Characterisation of TCR$\alpha\beta^+$, CD4$^-$, CD8$^-$ Double-Negative T Cells in Murine Homeostasis and Cancer

A dissertation submitted to Trinity College Dublin in candidature for the degree of Doctor of Philosophy

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2026

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Declaration

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For my Family and Friends

Abstract

Unconventional T cells bridge innate and adaptive immunity, combining features of both arms of the immune system. While iNKT, MAIT and γδ T cells have been extensively studied as innate T cells in both homeostasis and disease, less is known about non-CD1d/MR1-restricted TCRαβ$^+$ CD4$^-$CD8$^-$ double-negative T cells (DNTs). Here, we characterize non-MAIT/iNKT/γδ DNTs across murine tissues and investigate their potential innateness in comparison to other unconventional T cells. Combined single-cell TCR and RNA sequencing revealed a largely polyclonal and heterogeneous TCRαβ$^+$ DNT population in the spleen and adipose tissue. Flow cytometric analysis shows DNTs account for 1–3% of T cells in secondary lymphoid organs but expand to 10–20% in non-lymphoid tissues such as adipose. We identify two dominant subtypes: type-1 DNTs (NK1.1$^+$, T-bet$^+$) and type-17 DNTs (CXCR6$^+$, RORγt$^+$), with broad tissue distribution. Phenotypically, DNTs share conserved innate T cell features, including expression of PLZF and Helios, a pre-activated effector-memory–like state, and rapid cytokine-driven effector function independent of TCR engagement. Subtype-specific enrichment of DNTs across various organs suggests specialised roles for DNTs in certain tissue environments, furthermore DNTs expand and “fill the T cell niche” in knockout models which lack other T cell populations, supporting their tissue role. Additionally, we present evidence of distinct developmental requirements for both type-1 and type-17 DNTs in the thymus. DNT1 are MHC-I and -II independent, whereas DNT17 require a B2M-dependent restriction molecule that is Tap-independent, supporting distinct lineages for DNTs from other known T cell members. Finally, we demonstrate that type-1 DNTs, the most abundant subtype throughout murine tissues, and produce increased IFNγ compared to other innate T cells in response to cytokine stimulation. DNT1s comprise the largest innate T cell population infiltrating multiple syngeneic tumour models. After ex-vivo expansion and adoptive transfer, DNT1s reduced tumour growth in the MC38 model, and induced an overall greater anti-tumour immune response encompassing many other immune cells, suggesting DNT1 cells facilitate increased immunogenicity. Together, our findings define DNTs as a

thymically derived, innate-like T cell population with broad tissue distribution, distinct subtypes, and functional relevance in tumour immunity.

Acknowledgements

It's a very surreal moment finally writing the acknowledgements section of my PhD thesis, there are so many people I'm grateful for and would love to thank for their support, guidance and friendship over the past four years. First and foremost, I'd like to thank my mentor and supervisor, Lydia who gave me the chance to conduct research in a working environment that always fosters creativity, collaboration, passion for discovery and fun while doing it. Your excitement for science and positive outlook has always been very motivating and inspiring, thanks for supporting me throughout and taking me cross-Atlantic to your labs in the US.

Speaking of which, I have had the unexpected privilege of carrying out my PhD in not one, not two, but three different labs across Dublin, Boston and New Jersey! Aside from the obvious travel bonus, it's been amazing to get to know and work with everyone that has been in the Lynch lab over the past four years, and on both sides of the pond. I started my degree in Trinity College Dublin at the tail end of COVID, and despite pandemic restrictions, I couldn't have picked a better time to join. The post-docs in the lab at that time, Karen and Katie, and senior PhD students, Aaron, Stephen and Hannah really set the tone for the lab and made it a really nice place to get stuck into things. To Barry, Joyce and John, it was great starting our PhD journeys around the same time, and I'm so happy for all of you to either be finished already or on route to soon. Many thanks to my thesis committee at Trinity College Dublin, namely, Prof. Mills and Prof. O'Farrelly, I really appreciated your advice and guidance throughout.

My second home during my PhD was Boston where after two years, I finally got to know the second half of the Lynch lab, that previously only existed on a zoom screen every week for lab meeting. To Jaclyn, Katie and Bren, I couldn't have done it without you and so happy to have made such good friends in you, so many good memories with you all! Claire, you're one of the best people to work with. No matter how busy you are, you always find time to help people, regardless of how big or small the request. Thank you for all of your advice over the years, and genuine care in seeing everyone in the lab succeed.

Finally in June of 2024, I packed up my bags for the last time and moved down to Princeton, NJ. Probably, the most unexpected part of my PhD, but again it turned out wonderfully. The molecular bio department at Princeton was extremely welcoming and it's been a treat to get to work with a whole new team of people. I'm often asked which lab was my favourite to work in and the honest answer is I've loved them all equally, because each group of people has been so easy to get along with, to work with and to enjoy time with in and out of the lab. I think a huge part of that is a credit to Lydia, who fosters that environment from top down.

Outside of the lab, I'd like to thank all my friends who've had to listen to a fair amount of complaining and rants over the past four years, thanks especially to Noelle, Lydia, Dion, Chris, Sarah & Rebecca, we've known each other so long and can't wait to spend more time with you all back home. Of course, a massive thanks to my family who are probably happier than I am to be finished. Special thanks to my Mam, Dad, Michael, Maeve, Stephen and Sarah for your continued support and love.

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Rhythmic IL-17 production by γδ T cells maintains adipose de novo lipogenesis. Aaron Douglas, Brenneth Stevens, Miguel Rendas, Harry Kane, Evan Lynch, Britta Kunkemoeller, Karl Wessendorf-Rodriguez, Emily A. Day, Caroline Sutton, **Martin Brennan**, Katie O'Brien, Ayano C. Kohlgruber, Hannah Prendeville, Amanda E. Garza, Luke A. J. O'Neill, Kingston H. G. Mills, Christian M. Metallo, Henrique Veiga-Fernandes & Lydia Lynch. *Nature, 2024*.

Uptake of lipids from ascites drives NK cell metabolic dysfunction in ovarian cancer. Karen Slattery, Cong-Hui Yao, Eimear Mylod, John Scanlan, Barry Scott, Joseph Patrick Crowley, Orla McGowan, Gavin McManus**, Martin Brennan**, Katie O'Brien, Kate Glennon, Edward Corry, Ann Treacy, Rafael J. Argüello, Clair M. Gardiner, Marcia C. Haigis, Donal J. Brennan, and Lydia Lynch. *Science Immunology, 2025.*

The source of dietary fat influences anti-tumour immunity in obese mice. Britta Kunkemoeller, Hannah Prendeville, Claire McIntyre, Ayantu Temesgen, Róisín M. Loftus, Conghui Yao, Lydia Dyck, Linda V. Sinclair, Christina Rollings, Aaron Douglas, Gerard Pernes, Kathleen A. J. Mitchelson, Cathal Harmon, Mathilde Raverdeau, Ross Ward, Harry Kane, Jaclyn Kline, Katie L. O'Brien, **Martin Brennan**, Frances Smith, Brenneth Stevens, Helen M. Roche, Ed C. Lavelle, David K. Finlay, Doreen A. Cantrell, Edward T. Chouchani, Susan Kaech, Evanna L. Mills, Marcia Haigis & Lydia Lynch. *Nature Metabolism, 2025.*

TCRαβ double-negative T cells possess conserved innate features across murine tissues and promote enhanced immunogenicity in the tumour microenvironment. Martin Brennan, Harry Kane, Brenneth Stevens, Calvin Xu, Katie Healy, Claire McIntyre, Jaclyn Kline, Brittany Bamonte, Aaron Douglas, Barry Scott, Ayantu Temesgen, Adiba Azad, Karen Slattery, Katie O'Brien, Aine Ni Scannail, Mieszko Wilk, Hannah Prendeville, Hui-Fern Koay, Lydia Lynch. *Under revision - Journal of Experimental Medicine, 2026*

Abbreviations:

αGalCer	α-galactosylceramide
ALPS	Autoimmune Lymphoproliferative Syndrome
B2M	β-2-microglobulin
Btnl	Butryophilin-like
cRPMI	Complete Roswell Park Memorial Medium
CAR	Chimeric Antigen Receptor
CCL	Chemokine (C-C motif) ligands
CLIP	Class-II associated Invariant Chain Peptide
CTLA-4	Cytotoxic T-Lymphocyte Associated Factor 4
CXCL9	C-X-C motif chemokine ligand 9
CXCR6	CXC Motif Chemokine Receptor 6
ConA	Concanavalin A
cTEC	Cortical thymic epithelial cells
DC	Dendritic Cell
DEG	Differentially Expressed Gene
DETC	Dendritic Epidermal T cells
DNT	Double-negative T cells
DP	Double-positive
EOMES	Eomesodermin
EPCR	Endothelial Protein Receptor C
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
GZMB	Granzyme B
GZMK	Granzyme K
GvHD	Graft versus Host Disease
gWAT	Gonadal White Adipose Tissue
ICB	Immune checkpoint blockade
Id2	Inhibitor of DNA Binding 2
IELs	Intraepithelial Lymphocytes
IFNγ	Interferon gamma
iNKT	Invariant Natural Killer T cell
IRF	Interferon Regulatory Factor
IL	Interleukin
IKZF2	IKAROS Family Zinc Finger 2
KLR	Killer cell Lectin-like Receptors
KLRA1	Killer cell lectin-like receptor, subfamily A
KLRBC1	Killer cell lectin-like receptor, subfamily B
KLRC1	Killer cell lectin-like receptor, subfamily C
KLRK1	Killer cell lectin-like receptor, subfamily K
Lag3	Lymphocyte Activation Gene 3

LN	Lymph Node
LP	Lamina Propria
Lpr	Lymphoproliferation
MAITs	Mucosal Associated Invariant T cells
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
mTEC	Medullary thymic epithelial cells
NCR1	Natural Cytotoxicity Triggering Receptor 1
NK	Natural Killer
NKG2D	Natural Killer Group 2, Member D
pLN	Peripheral Lymph Nodes
PLZF	Promyelocytic Leukaemia Zinc Finger Protein
PMA	Phorbol-12-myristate-13-acetate
Pdcd1	Programmed cell death protein 1
PVR	Poliovirus Receptor
Kit	Protooncogene c-KIT
RAMP1	Receptor Activator Modifying Protein 1
RAE-1	Ribonucleic Acid Export 1
RORC	RAR-related Orphan Receptor C
RORγt	RAR-related Orphan Receptor
S1P1	Spingosine-1-phophate receptor 1
Sc	Single-cell
SI	Small Intestine
SLE	Systemic Lupus Erythematosus
STAT1	Signal Transducer and Activator of Transcription 1
TAM	Tumour associated macrophages
TAP	Transporter Associated with Antigen Processing
TIF	Tumour Interstitial Fluid
TCR	T-cell receptor
TRAV	TCRα-chain variable region
TNF	Tumour Necrosis Factor
T-reg	Regulatory T cell
TRBV	T cell beta variable
UMAP	Uniform manifold approximation and projection
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
XCR1	X-C Motif Chemokine Receptor 1

Chapter 1

Introduction

1.1 Unconventional T cells: Bridging innate and adaptive immunity

1.1.1 The growing family of unconventional T cells

Conventional T cells are defined by their expression of a diverse αβ T cell receptor (TCR), accompanied by either CD4 or CD8 s that direct their Major Histocompatibility Complex (MHC)-class-II or class-I restriction, respectively. In contrast, unconventional T cells are $CD3^+$ TCR^+ lymphocytes that fall outside of these canonical definitions. The unconventional T cell family is comprised of invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and a growing number of less well-defined subsets such as MR1T cells, H2-M3-restricted T cells, QA-1-restricted T cells, CD8αα intraepithelial lymphocytes, Tc17 cells, and other MHC class Ib–restricted populations[1]. These T cell subsets are considered unconventional as they typically do not recognise peptide antigens presented by classical restriction molecules; MHC-I and MHC-II. For instance, the semi-invariant NKT and MAIT cells recognise lipid and microbial metabolite antigens, respectively, through recognition of non-polymorphic MHC-Ib or MHC-I-like antigen presenting molecules, namely CD1d for iNKT, and MR1 for MAIT cells[2]. In contrast, γδ T cells are often MHC-I, MHC-Ib and MHC-II independent, but certain subsets have been shown to recognise specific restriction elements. In mice, examples include the non-classical MHC-Ib molecules T10/T22, recognised by a small proportion of murine γδ cells[3], and Skint1, which is essential for thymic selection of dendritic epidermal T cells (DETC). Butyrophilin-like (Btnl) family members such as Btnl1/Btnl6 also shape the repertoire of intestinal γδ T cells. In humans, the most prominent restriction system is the butyrophilin 3A1/BTN2A1 complex, which presents phosphoantigens to Vγ9Vδ2 cells in the blood[4,5]. Other human γδ subsets have reported recognition of lipid-binding non-classical molecules such as endothelial protein C receptor (EPCR) or CD1 family members (CD1c, CD1d, CD1b), and in rarer cases, γδ T cells have been reported to react with MR1[6,7].

Despite differences in origin, TCR-selection, and ligand specificity, iNKT, MAIT and γδ T cells share key immunological hallmarks of innate immunity. Each of these subsets are thymically pre-programmed with a “ready-to-go” effector state that allow these cells to respond quickly and efficiently to environmental stimuli. As such, unconventional T

cell subsets are commonly enriched in barrier and metabolic tissues, where their rapid effector responses, often mediated in a TCR-independent manner, enable them to act as first responders in immune surveillance, tissue repair, and host defence[8-10]. By initiating early targeted effector functions, which can in turn engage the adaptive immune response, they are often described as critical immune mediators that can efficiently bridge innate and adaptive immunity[11]. However, not all unconventional T cells share these innate qualities, with certain minor subsets lacking the TCR characteristics and transcriptional expression associated with innate immunity[1].

