactive T cells following immunisation. This appeared contradictory to the findings of apparent overall increased autoreactivity of T cells in these mice, which led the researchers to predict that miR-181a/b-1-deficient mice may have had defects in migration. In response to our data showing that miR-181a/b-1-deficiency results in the enhanced suppressive capacity of Tregs, it is possible that this may contribute to the increased protection against EAE, despite the presence of more auto-reactive T cells. Mice deficient in miR-181a/b-1 have broad defects in various T cell populations, therefore appropriate analysis of single subsets would require the isolation of the specific subset and transfer into a miR-181a/b-1-sufficient setting. Furthermore, it is probable that phenotypes observed in a T-cell mediated autoimmunity model in the absence of miR-181a/b-1 would be a result of a developmental legacy from altered thymic selection, due to very low expression levels in peripheral T cells. In support of this, peripheral miR-181a/b-1-deficient T cells exhibit WT levels of TCR signalling as indicated by ERK phosphorylation and calcium signalling[4].

Unlike EAE experiments, studies involving the onset of GvHD in miR-181a/b-1-deficeint mice, on initial glance do not appear to follow this trend. Upon overexpression of miR-181a/b-1, mice were protected from the onset of GvHD whilst loss of miR-181a/b-1 led to increased severity and onset of the disease[324]. It is important to note that some models of autoimmunity form due to a handful of reactive T cell clones to self-antigen, whilst GvHD involves a large repertoire of TCR clones that are specific for a broad distribution of PTAs. In this particular model it is possible that skewing the peripheral T cell pool in the direction of autoreactivity would be disastrous in a GvHD setting and regardless of the heightened suppressive capacity of the Tregs generated, they most likely could not keep up with the level of systemic autoimmunity generated. On the opposite end of the spectrum, increasing miR-181a/b-1 levels, would likely heighten the TCR sensitivity of developing thymocytes further, subjecting a larger portion of normally benign TCR clones to negative selection, resulting in a peripheral T cell pool with less reactivity to pathogens and self-alike. Furthermore, agonist-selected iNKT and MAIT cells, which are ablated in miR-181a/b-1 mice, have regulatory roles and provide protection against GvHD[385–387]. Therefore, an increase in GvHD severity in these mice may be a result of lack of regulation by iNKT and/or MAIT cells. Furthermore, miR-181a/b-1 overexpression models would generate an increase in TCR sensitivity that may divert more cells into these agonist-selected lineages. This would work towards theoretically regulating any aberrant immune responses that did develop, preventing the onset of GvHD.

## Discussion

In general, our mouse model carrying a germline deletion of miR-181a/b-1 does not develop any overt signs of autoimmunity. A more in-depth analysis via microscopic imaging of organs sections would provide more insight into the autoimmune-status of the mice. Regardless, in other loss-of-function models where regulators of thymic selection are missing, the result is multi-organ autoimmunity. Such is the case for Aire-deficient mice, which no longer produce self-peptides in the medulla, allowing a large fraction of autoreactive T cells to bypass negative selection[94,88]. In our case we do not witness any signs of autoimmunity, despite a large proportion of conventional T cells also escaping autoimmunity, which could in part be due to the increased presence of Tregs with a heightened suppressive function. It is pure speculation, but perhaps the thymus has its own in-built mechanism to enhance peripheral tolerance in case of a failure of central tolerance due to altered TCR signalling during thymic development. For example, we see an increased proportion of self-reactive T cells develop in the absence of miR-181a/b-1, but simultaneously due to the altered TCR signalling we gain a higher proportion of more suppressive T cells which then work towards regulating aberrant responses to self in the periphery.

To strengthen the hypothesis that more auto-reactive T cells exist in the absence of miR-181a/b-1 but are subdued by Tregs with a heightened capacity at the steady-state, further experiments need to be performed. One hypothetical example could be to deplete Treg cells in a miR-181a/b-1 mouse model, which should generate multi-organ autoimmunity and compare the disease onset and severity to control mice. An alternative which may be more physiological and cause less stress to the mice would be to deplete Tregs (perhaps using an inducible Foxp3 deletion model) in either miR-181a/b-1-deficient or WT mice and then re-transfer WT Treg cells back into mice. Mice would then be monitored for signs of autoimmunity and T cell activation, with the idea that autoreactive miR-181a/b-1-deficient T cells may escape peripheral tolerance in the presence of WT Tregs. Additionally, via using a model of diabetes induction the self-reactivity of peripheral T cells developed in the absence of miR-181a/b-1 could also be assessed. The pancreatic cells of transgenic RIP-mOVA mice express OVA-antigen. Transferring OVA-specific OT-I T cells into RIP-mOVA recipient mice, results in transgenic OT-I TCR-mediated autoimmunity against the pancreatic cells, thereby inducing diabetes[388]. Monitoring for diabetes induction and severity through blood glucose levels, pancreatic immune infiltration and activation/proliferation markers on T cells in recipients who have received either miR-181a/b-1-deficient or -sufficient naïve CD8 T cells, may also provide and insight into their inherent autoreactivity.