Aside from the unconventional T cells mentioned above, another subtype has been identified in TCRαβ+ double-negative T cells[12]. Significantly less research has focused on understanding non-MAIT/NKT TCRαβ+ double-negative T cells. For simplicity, these cells will be referred to as double-negative T cells (DNTs) from now on. Throughout this introduction, I will provide an overview of the shared features of unconventional T cells, with a focus on the innate T cells; iNKT, γδ and MAIT cells, highlighting the specific functionalities that allow these cells to bridge innate and adaptive immunity. In addition, I will discuss the field's current understanding of DNT cells, as well as the primary gaps in knowledge.

1.1.2 TCR selection and thymic development

T cell development in the thymus is a tightly regulated process that ensures the generation of diverse, self-tolerant lymphocyte populations capable of mounting effective immune responses while avoiding self-reactive TCRs and autoimmunity[13]. While the early stages of thymocyte differentiation are shared between both unconventional and conventional T cells, their divergence during selection reflects key differences in antigen recognition, TCR signal strength and the restriction molecules required[14]. Firstly, hematopoietic progenitors seed the thymus, with commitment to the T cell lineage driven by Notch signalling from thymic epithelial cells. Thymocytes progress through four double-negative (DN) stages, rearranging the TCRβ locus and expressing a pre-TCR, which signals the transition to the double-positive (DP) stage. At the DP stage, TCRα rearrangement completes the αβ TCR, enabling the cells to undergo the selection processes that determine antigen specificity and TCR restriction[15,16].

In the conventional pathway, DP thymocytes within the cortex, scan peptide-MHC complexes presented by cortical thymic epithelial cells (cTECs). Positive selection occurs when the TCR engages self-peptide-MHC complexes with intermediate affinity[17]. For $CD8^+$ T cell development, the selecting ligands are peptides bound to classical MHC-I molecules, whose surface expression depends critically on the antigen processing machinery present. Transporter associated with antigen processing 1 (TAP1), forms a complex with TAP2 to translocate proteasome-derived peptides from the cytosol into the endoplasmic reticulum (ER), where they are loaded onto MHC-I molecules. β2-microglobulin (β2M), a non-polymorphic light chain, binds the MHC-I complex and plays a critical role in ensuring stable surface expression and efficient MHC-I presentation[18].

CD4 T cell development progresses similarly to CD8 T cells, with the major difference being the peptide-MHC complex presented by cTECs is MHC-class-II. Unlike $CD8^+$ T cells, $CD4^+$ T cells do not require Tap1 or β2M for development, as MHC-II peptide loading occurs within the endosome compartment. MHC-II associates with the invariant chain (Ii), which acts as a chaperone guiding MHC-II molecules to the endosomal pathway and prevents premature peptide binding. Within the endosome, Ii is degraded to generate class-II associated invariant chain peptide (CLIP). CLIP is then exchanged for antigenic peptides by HLA-DM (H2-DM in mice), DM essentially acts as a peptide editor, releasing CLIP and promoting binding of peptides for presentation[19]. Following positive selection, $CD4^+$ and $CD8^+$ T cells migrate to the medulla for negative selection to remove autoreactive clones, with mature $CD4^+$ or $CD8^+$ T cells then exiting the thymus as naïve T cells[16,20].

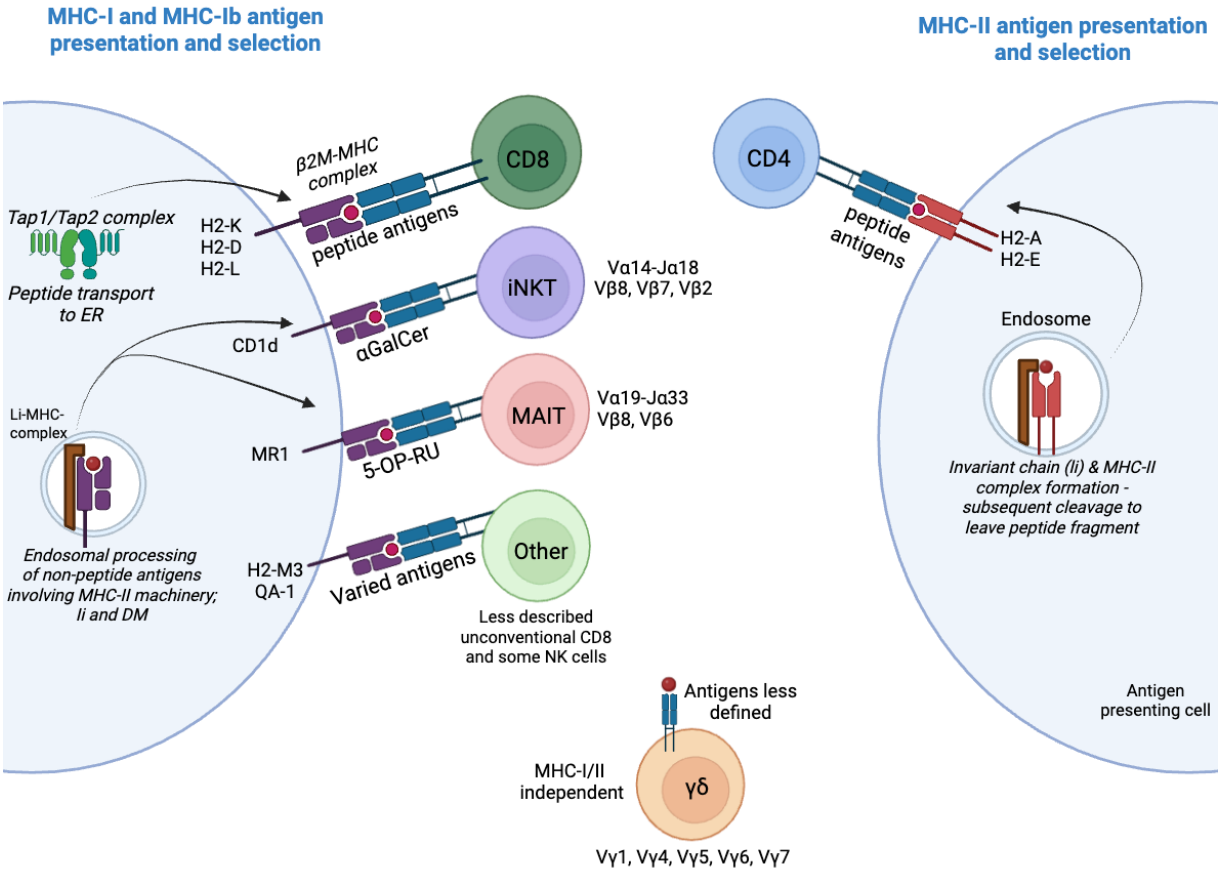
By contrast, unconventional T cells diverge from the conventional pathway at the DP thymocyte stage, often relying on MHC class-I-like molecules. These molecules present alternative antigens such as lipids, metabolites, or stress-induced ligands, and their antigen-loading pathways are often independent of TAP1. iNKT cells recognise the prototypic NKT lipid antigen; α-galactosylceramide (α-GalCer) presented by CD1d. CD1d surface expression requires β2M but not TAP1-mediated peptide transport. Alternatively, it binds lipid antigens delivered through endosomal trafficking via use of MHC-II antigen processing machinery, whereby CD1d associates with Ii to enhance antigen presentation[21]. MAIT cells recognise a limited array of microbial-derived

vitamin B metabolites presented by MR1, which is also β2M-dependent and uses a chaperone-assisted, TAP1-independent pathway for ligand loading in the endosome[22]. In both iNKT and MAIT cells, selection often occurs on DP thymocytes themselves, through agonist interactions that drive strong TCR signalling. This 'agonist selection' contrasts with the more moderate/intermediate signalling of conventional positive selection and contributes to the innate-like effector phenotype imprinted on these cells during thymic development.

γδ T cells develop much earlier in the thymus, and before the appearance of MAIT and iNKT cells, typically arising at the $CD3^{-}CD4^{-}CD8^{-}$ DN stage. DN thymocytes proceed through four distinct stages, based on CD44 and CD25 expression, with TCRδ first observed in DN1 ($CD44^{+}CD25^{-}$). Selection for γδ T cells is less defined but usually does not require classical MHC-I or -II and often appears to be mediated by ligand-independent programming. However, subset-specific ligands exist, such as the butyrophilin family member SKINT1 for DETCs, presented by thymic epithelial cells rather than DP thymocytes. Similarly, to MAIT and iNKT cells, γδ T cells leave the thymus as functionally defined subsets with a pre-programmed effector phenotype[23-25].

Egress from the thymus is a regulated step marking the completion of central development. Conventional T cells downregulate retention markers such as CD69 and upregulate sphingosine-1-phosphate receptor 1 (S1P1), enabling migration into the bloodstream[26]. They leave as naïve cells that will encounter antigen and undergo functional differentiation in secondary lymphoid organs. In contrast, unconventional T cells exit earlier and seed the periphery in pre-activated memory-like state. This is attributed to the strong TCR signals and agonist selection which initiates effector program transcription and functional maturation in unconventional T cell subsets before they egress from the thymus[27]. Furthermore, TCR signal strength also determines functional subset selection within unconventional T-cells, with a weaker TCR signal promoting IL-17-producing γδ, and a stronger signal driving development of IFNγ-producing γδ T cells[28,29]. These differences in timing and maturation state at

exit highlight the functional divergence between conventional and unconventional subsets.



*1***Figure 1.1:** Thymic selection of unconventional and conventional T cells

Schematic representation of MHC-I, MHC-Ib, and MHC-II antigen processing and presentation, and their associated T cell populations. **Left:** Classical MHC-I molecules (H2-K, H2-D, H2-L) present peptide antigens, transported to the endoplasmic reticulum via the Tap1/Tap2 complex, to conventional $CD8^+$ T cells. MHC-Ib molecules present non-peptide or specialised antigens to semi-invariant and unconventional T cells: CD1d presents αGalCer to invariant NKT (iNKT) cells (Vα14-Jα18 with Vβ8, Vβ7, or Vβ2); MR1 presents 5-OP-RU to mucosal-associated invariant T (MAIT) cells (Vα19-Jα33 with Vβ8 or Vβ6). Non-peptide antigens presented by CD1d and MR1 are processed in endosomal compartments using MHC-II–associated machinery (invariant chain [Ii], DM). H2-M3 and QA-1 present varied antigens to less-defined unconventional cells. **Right:** MHC-II molecules (H2-A, H2-E) present peptide antigens derived from endocytosed proteins to $CD4^+$ T cells following invariant chain association, cleavage, and peptide loading in endosomes. **Bottom:** γδ T cells recognise ligands in an MHC-I/II–independent manner, with less defined antigens and varied TCR usage including Vγ1, Vγ4, Vγ5, Vγ6, and Vγ7.

The dependency on classical and non-classical MHC, TAP1 and B2M is a critical distinction between conventional and unconventional T cells. In the conventional $CD8^+$ pathway, TAP1 is indispensable and without it, MHC-I surface expression is greatly reduced to absent, resulting in a severe loss of CD8 T cells in the periphery[30]. However, for many cells selected by MHC-I-like molecules, TAP1 is redundant for processing of non-peptide antigens. Nonetheless, B2M is universally required for structural stability and surface expression of both classical and non-classical MHC-I-restricted T cells. γδ T cells are unique in that they typically don't require the above antigen processing machinery and continue to develop normally in their absence. As such, γδ T cells have been shown to persist in the periphery in the absence of a thymus in both nude mice and thymectomised mice[31]. The contrasting antigen presentation requirements and thymic selection mechanisms have clear functional implications. While conventional T cells are optimised for recognising a diverse array of peptides on classical MHC-I and -II, unconventional T cells are often more constrained to TCR-recognition of specific conserved microbial metabolites or self-lipids that arise during infection or cellular stress.

1.1.3 Innate features and transcriptional programming of unconventional T cells

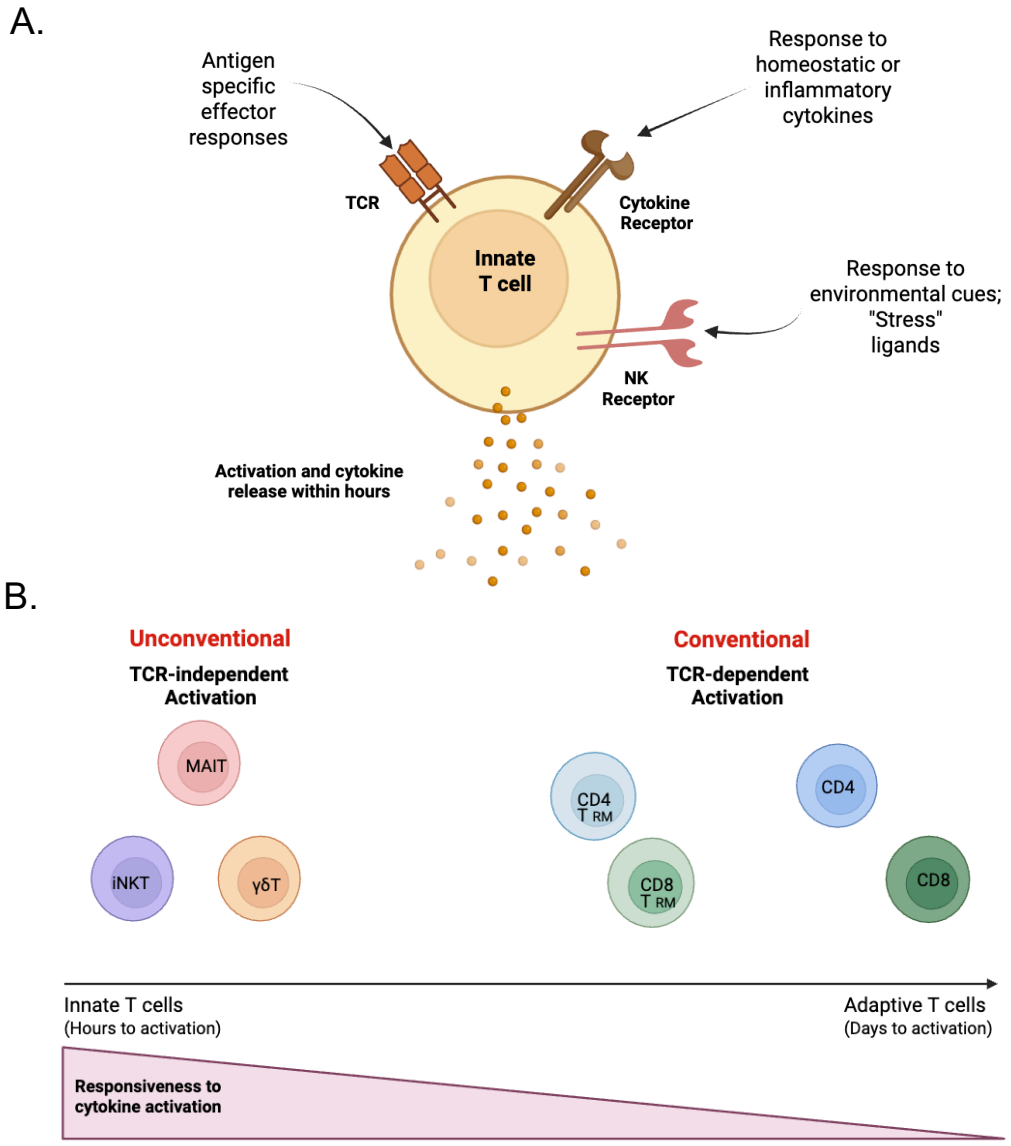
The innate properties of γδ, iNKT and MAIT cells arise not only from TCR recognition of conserved antigens but, also from early transcriptional programming that imprints effector potential. Key to this process, is the transcription factor promyelocytic leukaemia zinc finger (PLZF), encoded by *Zbtb16*. PLZF acts as a master regulator of the innate-like phenotype observed across multiple unconventional T cell subsets. PLZF expression is induced following agonist selection in the thymus and drives the acquisition of an effector-memory-like state, which includes constitutive expression of activation markers, chemokine receptors, and transcription factors necessary for cytokine production, such as T-box transcription factor 21 (*Tbx21*) and RAR-related orphan receptor C (*Rorc*) [32]. For iNKT cells, this programming ensures they leave the thymus poised to produce IFNγ, IL-4, or IL-17A depending on their subset identity, NKT1, NKT2, NKT17, respectively[33]. Similarly, for MAIT cells, thymic PLZF expression coordinates their effector potential, allowing for rapid release of IFNγ, TNFα, or IL-17A upon microbial challenge or cytokine stimulation. Interestingly, γδ T cells display subset specific PLZF activity, where it is only associated with $CD27^-$ IL-17-producing

γδ T cells (γδ17), and not CD27^{+} IFNγ producers (γδIFN), where it again contributes to their innate effector status[8,32,34]. PLZF+ innate T cells can exhibit high basal expression of chemokine receptors such as CCR6, CXCR6, and CCR5, enabling their localisation to barrier and metabolic tissues where early responses are most advantageous[35-37]. They also display constitutive chromatin accessibility at loci encoding cytokines[35,38], supporting their ability to release effector proteins within hours of stimulation, as opposed to days for conventional T cells. Importantly, these features are maintained in the periphery, even without recent antigen experience, granting their "ready-to-go" phenotype.

While TCR-specificity and PLZF expression are defining feature of innate immunity, unconventional T cells can also express innate receptors of the natural killer (NK) family that broaden their activation potential[39,40]. Through these receptors, UTCs can sense stress ligands presented by other cells, bypassing the need for classical antigen presentation via MHC. Activating NK receptors such as NKG2D recognize ligands upregulated under cellular stress such as Ribonucleic Acid Export 1 (RAE-1) or Poliovirus Receptor (PVR)[41,42], enabling γδ T cells and iNKT cells to exert cytotoxicity or secrete cytokines independently of their TCR. Conversely, inhibitory receptors including NKG2A help calibrate responses and maintain tolerance[43]. This alternative activation pathway enhances unconventional T cells adaptability in changing tissue environments.

The innate imprinting of unconventional T cells equips them not only for host defence but also for homeostatic and reparative roles. γδ-17 T cells and MAIT cells help maintain barrier integrity by promoting epithelial proliferation and antimicrobial peptide release[44]. iNKT1 cells contribute to macrophage activation and tissue inflammation, while iNKT2 can also promote resolution and repair[45]. In the adipose tissue, iNKT cells influence inflammation and metabolic health by skewing macrophage populations toward pro- or anti-inflammatory states[45-47]. γδ-17 T cells in the adipose, can also maintain metabolic homeostasis by regulating thermogenesis and de novo lipogenesis[48]. MAIT cells in the liver similarly act as tissue sentinels by protecting against microbial translocation from the gut[49]. Overall, the combined effect of PLZF-mediated transcriptional programming, NK receptor expression, and cytokine responsivity establishes iNKT, MAIT, and γδ T cells as rapid responders and critical

protectors of tissue homeostasis[34]. By bridging innate speed with adaptive specificity, they ensure that tissues are protected not only from infection but also from dysregulated inflammation and metabolic imbalance.



2 **Figure 1.2:** Rapid Effector Function of Innate T cell

(A) Innate T cells can be activated through multiple mechanisms; TCR-antigen specific activation, cytokine-induced activation, or stress-ligand recognition on activating NK cell receptors. (B) Innate T cells, including MAIT, iNKT and γδ T cells, are capable of rapid, TCR-independent activation, often responding within hours via cytokine-mediated pathways. In contrast, conventional $CD4^+$ and $CD8^+$ T cells, typically require antigen recognition through the TCR and exhibit slower activation kinetics over several days. Resident-memory conventional T cells, with previous antigen exposure can respond to cytokines in a more timely manner than naïve T cells but with decreased responsiveness compared to innate T cells. *Adapted from: Hackstein & Klenerman. Clinical & Experiemental Immunology. 2023*

1.1.4 Unconventional T cells in tumour immunity

Although previously overlooked compared to conventional T cells, γδ T cells, MAIT and iNKT cells have emerged as key players in both promoting and suppressing tumour progression, despite their lower frequencies in the tumour. A defining feature of their anti-tumour activity is the ability to combine direct cytotoxicity with modulation of other immune subsets, thereby shaping the tumour microenvironment (TME)[50]. Additionally, multiple means of activation give them an advantage over their conventional counterparts within the tumour, for example, detection of stress-induced antigens such as MICA/B or Rae-1 through innate receptors like NKG2D, enables them to recognise transformed cells independently of MHC. This is of particular benefit in the tumour setting, where MHC can often be downregulated. In early disease stages, skin- and gut-resident γδ T cells mediate cancer surveillance through IL-13 or CD103–EpCAM interactions, while cytotoxic γδ subsets exert potent effector functions via perforin, granzymes, FasL, TRAIL, and IFNγ[51,52 53]. IFNγ, which can produced by all type-1 innate T cells, enhances antigen presentation, promotes Th1 polarisation, and supports cytotoxic lymphocyte recruitment. Moreover, activated γδ T cells within the tumour secrete chemokines that amplify infiltration of effector lymphocytes, further strengthening tumour control[54,55]. Additionally, γδ-derived GM-CSF can recruit dendritic cells and boosts cross-priming of tumour-specific $CD8^+$ T cells[56]. MAIT cells, when stimulated with synthetic MR1 ligands such as 5-OP-RU leads to a robust upregulation of IFNγ, CD69, and DNAM-1 while downregulating inhibitory receptors such as CD96. In turn, activated MAIT cells boost NK cell responses and IFNγ production, resulting in slowed tumour growth in E0771 mammary carcinoma[57,58]. iNKT cells exhibit particularly versatile anti-tumour functions. Through recognition of CD1d, iNKT cells can directly lyse tumour cells using perforin and granzymes. More significantly, they remodel the TME by regulating certain myeloid populations. iNKT cells upregulate CD40L and secrete IFNγ, which activates dendritic cells, enhances epitope spreading and tumor-specific responses by cytotoxic lymphocytes[59]. Additionally, iNKT can assist in eliminating suppressive populations, including tumour-associated neutrophils and macrophages via the CD40–CD40L axis[60,61]. Overall,

innate T cells can rapidly and efficiently engage a targeted anti-tumour response while contributing to sustained activation of adaptive T cells.

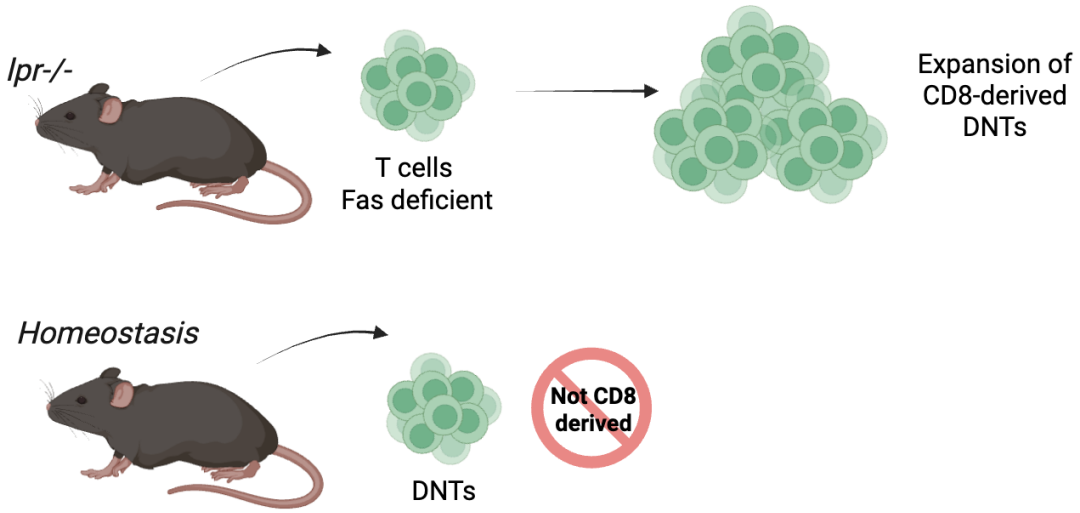
1.2 Double-Negative T cells (DNTs)

As demonstrated, significant research has been carried out to characterise and examine the roles of unconventional T cell subsets, with particular emphasis on γδ T cells, iNKT and MAIT cells in both homeostasis and disease. However, our understanding of non-MAIT/iNKT/γδ DNTs is much more limited. DNT cells account for approximately 1–5% of all CD3+ T lymphocytes in the peripheral blood of both humans and mice[62,63]. They are also present in secondary lymphoid organs, but appear more abundantly in non-lymphoid tissues, such as the kidneys, gut, and female genital tract[64-67]. In the existing literature, DNT cells have been attributed to pathogenic roles in autoimmunity, regulatory roles in reproductive immune tolerance, and both pro- and anti-tumour roles in the cancer setting[68-71]. Whilst, these studies and review articles can provide insightful information on potential roles of DNT cells, a complication exists in that the identification and gating strategy of DNT cells in these settings, vary widely by study and topic. For example, studies outlining regulatory roles of DNT cells in both humans and mice, commonly identify DNT cells as $CD3^+CD4^-CD8^-$, whilst including both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells, without exclusion of MAIT or iNKT cells from the analysis[70]. Similarly, in autoimmunity, DNT cells are described as $TCR\alpha\beta^+CD3^+CD4^-CD8^-$, whilst accounting for $\gamma\delta^+$ cells, there is still no separation of the heterogenous unconventional T cell pool within the $\alpha\beta^+$ cells[69]. Some studies use NK1.1 (in mice) or CD56 (in humans) as negative markers to delineate double-negative T (DNT) cells. However, neither marker is uniformly expressed on all MAIT or iNKT cells, and without direct characterisation of DNT cells, this approach risks excluding important subsets and may mislead interpretation of results. Furthermore, unconventional T cell subsets can be preferentially enriched in several tissue sites across both mice and humans and can often be expanded and/or carry out unique effector functions across disease settings. Therefore, accurate labelling of all unconventional T cells, including DNTs is critical for assessing differences in their roles in both homeostatic and pathogenic states. Additionally, DNT cells appear to have relatively low frequencies in the periphery and tissues of both mice and humans, which contributes to our lack of understanding of these atypical T cells. Despite this,

there have been key insights into the origin and functionality of DNT cells throughout the years, which provide a useful framework for assessing the current understanding and critical questions that still need to be addressed.