An interesting observation is that all T cell subsets studied appeared to be sensitive to regulation by miR-181a/b-1, except for γδ T cells[326]. γδ T cells are a unique exception, because they divert from normal development at the DN3 stage. miR-181a has been shown to be highly expressed in the DN3 stages where γδ T cell selection and diversion occurs[16,257,23]. Interestingly, when analysing the overall expression of global miR-181a target transcripts in the various stages of thymocyte development, DP

cells had an expected overall repression of targets, whilst the DN3 subset actually contained a derepression of targets[298]. While overall copy number of miR-181a in DN3 cells is relatively high in comparison to DP cells, the fraction of miR-181a in relation to the whole miRNA pool is quite low. Higher ratios seen in the DP stage are most likely responsible for its biological effect, due to an increased concentration with its targets[298]. This data combined with the alternate differentiation pathway taken by γδ T cells, may account for their lack of regulation by miR-181a/b-1.

The understanding of regulatory landscapes exhibited by a miRNA family or subset is non-trivial. Individual miRNAs target a network of transcripts normally resulting in weak to moderate target repression and therefore modulating multiple biological processes. With current methodology for accurate miRNA target identification lacking, there is lots of unvalidated data available surrounding the potential targets of miR-181a/b-1. The predicted targetome of miR-181a/b-1 is currently derived from combing *in situ* miRNA target software tools with experimental evidence involving miR-181a/b-1 knock-out mouse models, plus *in vitro* overexpression and/or knock-down models. Unfortunately, *in vitro* experiments come with their caveats. Various knock-down efficiencies and supraphysiological miRNA concentrations that oversaturate potential targets can obscure results and not necessarily reflect physiology. On top of this, recent research revealed that an individual's miRNA response elements alter between cell-types, through yet unidentified targeting mechanisms[296]. To date, several candidates have been experimentally examined as potential targets of miR-181a/b-1 in developing thymocytes. The negative regulators of TCR signalling: SHP-2, PTPN22 and DUSP5/6, are some of the likely candidates[1,2,4]. PTEN was examined[325], but differential expression could not be validated in alternate miR-181a/b-1 knock-out mouse models[3,4]. Some groups conclude the anti-apoptotic molecule Bcl-2 as a direct target of miR-181 family members[298,389,390] as well as a CD69, which is involved in thymic retention and T cell migration[298]. Furthermore, negative regulators of NOTCH signalling have been implicated as targets of miR-181a/b-1[373]. This wealth of research that relies mostly on correlation in *in vitro* cell-types is quite frankly overwhelming and confusing. Until we come up with better methods to accurately identify and quantify miRNA:mRNA binding and what causes miRNAs to target certain transcripts under specific conditions or cellular environments, studies of individual miRNAs will remain largely descriptive and should tread carefully when making bold mechanistic conclusions from correlations. Current methods suffer due to the apparent lack of efficient and/or commercially available Ago2 antibodies for miRNA-RISC:mRNA pulldown experiments. A recently developed transgenic mouse model containing a Flag-tagged Ago2 may aid in the quest to find *bona fide* miRNA targets in specific cell types[318]. The only report using Flag-tagged Ago2 mice identified miRNA binding sites using *ex vivo* individual miRNA overexpression[318]. As mentioned oversaturation may not be physiological, but a method to circumvent overexpression would be to cross these mice with individual miRNA knock-out's and then compare total bound miRNAs to control

mice. Combinations of RNA-seq and ribosome profiling can also provide insights into mRNA transcripts that undergo degradation and also translational repression in miRNA-deficient models, although this method is not without its drawbacks. Preserving an accurate picture of ribosome mediated translation requires ribosomes to be frozen to mRNA rapidly after removing cells from the living organism, which prevents isolation of specific cell populations through FACS or tissue digestion methods. Hopefully future research will be able to accurately decode miRNA regulatory networks and targeting mechanisms and allow us to precisely explain observed phenotypes in miRNA-deficient models.