1.2.1 Initial discovery of DNTs and misrepresentation as “abnormal” T cells

A major limiting factor in the research progress of DNT cells, arose from their initial discovery in autoimmune settings, and as a result, having long been considered “abnormal” T cells which negatively contribute to disease. This misinterpretation came from their initial discovery in 1982 in *lpr* knockout mice, where DNTs were found to be expanded in secondary lymphoid organs, resulting in splenomegaly and lymphadenopathy[72,73]. The *lpr* mouse model is reflective of autoimmune lymphoproliferative syndrome (ALPS) disease in humans, where patients display mutations within the Fas Cell Surface Death Receptor (Fas) gene, and an expansion of peripheral DNTs[74,75]. It is now understood, that in Fas pathway deficiency (lpr/ALPS) and related autoimmune contexts, defective Fas-mediated deletion permits accumulation of chronically stimulated, self-reactive clones that convert from CD8+ T cells to DNT cells[76].



3 **Figure 1.3**: DNT Origins

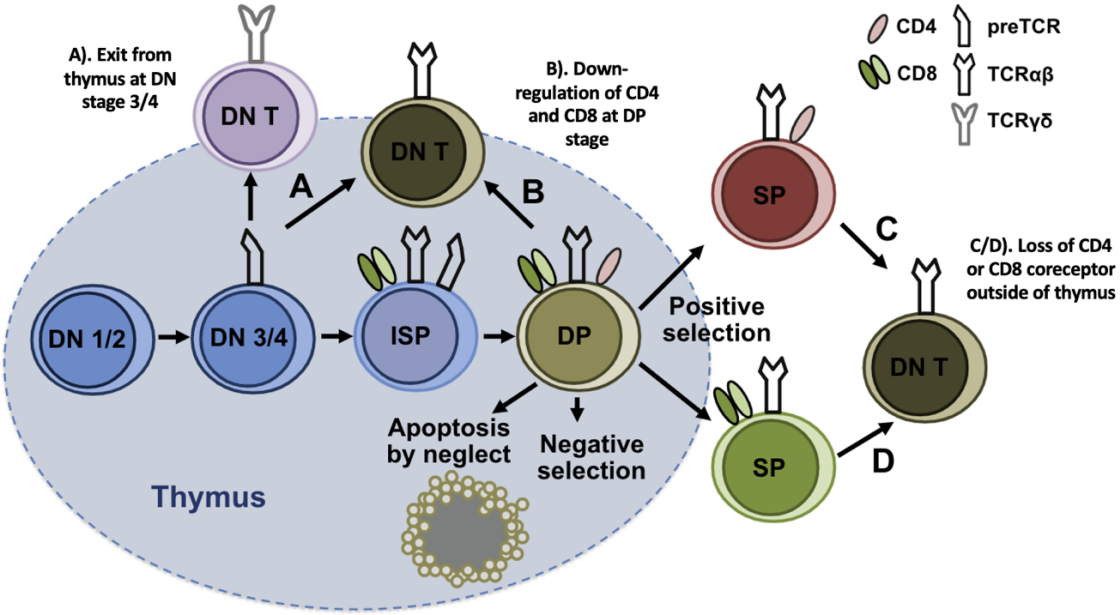
DNT cells were initially discovered in *lpr* knockout mice, a model of lymphoproliferative disease, resulting in expansion of CD8-derived T cells due to impaired FAS-mediated apoptosis. More recent work has pointed to non-pathogenic roles for DNT cells in homeostasis and alternative origins to that in lpr-/- mice.

In systemic lupus erythematosus (SLE) and Sjögren's syndrome, DNT cells have been shown to originate from $CD8^+$ T cells that have lost CD8 expression in the peripheral tissues, rather than representing a distinct thymic lineage. Clinical and experimental observations in both SLE and Sjögren's patients further support this, as DNT cells are expanded in these autoimmune conditions and often share TCR clonotypes and specificity with $CD8^+$ counterparts[77]. DNT cells gain the ability to secrete IL-17 and infiltrate target organs such as the kidneys, thereby exacerbating autoimmune inflammation[78]. In both SLE and primary Sjogren's syndrome, DNT cells are believed to be the primary producers of IL-17, contributing to disease progression[79,80]. In healthy settings, DNTs do not reflect the same ontogeny as the expanded, Fas-deficient, self-reactive CD8-derived DNTs seen in autoimmune lymphoproliferative states. Under homeostasis, they are not derived from CD8+ T cell precursors, instead representing a developmentally and functionally separate T cell population[81]. Steady-state DNTs are reported to be enriched in normal mammalian kidney and human kidney, where they can act early after injury and exhibit anti-inflammatory functions, and they also exist as pre-activated, resident memory-like DNTs in the lung that participate in antiviral responses. In summary, although DNT cells in SLE and Sjögren's syndrome emerge through peripheral plasticity of $CD8^+$ T cells under chronic autoimmune stimuli, DNTs are no longer believed to only exist in an "abnormal" or disease settings, with many studies suggesting important roles in maintaining immune homeostasis[82-84].

1.2.2 Potential DNT development routes

Traditionally, T lymphocytes originate from progenitors in bone marrow, which migrate to the thymus for TCR rearrangement, maturation, selection, and subsequent egress to the periphery. There has been much debate as to which stage of thymus maturation DNTs arise, or whether they develop outside of the thymus due to loss of CD4 or CD8 coreceptors on conventional T cells[68]. In the thymus, T cells undergo several differentiation stages, including four double negative (DN) stages in mice (three in

humans), before progressing to SP and DP stages. Here, they lose one receptor committing to either the CD4 or CD8 lineage as they exit the thymus(**Figure 1.4**)[85].



4 **Figure 1.4**: Thymocyte maturation with proposed DNT exit routes

Developing T cells pass through 4 double-negative (DN) stages, before becoming single-positive, double-positive, and finally committing to coexpression of a single-positive CD4 or CD8 receptor. DNTs have four possible routes of leaving thymocyte maturation; A) Exit at DN stage 3/4; B) Down-regulation of CD4 and CD8 at DP stage. C) Loss of CD4 coreceptor outside of thymus D) Loss of CD8 coreceptor outside of thymus. *Figure adapted from: Brandt et al., 2018*

Studies have suggested possible exit routes for DNT cells at various stages of thymocyte maturation. Similarly to γδ-T cells, DNTs could depart from αβ T cells at DN stage 3, and egress from the thymus at DN stage 4 before entering the single positive stage, to seed peripheral tissues. Factors which have been suggested to influence continuation of DNT thymic development, or egress at DN stage 3 include the strength of the TCR signal or sex steroid induced release[86,87]. However, given that TCRαβ rearrangement occurs at the DP stage, earlier exit seems unlikely. Studies involving fate-mapping using transgenic mice have indeed traced DNT cells to the DP stage of thymic development. It is hypothesised that they could also escape as DNTs at this

stage by downregulating CD4 and CD8 due to interactions between DP thymocytes and high affinity antigen presentation[88,89]. Outside of the thymus, DNTs can arise from peripheral mature T cells containing an intrinsic defect which results in the loss of CD4 or CD8 coreceptor expression[12]. One study showed that CD4 gene silencing could convert mature murine peripheral CD4+ T cells into DNT cells, with unique cell surface markers and molecular profiles that are separate from CD4+ T cells[90]. Although, it is less apparent if DNT cells can develop from CD4 or CD8 in unmanipulated, steady-state conditions. Therefore, a clearer understanding of the molecular mechanisms involved in DNT developmental pathways and how they are selected for in the thymus, is required to distinguish them from both conventional T cell subsets.

1.2.3 Suggested roles of DNT cells in health and disease

Several studies have identified regulatory roles for $CD3^+CD4^-CD8^-$ DNTs, with the sub-type often referred to as DN Tregs. Unlike $CD4^+$ Tregs, human and mouse DN Tregs do not express FoxP3[91,92]. Although, DN Tregs have been shown to express common regulatory markers such as CD25, CD30, CD28 and CD442, a single cell specific marker to distinguish DN Tregs remains to be identified[62]. DN Tregs display high expression of perforin, granzyme B (GZMB) and FasL, in addition to exhibiting strong suppressive activity toward $CD4^+$ and $CD8^+$ T cells[19,63,90], as well as B cells, dendritic cells, and NK cells[93-95]. DN Tregs have been reported to employ multiple mechanisms to exert antigen-specific control over immune responses. Immature/tolerogenic dendritic cells secreting EBI3 can elicit IFNγ from DN Tregs, resulting in allograft tolerance by an unknown mechanism[96]. Mature or immature dendritic cells presenting alloantigen to DN Tregs are susceptible to apoptosis in part due to the Fas pathway[94]. CTLA4 expression by DN Tregs also interacts with CD80/CD86, similar to CD4+ Tregs, on mature DCs causing downregulation of CD80/CD86 decreasing the ability of dendritic cells to prime allogeneic T cell responses[94]. DN Tregs can acquire alloantigen from DCs via trogocytosis and present them to T cells bearing the same TCR specificity as the DN Tregs, subjecting allogenic T cells to Fas-mediated apoptosis[97]. This mechanism has only been demonstrated for CD8+ T cells, but CD4+ T cells are also susceptible to killing by DN Tregs. Similarly, B cells presenting specific antigen through MHC class II are susceptible to killing by DN Tregs[91], mediated via a

perforin/granzyme-mediated release[97]. NK cells can also be killed by DN Treg perforin/granzyme[98]. IL-10 secreted by DN Tregs can inhibit Type-1 diabetes development while also limiting DN Treg expansion[99]. A recent 2025 study demonstrated DNT cells can tolerise naïve CD4+ T cells in the gut by presenting intestinal antigens via MHC-II, though without CD8α exclusion, it's difficult to ensure separation from the gut-abundant CD8αα population[100]. These studies indicate the diverse mechanisms by which DNTs can exert immune suppression on various immune cell types, highlighting their potential importance in immune homeostasis, however it's unclear as to the extent of contribution from other unconventional T cells within the $CD4^{-}CD8^{-}$ double-negative pool in these settings.

DN Tregs have been shown to provide significant protection against allograft rejection and graft-versus-host disease (GVHD). Low numbers of DNTs have been correlated with an increased disease severity and chronic GVHD[101]. In murine models, adoptive transfer of DNTs inhibits the onset of GVHD by generating tolerance and inhibiting the anti-host $CD8^{+}$ T cell population that contribute to pathology in GVHD[102]. Additionally, ex-vivo expansion of human DNTs have also been shown to suppress proliferation of T and B cells attenuating GVHD, showing the promising therapeutic value of DNT therapies for GVHD patients[82]. A study conducted in the murine liver demonstrated that CD4-derived DNT cells can protect against diet-induced fat accumulation and prevent the development and progression of non-alcoholic steatohepatitis. Through adoptive transfer of DNT cells in this setting, they highlight immunoregulatory mechanisms by which DNT cells suppress Th17 and macrophage populations, reducing overall proinflammatory cytokine secretion in the liver[103]. However, it is important to note, that the DNT cells examined here are derived from CD4 T cells that are treated with Concanavalin A (ConA) which directly induces down-regulation of CD4 co-receptor expression[104]. ConA is a lectin extracted from jack beans, and a potent anti-hepatoma therapeutic, with no known endogenous presence in humans or mice[105]. Additionally, there is a lack of evidence that DNT are CD4-derived in the steady-state mouse.

DNTs are reported to play both protective and pathogenic roles in a number of diseases, depending on the effector cytokines they produce, including IL-17, TNF, IFNγ, and GZMB. Most notably, as pathogenic drivers of autoimmunity, driven by IL-

17 production. However, in certain infectious disease models, DNTs have been shown to maintain both innate and adaptive responses, regulating the functions of conventional CD8s, B-cells and macrophages[106-108]. DNTs have also been identified as tumour-infiltrating lymphocytes (TILs) in various cancer types, including non-small-cell lung cancer, liver cancer, glioma and pancreatic tumours[109-113]. While these studies suggest both pro- and anti-tumour roles for DNTs in mice, they do not clearly differentiate between the various unconventional T cells within the double-negative pool[71]. However, more recent work has examined the involvement of NK1.1$^-$ TCRαβ$^+$ non-MAIT/iNKT DNTs in anti-tumour immunity, providing interesting insight into the mechanistic action of DNT cells in this setting. In 2019, Ponzetta et al., described a subset of DNT cells which infiltrated a murine sarcoma model and contributed to reduced tumour growth through high production of IFNγ. Of note, the DNT cells in this study were polarised towards type-1 immunity by IL-12 production of tumour-associated macrophages (TAMs) in a neutrophil-dependent manner. The DNT cells in this setting, were NK1.1$^-$, whilst expressing other NK-associated surface markers such as NKG2, Ly49G2 and Ly49A. Accordingly, they expressed T-bet and EOMES cytotoxic transcription factors, and responded to cytokine-induced IFNγ production within the tumour[114]. A second seminal paper in 2019, by Hundeyin et al., again described tumour-infiltrating NK1.1$^-$ DNT cells in a model of pancreatic ductal adenocarcinoma, whereby DNT cells promote immunogenic macrophage polarisation, as seen by increased CCR5$^+$ macrophages in the tumour[115]. Both papers used NK1.1 as a negative marker to remove NKT and MAIT cells from their analysis, while retrospectively including CD1d- and MR1-tetramer staining in later experiments to confirm an absence of their presence in the respective tumour model[114,115]. Overall, despite their low frequencies and previous reputation of being ‘abnormal’ cells, more recent developments have extended our understanding of the potential origin, effector phenotypes and both pro- and anti-inflammatory roles of DNT cells in health and disease.

1.3 Thesis Aims

Throughout this introduction, I have introduced the key family members of unconventional T cells, and an understanding of their ability to bridge innate and adaptive immunity. Additionally, I have provided an overview of what is currently known about the less well-characterised αβ+ DNT cells, outlining potential importance for these cells in homeostasis and disease. Thus, to allow this population of cells to receive wider attention in the scientific community and to warrant further research, we must clearly define a uniform gating strategy for DNT identification, examine phenotypic markers across tissues and better understand their functional role within the unconventional T cell compartment. In this project, we hypothesised the DNTs would be comprised of a heterogenous population of cells with shared features of innate immunity across murine tissues and be of functional importance in disease states.

Specific Aims

1) Investigate shared clonality and transcriptional phenotypes of DNTs from lymphoid and non-lymphoid tissues to define clear subtypes
2) To determine the innateness of DNT cells in comparison to other unconventional T cells
3) To elucidate presence and functional roles for identified DNT subtypes within the tumour setting

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Solutions and Buffers

Buffer	Formulations
cRPMI Media	500mL: 50 ml FCS, 5ml PS, 5 ml sodium pyruvate, 5ml NEAA, 5ml glutamax, 500ul 1000x B-mercaptoethanol
DMEM Media	500mL: 50 ml FCS, 5ml PS, 5 ml HEPEs buffer
FACs buffer	50mL: 49 ml PBS + 1mL FCS
RBC lysis buffer	For 1L: 1L dH20 + 8.7g NH4CL
1X Perm buffer	50mL: 45 ml dH20 + 5ml 10X permeabilisation buffer

2.1.2 Chemicals/Reagents

Name	Catalogue #	Supplier
Brefeldin A	B765	Merck
Collagenase II	17101015	Merck
Foxp3/TF staining kit	00-5523-00	Invitrogen
Intracellular fixation and permeabilization buffer kit	00-5523-00	Invitrogen
Permeabilization buffer	00-8333-56	Invitrogen
Foetal Calf Serum		Gibco
Ionomycin	I0634	Merck
OneComp eBeads Compensation Beads	01-1111-42	Fisher Scientific
PBS tablets pH 7.4	TA02T9181	MSC
Penicillin-Streptomycin	P433	Merck
PMA	P1585	Merck
Precision Count Beads	42902	Biolegend
RPMI-1640 Media	R0883	Merck
Percoll	GE17-0891-02	Sigma
FITC Ab conjugation kit	ab102884	Abcam
EDTA 500mM	10306983	Fisher Scientific
Sodium Pyruvate	11530396	Fisher Scientific
Corning HBSS +/+ Ca/Mg	15353571	Fisher Scientific
Glutamax	1157446	Fisher Scientific

Sytox green	S7020	Fisher Scientific
Draq5	424101	Biolegend
CTV	C34571	Fisher Scientific
DMEM media	11995073	Fisher Scientific
HEPEs buffer	15630080	Fisher Scientific
CD3 Isolation kit	130-095-130	Miltenyi
DNase I	D4263-5VL	Sigma
Collagenase D	11088882001	Sigma
Cell stimulation cocktail	00-4970-03	Fisher Scientific
Brefeldin A	420601	Biolegend

2.1.3 Cell culture stimulants

Name	Catalogue #	Supplier
Mouse recombinant IL-7	78054.1	Stemcell technologies
Mouse recombinant IL-2	78081	Stemcell technologies
Mouse recombinant IL-12	577002	Biolegend
Mouse recombinant IL-15	566302	Biolegend
Mouse recombinant IL-18	767002	Biolegend
Mouse recombinant IL-1b	130-101-680	Miltenyi Biotec
Mouse recombinant IL-23	130-096-676	Miltenyi Biotec

2.1.4 Flow Cytometry Antibodies

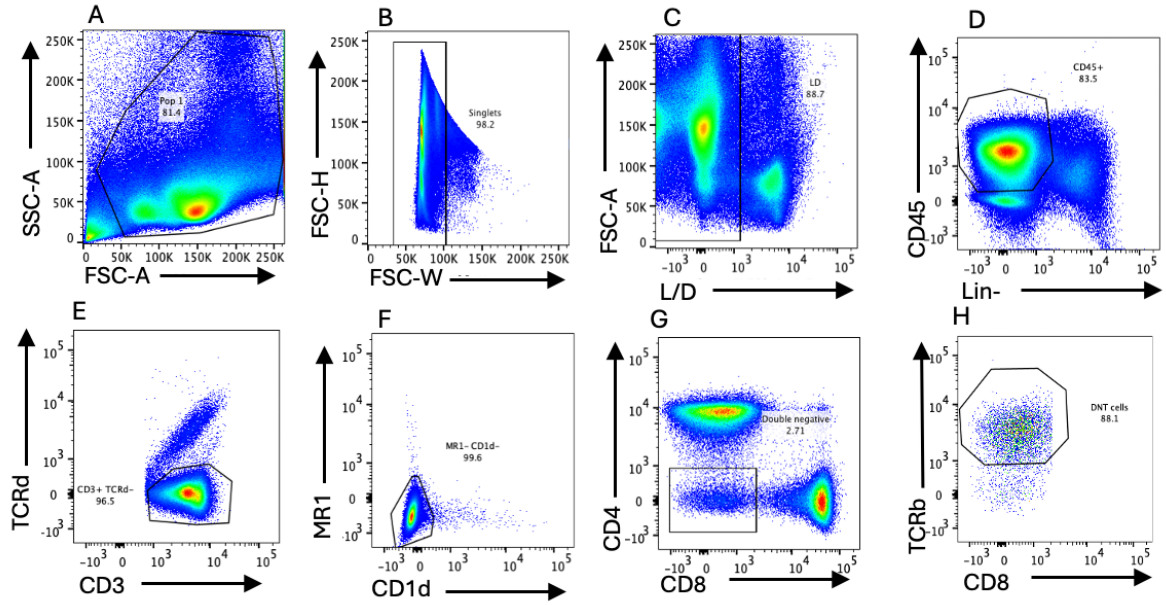
Target	Fluorochrome	Catalogue #	Supplier
Zombie Aqua Viability Dye	Zombie Aqua (BV510)	423102	Biolegend
TruStain FcX (anti-CD16/32)	N/A	101320	Biolegend
B220	BV480	565631	BD Biosciences
CCR5	BV786	743700	BD Biosciences
CCR7	BUV615	752519	Biolegend
CCL5 (RANTES)	149103	149103	Biolegend
CD1d-tetramer	PE/APC/BV421		NIH Tetramer Core
CD103	BV421	562771	BD Biosciences
CD11b	BV480	566117	BD Biosciences
	BV421	562605	BD Biosciences
	BUV563	741242	BD Biosciences
	PerCP/Cy5.5	101228	Biolegend
CD11c	APC-eF80	47-0114-80	Fisher Scientific

CD19	BV480	566167	BD Biosciences
	BV421	115537	Biolegend
CD206	PE/Fire 700	141741	Biolegend
	FITC	141741	Biolegend
CD27	PE	124216	Biolegend
CD3	Spark Blue 550	100259	Biolegend
	BUV395	740268	BD
	FITC	48-0032-82	eBioscience
CD4	APC-Fire810	100479	Biolegend
	PE-Cy7	50-154-34	Fisher Scientific
CD8a	APC-eF780	47-0081-82	Invitrogen
	BV786	563332	BD Biosciences
CD8b	RB780	569478	BD Biosciences
CD45	BV570	103136	Biolegend
	BV605	103155	Biolegend
	AF700	103127	Biolegend
CD45.1	BUV661	741517	BD Biosciences
	BV785	110743	Biolegend
CD62L	PE-Fire810	161205	Biolegend
CD64	BV605	139323	Biolegend
CD69	BUV496	741063	BD Biosciences
CD86	PE	105008	Biolegend
CXCR6 (CD186)	BV711	151111	Biolegend
DNAM-1	BV605	133613	Biolegend
EOMES	PE-eF610	61-4875-80	Fisher Scientific
F4/80	BV711	123147	Biolegend
Granzyme B	PerCP-eF710	46-8898-80	Fisher Scientific
	FITC	422302	Biolegend
Granzyme K polyclonal Ab	N/A	PA5-50980	Fisher Scientific
Helios (Ikzf2)	PE	137206	Biolegend
I-A/I-E (MHC-II)	BV650	563415	BD Biosciences
IL10	BV605	564082	BD Biosciences
IL17A	BV650	506929	Biolegend
IL17F	AF647	517004	Biolegend
IL-18Rα	PE	157903	Biolegend
IFNγ	PerCP-Cy5.5	505822	Biolegend
Ly-6C	BUV805	755203	BD Biosciences
Ly-6G	PE-Cy7	127618	Biolegend
Ly-49F	APC-Vio770	130-117-270	Miltenyi Biotec
Ly-49G2	RB780	755977	BD Biosciences
MR1-monomer			NIH Tetramer Core
NK1.1	BUV615	751111	BD Biosciences

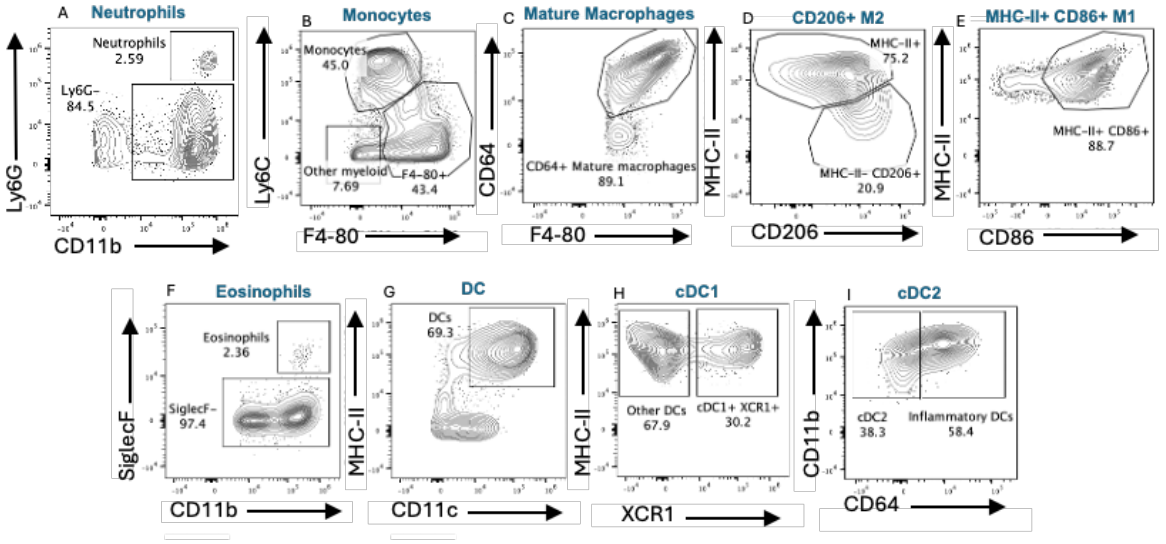
	BV605	563220	BD Biosciences
NKG2A/C/E	BV605	564382	BD Biosciences
NKG2D (CD314)	PE-CF594	562614	BD Biosciences
	BV711	563694	BD Biosciences
NKp46 (CD335)	PE-Cy7	137617	Biolegend
Perforin	Pac Blue	145311	Biolegend
	FITC	154309	Biolegend
PD-1 (CD279)	PE-Fire810	135253	Biolegend
	BV785	135225	Biolegend
PLZF	AF488	53-9320-80	Invitrogen
	PE-Cy7	145805	Biolegend
RORγt	BV650	564722	BD Biosciences
SiglecF	APC	155508	Biolgend
T-bet	RB780	569090	BD Biosciences
TCRαβ	BUV805	748405	BD Biosciences
TCRγδ	PE-Cy7	25-5711-82	Fisher Scientific
	BUV737	748991	BD Biosciences
	PerCP-eF710	46-5711-82	Invitrogen
TNF	APC	506307	Biolegend
	AF700	506338	Biolegend
XCR1	PE/Dazzle594	148233	Biolegend

2.2 Methods

2.2.1 Representative Gating



5**Figure 2.1:** Representative Gating: Double-negative T cells in the spleen. **(A)** Leukocytes **(B)** Singlets **(C)** Live cells **(D)** CD45^{+} **(E)** CD3^{+}, TCRδ^{-} **(F)** non-MAIT/iNKT **(G)** CD4^{-}, CD8^{-} **(H)** TCRβ^{+} DNT



6 **Figure 2.2:** Representative Gating: Myeloid cells in the tumour

2.2.2 Mice

Male C57BL/6J mice between 7-10 weeks of age were used for most experiments, female mice were used for E0771 tumour models. Mice used for experiments profiling identifying DNT TCR-restriction, expansion protocol or involvement in tumour models were purchased from Jackson Laboratory, and housed in the genomics animal facility at Princeton University; *B2M-/-* (RRID:IMSR_JAX:002087), *MHC-II-/-* (RRID:IMSR_JAX:003584), CD8-/- (RRID:IMSR_JAX:002665). Jaxboy CD45.1+ mice were purchased from Jackson Laboratory (RRID:IMSR_JAX:033076), and housed in the genomics animal facility at Princeton University. *Tap1-/-* mice were kindly gifted by Kenneth Rock (University of Massachusetts). All experiments were conducted under the lynch lab Institutional Animal Care and Use Committee (IACUC). Mice used for experiments examining TCR clonality, RNA transcriptional profiling, tissue phenotyping and cytokine responsivity were conducted in the animal facility at Brigham Women's Hospital, in accordance with the Lynch lab IACUC protocol. Mice were euthanised by CO_2 inhalation, and tissues were immediately harvested into HBSS.