Finally, I would like to share a quote from John Maraganore, CEO of the pharmaceutical company Alnylam:

> *'After 16 years, tireless perseverance through near-death moments, billions of dollars in investment, we've finally succeeded in advancing RNAi therapeutics as a whole new class of innovative medicines.'*

After winning the Nobel Prize in Physiology or Medicine in 2006, the heavily invested pharmaceutical development of RNA interference (RNAi) gene silencing techniques faced sudden withdrawal from pharmaceutical companies *en masse* following devastating clinical trial results. In 2018, RNAi therapeutics rose from the dead when the US Food and Drug Administration approved the first RNAi therapy to treat polyneuropathy caused by transthyretin amyloidosis[391]. With academic research into approving delivery methods and localised RNAi targeting showing no signs of slowing, it could well be that the problematic youth of RNAi therapies is over. Research into the functions and regulatory networks of individual miRNA in various tissues and cell-types has therefore solidified its importance and previous and/or future research may be harnessed to provide clinical therapeutic benefits. A quantitative understanding of the miR-181 family's role in thymic selection may open the doors to therapeutically modulating peripheral T cell repertoires through miR-181 RNAi. Perhaps one day skewing of T cell repertoire either towards or away from auto-reactivity may be clinically beneficial in the treatment of immunopathology.

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## EDUCATION

02/2016 – present
**Goethe University Frankfurt, *PhD in Biology***
Frankfurt, Germany
- Worked in an immunology lab under Prof. Dr. Andreas Krueger, focusing on small non-coding RNAs in T cell development.
- Thesis: The role of miR-181 in thymic development

01/2014 – 11/2014
**Murdoch University, *BSc (Hons) in Biomedical Science***
Perth, Australia
- Worked in a tumour immunology lab, focusing on immune cell regulation in melanoma.
- Thesis: The role of tissue resident memory T cells in cutaneous melanoma

02/2010 – 07/2013
**Murdoch University, *BSc in Biomedical Science***
Perth, Australia

## PROFESSIONAL EXPERIENCE

02/2016 – present
**Molecular Immunology, Doctoral Researcher**
**Institute for Molecular Medicine**
Frankfurt, Germany
- Laboratory techniques: Flow cytometry, molecular biology, animal experiments, cell culture
- Teaching: supervising master students

07/2013 – 01/2014
**Immunology**, **Participant**
**Lower Saxony International Summer Academy, MHH**
Hannover, Germany
- Intensive workshops on inflammation, regeneration and immunity – basic aspects, novel approaches and experimental models.

01/2014 – 11/2014
**Cancer Immunology**, **Honours Researcher**
**Telethon Kids Institute**
Perth, Australia
- Courses: animal handling and ethics, statistical analysis workshop
- Laboratory techniques: Animal tumour models, flow cytometry, confocal microscopy
- Data presentation

07/2013 – 01/2014
**Cancer Immunology**, **Research Assistant**
**Telethon Kids Institute**
Perth, Australia
- Mouse genotyping and phenotyping. Basic molecular biology

## CONFERENCES

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|---|---|
| **05/2018** | 12th German Meeting on Immune regulation • Oral Presentation • Berlin, Germany |
| **09/2017** | 47th Annual Meeting of the German Society of Immunology • Oral Presentation • Erlangen, Germany |
| **Annually 2016-2018** | RNA Integrated Research Training Group (IRTG) Symposium • Oral Presentation • Wernigerode, Germany |
| **Annually 2016-2018** | NK and T cell biology retreat • Oral Presentation • Kleinwalsertal, Austria |
| **07/2015** | Lower Saxony International Summer Academy • Poster Presentation • Hannover, Germany |
| **08/2014** | 24th Annual Combined Biological Sciences Meeting • Poster Presentation • Nedlands, Western Australia • *Australasian Society of Immunology (ASI) student poster presentation award* |
| **08/2014** | Telethon Kids Institute Student Symposium • Oral Presentation • Subiaco, Western Australia |
| **06/2014** | The Australian Society for Medical Research Western Australia Scientific Symposium • Oral Presentation • ECU Mt Lawley, Western Australia |

## PUBLICATIONS

**Winter SJ,** Kunze-Schumacher H, Imelmann E, Grewers Z, Osthues T, Krueger A (2019). MicroRNA miR-181a/b-1 controls MAIT cell development. *Immunology and Cell Biology* 97 (2), pp. 190–202.

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