2.2.3 10X Single-cell RNA Sequencing

Spleen or adipose tissue lymphocytes were isolated from C57BL6/J mice as described above and were then surface stained before being FACS sorted for total DNT cells ($CD45^+$, $CD19^-$, $B220^-$, $CD11b^-$, $TCR\gamma\delta^-$, CD1d-$tetramer^-$, MR1-$tetramer^-$, $CD4^-$, $CD8^-$, $CD3^+$, $TCR\alpha\beta^+$). CD1d-tetramers were provided by NIH tetramer core and pre-loaded with αGalCer. 5-OP-RU pre-loaded MR1 biotinylated monomers were provided by NIH tetramer core, with streptavidin-PE labelling performed before staining. Sorted DNT cells were then counted and supplied to the Brigham and Women's Hospital single cell genomics core for 10X scRNA sequencing and combined TCR sequencing. Sc seq data from spleen and adipose tissue were processed using the Seurat R package. Raw feature-barcode matrices were read with Read10X, and cells were filtered to include those with >200 and <5000 features and <5% mitochondrial reads. A Seurat object was generated, and data were normalized using SCTransform. Cells with fewer than 300 detected genes, fewer than 500 UMIs, or more than 10% mitochondrial content were excluded from the analysis. Putative doublets were removed using

DoubletFinder with per-sample parameter tuning based on expected doublet rates and neighbourhood size. Dimensionality reduction was performed via PCA (top 40 PCs), followed by UMAP visualization, and clustering with the Louvain algorithm (FindClusters, resolution = 0.5). TCR data (filtered_contig_annotations.csv) were loaded and cleaned to remove "-1" suffixes from barcodes. Barcodes were intersected with scRNA-seq data, and the filtered TCR annotations were aggregated per barcode. Metadata was added to the Seurat object (AddMetaData), including clonotype IDs, CDR3 sequences, and frequencies. Clonotype-level visualization was performed using DimPlot, FeaturePlot, and clonalOverlay from scRepertoire. Genes encoding ribosomal proteins (Rpl, Rps) and TCR V genes (Trbv) were removed using pattern matching. The dataset was subsetted and re-normalized. PCA and clustering were re-run, and clusters were renamed to reflect functional phenotypes (e.g., Effector memory, NK-like, Type-17) based on marker gene expression. Differentially expressed genes (DEGs) were identified using FindAllMarkers (Wilcoxon test, logFC > 0.25, adjusted $p < 0.05$). For heatmap visualization, the top 5 DEGs per cluster were selected using slice_max. For high-expression genes (HEGs), the top 100 DEGs per cluster were exported with Bonferroni correction using openxlsx. Seurat objects from spleen (Spleen_dn_2) and adipose (DN_adipose_1) were merged using merge, with distinct cluster identifiers prepended (e.g., "Spleen_Type-17"). UMAP embeddings were manually concatenated. Metadata was labelled with sample origin (orig.ident). For batch correction, Harmony integration was performed using RunHarmony on SCT-normalized data, and visualized with UMAP (RunUMAP, DimPlot).

2.2.4 Tissue Isolation and Processing

Mice were euthanised by CO_2 inhalation, and tissues were immediately harvested into HBSS. Lymph nodes and spleens were mashed through a 70µm filter and washed, spleens were subsequently lysed using RBC lysis buffer for 5min before being washed and ready for staining. Adipose tissue was minced and digested with 1mg/ml type II collagenase in RPMI for 25-30min in shaking incubator at 37C. Cells were passed through a 70µm filter, washed with RPMI and centrifuged at 300g to pellet the stromal vascular fraction from floating mature adipocytes. Liver tissue was mashed and washed through a 100µm filter. Hepatocytes were spun at 50g for 5 minutes, before

removing supernatant and spinning at 1500rpm for 5 minutes. Cells were resuspended in 25ml of 35% percoll and spun at 900g for 20 mins at RT. The remaining pellet was placed in RBC lysis buffer before a final wash and spin. Small intestines were cleared of peyers patches and faeces by using a curved scissors and flushing with PBS. The tissues were cut into 1-2cm pieces and placed into 20ml HBSS without $Ca^{2+/}Mg^{2+}$, and the addition of 5mM EDTA and 1mM DTT for 20min in shaking incubator at 37C. First fraction was collected over a 70µm strainer, and the tissue was placed into 20ml HBSS without $Ca^{2+/}Mg^{2+}$, and the addition of 5mM EDTA only for a 20min in shaking incubator at 37C. The second fraction was collected over 70µm strainer and pooled with fraction 1. The IELs were washed, centrifuged at 1400rpm and resuspended in RPMI to await staining. Tumours were dissected and weighed, before cutting into ~1cm pieces, and placing into pre-warmed 37C RPMI with digestion cocktail (100µg/mL DNase1, 30µg/mL Collagenase D) and incubating in a 37C shaking incubator for ~45 minutes. Subsequently, tumours were filtered through 100µM mesh and washed with HBSS and 2% FBS buffer x2 times, prior to stain or stimulation. Tissue processing for downstream applications was performed as per Lynch Lab protocols.

2.2.5 Ex Vivo Stimulations

After cell isolation from murine tissues, cells were stimulated for 4 hours with 10ng/ml phorbol 12-myrisatate 13-acetate (PMA), 500ng/ml ionomycin and 5ug/ml brefeldin A in cRPMI medium and incubated at 37C. For cytokine stimulations, cells were stimulated for 4-6hrs in IL-17 inducing conditions (10ng/ml IL-23, 10ng/ml IL1-β), or IFNγ inducing conditions (10ng/ml IL-12, 10ng/ml IL-15, 10ng/ml IL-18). For activation experiments on whole lymphocytes from spleen, some samples required TCR stimulations which involved coating a 24-well plate with 1ug/ml CD3 and adding 5ug/ml CD28 to cell suspension when plating. After all stimulations, cells were washed in PBS and resuspended in FACs buffer for staining.

2.2.6 Staining for flow cytometry

Cell suspensions from tissue isolations were either directly plated for staining or stimulated for downstream intracellular staining. Ex-vivo cells were stimulated for 4 hours with 10ng/ml phorbol 12-myrisatate 13-acetate (PMA), 500ng/ml ionomycin and 5ug/ml brefeldin A in cRPMI medium and incubated at 37C. For cytokine stimulations, cells were stimulated for 4-6hrs in IL-17 inducing conditions (10ng/ml IL-23, 10ng/ml IL1-β), or IFNγ inducing conditions (10ng/ml IL-12, 10ng/ml IL-15, 10ng/ml IL-18). Certain experiments required TCR stimulations which involved coating a 96-well plate with 1ug/ml CD3 and CD28. After all stimulations, cells were washed in PBS and resuspended in FACs buffer for staining. Cells were incubated in Live/Dead Aqua (1:800 in PBS) for 10min on ice before or after tetramer staining. Cells were stained with CD1d- and MR1-tetramers provided by the NIH tetramer Core for 30 minutes at RT. Cells were then washed with PBS and stained with surface antibodies and Fc-block (1/200 in FACs buffer) for 20min. Chemokine receptor antibodies, if included, were stained for 20 min at RT. Subsequently cells were washed with FACs buffer and fixed using 100 ul of eBioscience intracellular fixation buffer or eBioscience FoxP3 fix (if looking at transcription factors). Cells were incubated at RT for 20min and washed with 1X permeabilization buffer. Cells were then stained with intracellular antibodies for 30min at RT and washed in FACs buffer and resuspended for acquisition on the Cytek Aurora spectral cytometer. Flow cytometry staining was performed as per Lynch Lab protocols.

2.2.7 In-vitro activation of DNTs

Spleens from naïve C57BL/6J mice were harvested and isolated lymphocytes were stimulated overnight under various stimulation conditions. To assess the TCR-dependence of DNTs, DNTs were stimulated with CD3 alone or with CD28 co-stimulation (1 ug/mL). To assess innate effector function, DNTs were stimulated with the cytokines IL-12/15/18 (10 ng/mL) in the presence or absence of CD3 stimulation. To investigate the type-17 effector profile, DNTs were stimulated with the cytokines IL1B/IL23 (10 ng/mL) with or without CD3 stimulation. Additionally, some cells were stimulated with PMA/Iono as a positive control. Following overnight stimulation, cells were stained for DNTs and a selection of intracellular proteins. To further investigate the in vitro activation of DNTs without any potential interference from other cell types

in culture, DNT cells, and in specified experiments; CD4, CD8, iNKT and γδ T cells, were FACs sorted, using the Cytek Aurora sorter (Harvard Medical School core) from 20 pooled spleens and cultured overnight in either CD3 and CD28 or PMA/Ionomycin (cell stim cocktail), with and without type-1 and type-17 inducing cytokines, stated above. CD3 MACs enrichment by negative selection beads was performed prior to the sort to increase sorting speed and efficiency.

2.2.8 Ex-vivo expansion of DNTs

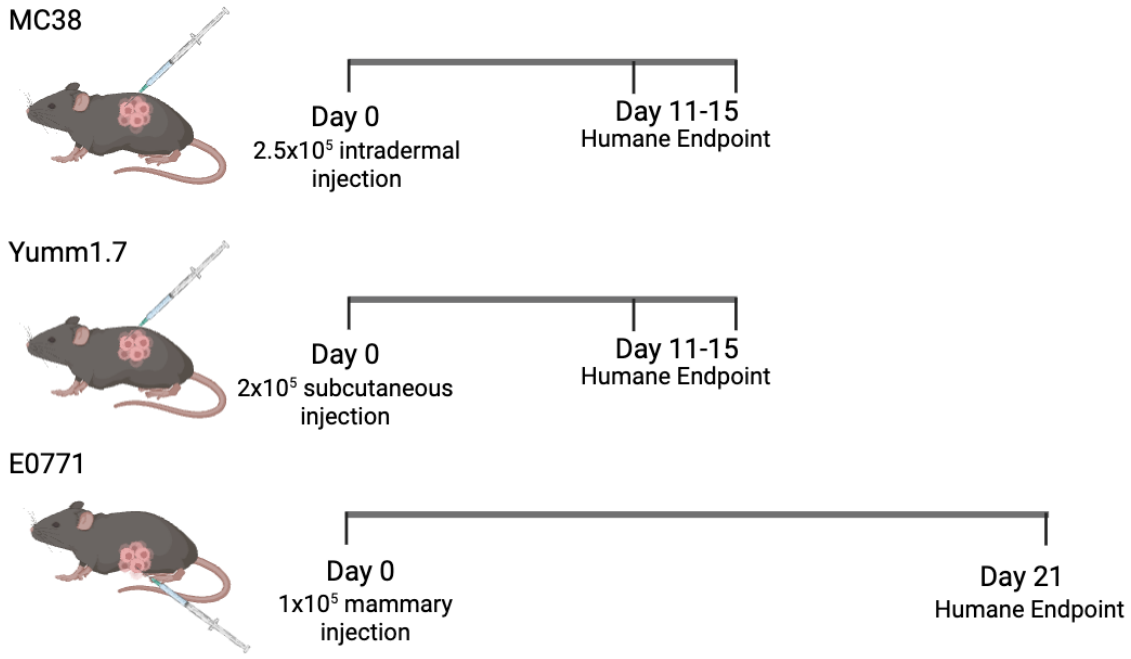
A single-cell suspension from 10 to 20 pooled spleens/inguinal LNs of CD45.1 (Jax boy) mice was generated as described above. CD3 MACs enrichment was performed (Miltenyi pan T cell isolation kit II) and DNTs were FACS sorted into Eppendorf tubes with the Aria Fusion sorter at Princeton University flow core ($CD45^+$, $CD3^+$, $TCR\alpha\beta^+$, CD1d-tetramer$^-$, MR1-tetramer$^-$, $CD4^-$, $CD8^-$). Cells were plated at 6 – 10 x 10^4 cells/well in a 96-well u-bottom, pre-coated with 1 μg/ml CD3/CD28 and soluble cytokines were added after plating (10 ng/ml IL-2, IL-7, and IL-15). Cells were cultured in cRPMI media, as listed in materials. Three days later, cells were split 1:2 and every two to three days following that. TCR stim was only used for first three days, cytokines were replenished at the same concentration on each split, with increased IL-7 (20 ng/ml) from day 14 onwards. Cells were expanded for two-three weeks to yield working numbers (~150-fold increase at 21 days). Prior to use of expanded cells for in-vitro or in-vivo work, an overnight stimulation with IL-12, IL-15 and IL-18 (10 ng/mL) was provided to induce IFNγ production. Expansion protocol was optimised through a series of trouble-shooting experiments assessing various concentrations of TCR and cytokine stimulations, DNT viability and growth was assessed over three weeks and optimum concentration was chosen for subsequent experiments.

2.2.9 In-vitro killing assays

MC38 cells were plated at 5000 cells/well in a 96-well flat bottom plate, and incubated at 37 degrees overnight. MC38 cells were stained with Draq5 nuclear permeabilising dye at 1/1000 for 5 minutes prior to being washed twice and replenished with DMEM media. Expanded DNT cells were restimulated with IFNγ-inducing cytokines as stated

above., and stained with Cell Trace Violet for 20 min at 37 degree Celsius prior to washing, and combination with DNTs at decreasing concentrations; 20:1, 10:1, 5:1, 1:1. Some experiments were run in Sartorius Incucyte live cell imager, and others in Agilent BioTek Lionheart, as indicated in the text.

2.2.10 Murine tumour Models and adoptive transfer



7 **Figure 2.3:** Tumour Models; Injection routes and endpoints.

Yumm1.7 melanoma cells were kindly provided by Prof. Hans R. Widlung of Brigham and Women's hospital. The E0771 murine breast adenocarcinoma cells, and MC38 murine colon adenocarcinoma cells were purchased from ATCC (Manassas, VA), and were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% (vol/vol) FCS (Gibco; Thermo Fisher Scientific), 1% (vol/vol) penicillin/streptomycin (Sigma-Aldrich) and 10 mM HEPES buffer (Gibco; Thermo Fisher Scientific). At ~8 weeks of age, mice were anesthetized and injected subcutaneously on the right flank with 2×10^5 (Yumm1.7) or intradermally in the back with 2.5×10^5 MC38 cells or in the mammary gland with 1×10^5 E0771 cells suspended in PBS. For Yumm1.7 or MC38 tumours, male mice were used, while female mice were used EO771. Once tumours were palpable, callipers were used to perform measurements of length and width every 1 to

2 days and tumour area was calculated by multiplying length x width. Mice were euthanized pre-humane endpoints, typically 11-15 days for MC38 and Yumm1.7 depending on rate of tumour growth, and by 21 days post-injection for E0771, as indicated in figure legends. Tumours never exceeded maximum tumour burden listed under the IACUC protocol of Princeton University (20 mm in any direction). In certain experiments, 5 x 10^6 ex-vivo expanded DNTs from Jaxboy CD45.1 mice were adoptively transferred intratumorally to C57BL6/J CD45.2 mice bearing MC38 tumours on two separate injections, two days apart, starting from when tumours were of size to be injected (3x3 mm), typically day 4 or 5.

2.2.11 TIF extraction and Luminex analysis

LNs and tumours were dissected from MC38 tumour bearing mice, treated with PBS or DNTs as described above. Weights were measured for both tissues, and the tumours were cut in half before transferring on top of filter paper in 1.5 ml Protein LoBind Eppendorf tubes (Cat #0030108442). LN and tumours were spun at 350 G for 15 minutes. For LNs, 1 to 3 ul of interstitial fluid was extracted and 8 to 23 ul for tumours. Samples were frozen at -80 prior to luminex. Tumour TIF samples were run on a sensitive detection micro-BCA protein assay kit (Thermo #23235) in a final dilution of 1/350. Tumour samples were normalised to a final conc. of 0.5 mg/ml for the Luminex assay. Total LN volume was used for the assay and normalised post-hoc to LN weight. A custom 26-procarta plex (Thermo #PPX-26), was run as per manufacturer's instructions (for serum/plasma samples) on the Bio-plex 200 system (Bio-rad).

2.2.12 Data Analysis and Statistics

The data are presented as mean ± SEM. A D'Agostino–Pearson omnibus normality test (or Shapiro-Wilk if n<6) was first performed to test whether the data were normally distributed (Gaussian distribution). If data were normally distributed, parametric testing was performed and Dunnet's multiple comparison test. If data were not normally distributed, non-parametric testing was performed with Dunn's multiple comparison. Statistical significance was determined using the student's t-test, one-way ANOVA, or

two-way ANOVA as indicated. For all experiments, a 95% confidence interval was used and a p-value of <0.05 was considered statistically significant and is presented as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or $p < 0.0001$. GraphPad Prism 10.5.0 was used for all statistical analysis. All diagrams and graphical representations were created using biorender.

Chapter 3:

DNTs are a clonally diverse population with conserved effector phenotypes across tissues

3.1 Introduction

Unconventional T cells often present with limited TCR diversity and clonality across tissues in both mice and humans, allowing them to have TCR-specificity and fine-tuned responses to conserved antigens. Accordingly, innate T cells preferentially localise to distinct barrier and metabolic tissues in both mice and humans, with subset composition and frequency shaped by tissue context. For example, iNKT cells express an invariant TCRα, consisting of TCRα-chain variable region (TRAV) and joining region (Vα14-Jα18 in mice, and Vα24-Jα18 in humans), coupled to a limited array of β chains (Vβ8, Vβ7 and Vβ2 in mice, and Vβ11 in humans)[116,117]. In mice, iNKT cells are strikingly enriched in the liver (~40% of hepatic T cells) and are also prominent in the thymus, spleen, lung, intestine, and visceral adipose tissue in humans, iNKT cells are typically rarer in blood (often <0.1% of T cells) but are detectable in liver, adipose tissue, mucosal sites, and secondary lymphoid organs. In humans, MAIT cells are especially abundant in the liver (often comprising ~20–40% of hepatic T cells) and present in blood (~1–8% of T cells), with substantial enrichment across mucosal and barrier tissues including the intestine, respiratory tract, female genital tract, oral mucosa, and skin. In mice, overall MAIT frequencies are much lower than in humans, the inverse of iNKT distribution, but subset and tissue biases are conserved. MAIT17 cells are detectable at mucosal sites such as lung and gut, whereas MAIT1 cells are present at low frequencies in liver and lymphoid tissues. Similarly, MAIT cells also possess an invariant TCRα with Vα19-Vα33 in mice and Vα7.2-Jα33 in humans[118].

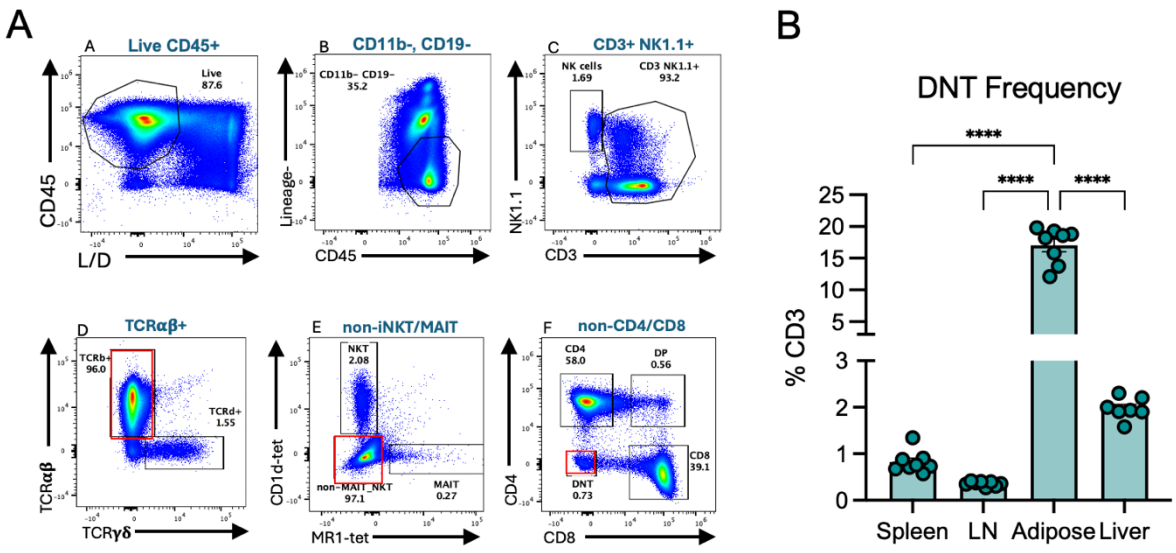
γδ T cells are predominantly tissue-resident, concentrating at epithelial and barrier interfaces. In mice, development occurs in waves that seed distinct tissues. γδ T cells display greater diversity in receptor usage than MAIT or iNKT cells but remain oligoclonal, with subsets skewed toward particular Vγ and Vδ chains that confer specificity for microbial phosphoantigens, stress-induced self-ligands, or metabolites[2]. Accordingly, γδ T cell subsets are named according to the TCR variable (V) segments used, with emphasis on TCRγ-chain use in mice and TCRδ-chain use in humans. In mice, γδ T cell subsets are defined by the V segments used within the δ chain, which also determine effector functions within subsets. γδ-17 cells producing IL-17, possess either Vγ4 or Vγ6 chains and tend to be found in the LN, lungs and intestinal lamina

propria[119]. DETCs are IFNγ producers, present in the skin and identified by Vγ5[120]. Gut specific IFNγ producers are found in the intraepithelial layer of the intestines and are Vγ7^{+}. The more widespread Vγ1+ cells are also type-1 IFNγ producers[121]. In humans, there are three main γδ subsets; Vδ1 which are primarily found in the skin, spleen, liver and gut epithelium, Vδ2, found mainly in adult blood, and the less described Vδ3 which can be found in the liver and gut[122-124].

The restricted TCR repertoire of unconventional T cells, focuses their antigen recognition on a limited range of conserved ligands. This constraint allows for finely tuned effector programs that are optimised for early host defence, tissue homeostasis, and rapid recall responses[45,125,126]. Additionally, TCR specificity of the unconventional subsets provides a reliable means of distinguishing them from one another, enabling studies that examine subset-specific roles for these cells across homeostasis and disease. We thus aimed to uncover the TCR landscape of DNT cells to delineate potential reliance on certain TCR rearrangements or chain usage. We investigated the clonality and transcriptional profile of DNTs in spleen and adipose tissue to determine presence of conserved clonotypes and identifiable DNT-specific signatures within the TCR. Additionally, to understand if DNTs present in lymphoid and non-lymphoid tissues share similar characteristics and appear to be descendent of the same lineage. We also adapted a tissue-wide flow cytometric analysis of DNT cells, to observe distribution and subtype-specific tissue adaptations. Combined single-cell (sc)-RNA/TCR sequencing demonstrated diverse polyclonality within the DNTs from both tissue sites, showing no clonotype consistency within the population. However, sc-RNA sequencing revealed seven distinct DNT clusters in the spleen, with five present and conserved in the adipose, characterised by known functional markers of T cell subtypes. Functional confirmation of DNT transcriptional identifiers and subsetting across mouse organs, identified a fluid expression of various type-1 markers that could collectively be described as DNT-1. DNT-1 can be identified by expression of NK1.1, T-bet & EOMES, whereas DNT-17 are classified by expression of RORγt, and higher CXCR6 and PLZF.

3.2 DNT identification and frequency in lymphoid and non-lymphoid tissue

Using flow-cytometry analysis, we defined the DNT population in murine tissues, as TCRαβ+ CD4-, CD8- double-negative T-cells and non-CD1d/MR1-tetramer reactive, as shown in representative gating from the spleen (**Figure 3.1A**). DNTs are present at low frequencies of total T cells ($CD3^+$) in secondary lymphoid tissues (<3%) but have substantial enrichment in the gonadal white adipose tissue (gWAT), where they can constitute up to 20% of the total CD3 population (**Figure 3.1B**).



8 **Figure 3.1:** DNT gating strategy and tissue distribution

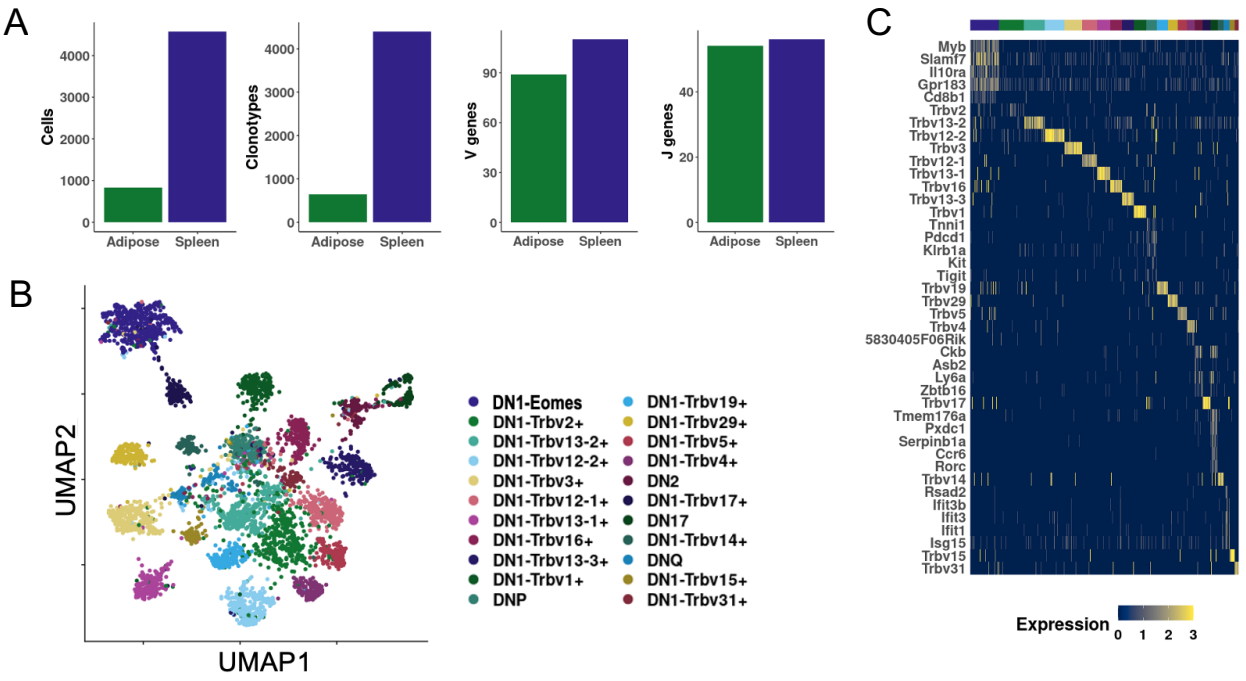
(A) Representative images of DNT identification by flow cytometry ($CD3^+$ $NK1.1^+$, excluding $CD3^-$ $NK1.1^+$, $TCR\alpha\beta^+$, CD1d/MR1-tetramer$^-$, $CD4^-$, $CD8^-$ double-negative).

(B) Frequency of DNT as a percentage of $CD3^+$ cells from flow cytometry analysis of ex-vivo murine spleen, iLN, gWAT, and liver.

Data are shown as mean ± SEM, N=2, n=8. Significance calculated using one-way anova. **** $p \leq 0.0001$

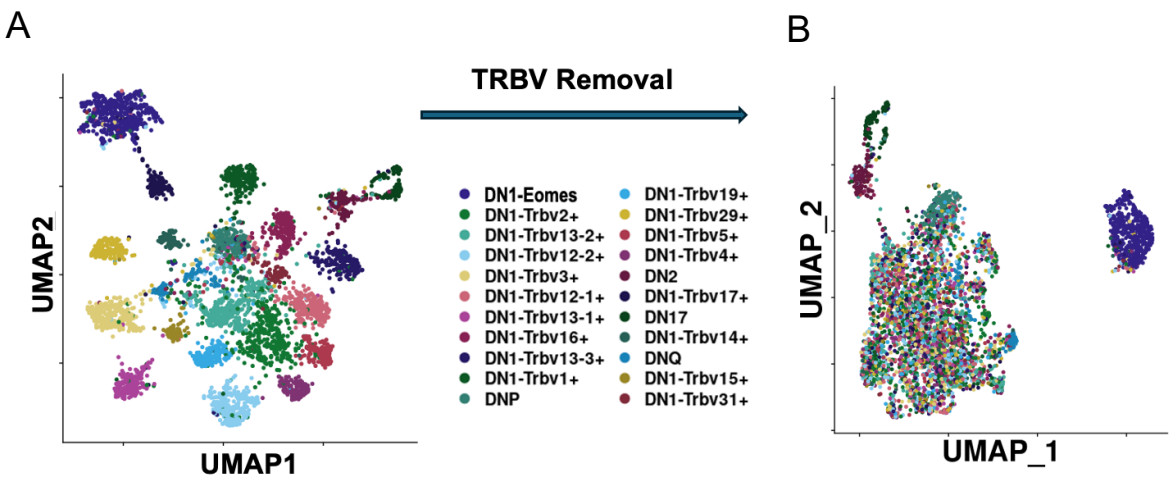
3.3 DNT cells display high polyclonality in spleen and adipose tissues

To identify potential clonality and transcriptional phenotypes of DNTs across tissues, we performed combined 10x single cell (sc)-RNA and TCR sequencing of isolated splenic and gWAT DNTs. A defining feature of unconventional T cells is their limited TCR diversity, via agonist-selection in the thymus on non-polymorphic antigen presenting molecules[2]. This selection process promotes oligoclonal or clonal TCR expansions, as exemplified by MAIT and iNKT cells that populate the periphery with semi-invariant or invariant TCRα chains, and restricted TCRβ pairing. This limited TCR repertoire confers fine-tuned responses to highly conserved antigens[1]. Contrary to this norm, the DNT population are highly clonally diverse with over 4000 clonotypes in the spleen and ~700 in the adipose, close to matching the total cell count in each tissue (**Figure 3.2.A**). Additionally, the DNT TCRs also have high variability in both V and J gene segments. Following batch correction and graph-based clustering, uniform manifold approximation and projection (UMAP) of splenic DNTs distinguished 21 clusters, at first indicating expansive heterogeneity within the DNT population (**Figure 3.2.B**). However, differential gene expression (DEG) analysis amongst the 21 clusters revealed most of the variation between clusters is due to variability in T cell beta variable genes (TRBV; **Figure 3.2.C**). To allow for accurate clustering based on transcriptomic differences across the DNT population, TRBV genes were removed from the sample of highly variable genes, with the resulting UMAP no longer skewed by V gene usage (**Figure 3.3A and B**). Importantly, removing TRBV genes from the gene-expression object does not delete the underlying VDJ calling from the paired TCR-seq, so TRAV/TRBV usage can still be quantified from contig annotations/scRepertoire outputs and visualised as V-gene distributions. Within the non-TRBV dictated DNT clusters, V and J gene distribution of splenic DNTs have a high degree of diversity and a lack of any dominant gene usage in either TCRα or TCRβ chains across seven different gene clusters (**Figure 3.4 and 3.5**). Additionally, Shannon diversity confirms high polyclonality and appears largely similar between groups (**Figure 3.6.C**). The seven subtypes do not present any dominant α or β clonotypes or altered Shannon diversity amongst clonotypes (**Figure 3.6.D and E**).



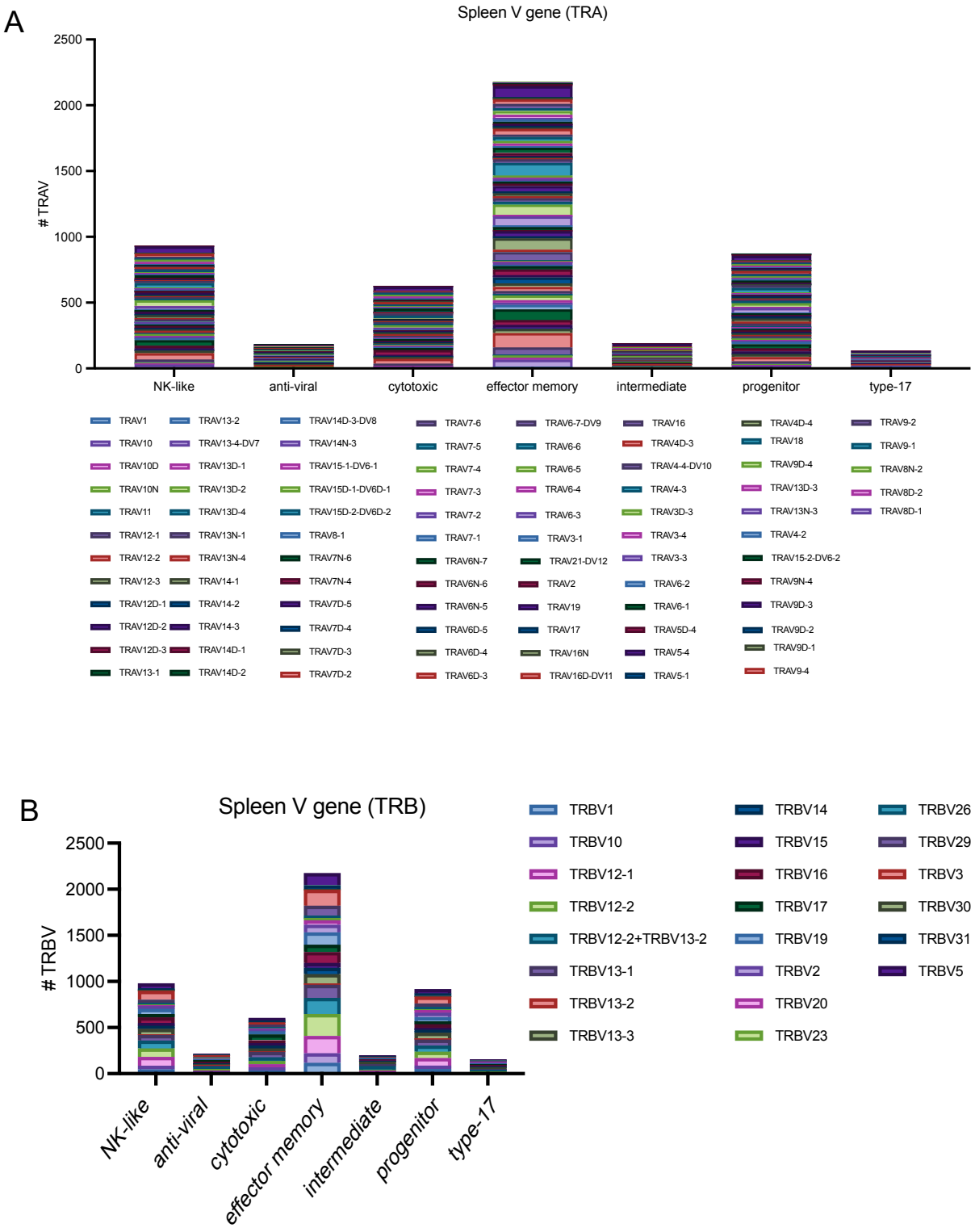
9 **Figure 3.2** Single-cell RNA/TCR sequencing of splenic DNTs reveals heterogenous population with high TRBV variability

scTCR-sequencing of spleen and adipose DNT cells **(A)** Bar plots of cell count, unique clonotype count, V & J gene count, of spleen and adipose DNTs. **(B)** UMAP of splenic DNTs pre-*Trbv* gene removal; unsupervised clustering results in segregation by *Trbv* usage. **(C)** Heatmap of top DEGS in 21 cluster pre-*Trbv* gene removal. sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio. *Analysis in this figure generated by Harry Kane, PhD.*



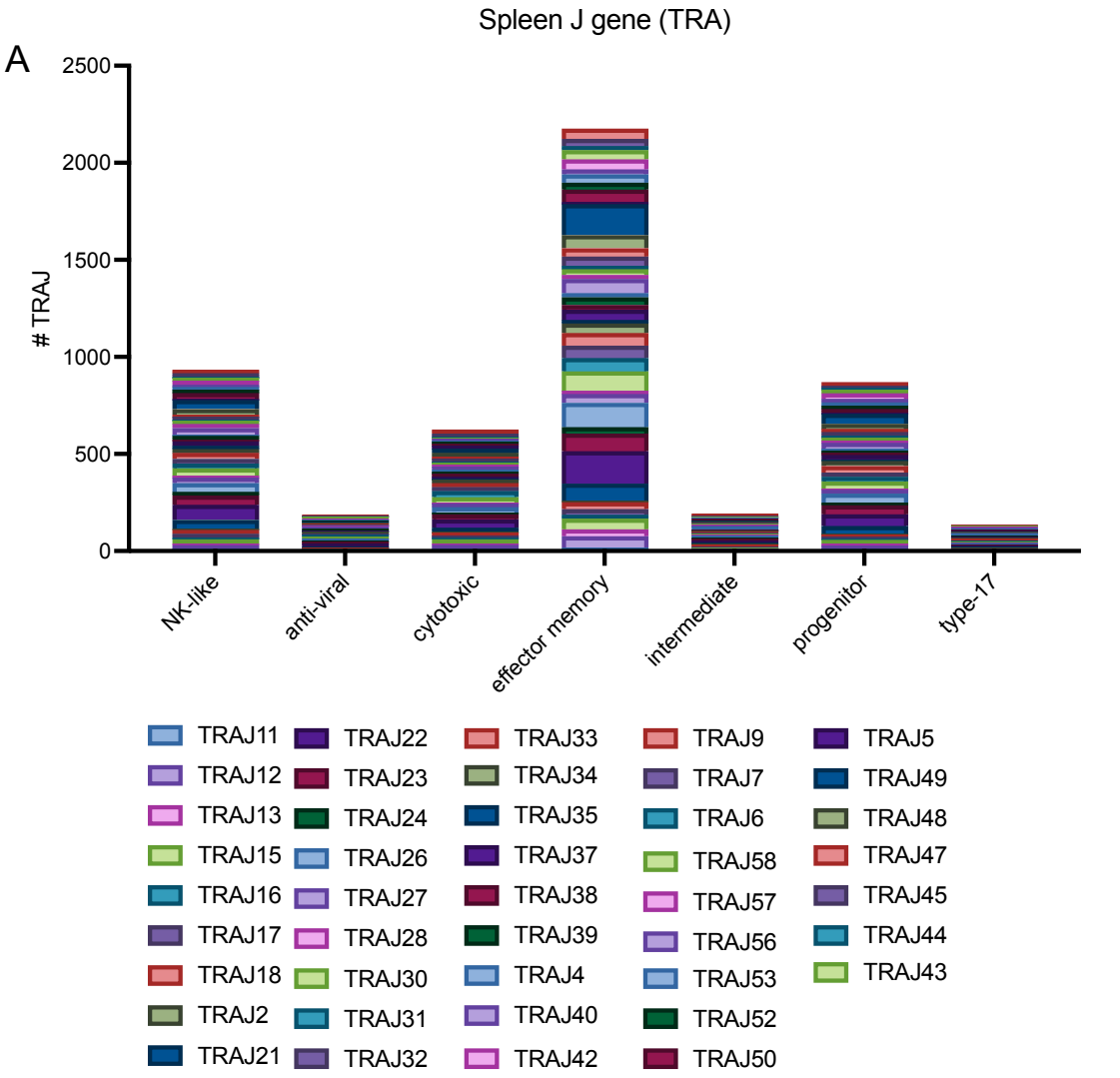
10 **Figure 3.3** Single-cell RNA/TCR sequencing of splenic DNTs reveals heterogenous population with high TRBV variability

sc-TCR-sequencing of splenic DNTs. **(A)** UMAP of splenic DNTs pre-*Trbv* gene removal; unsupervised clustering results in segregation by *Trbv* usage. **(B)** UMAP of splenic DNTs post-*Trbv* gene removal; loss of cluster separation by TCR differences. sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio. *Analysis in this figure generated by Harry Kane, PhD.*



11 **Figure 3.4:** Diversity in V gene distribution in splenic DNT cells.

sc-TCR-sequencing of splenic DNTs, clusters annotated post-Trbv removal. **(A)** V gene distribution in T cell receptor alpha. **(B)** V gene distribution in T cell receptor beta.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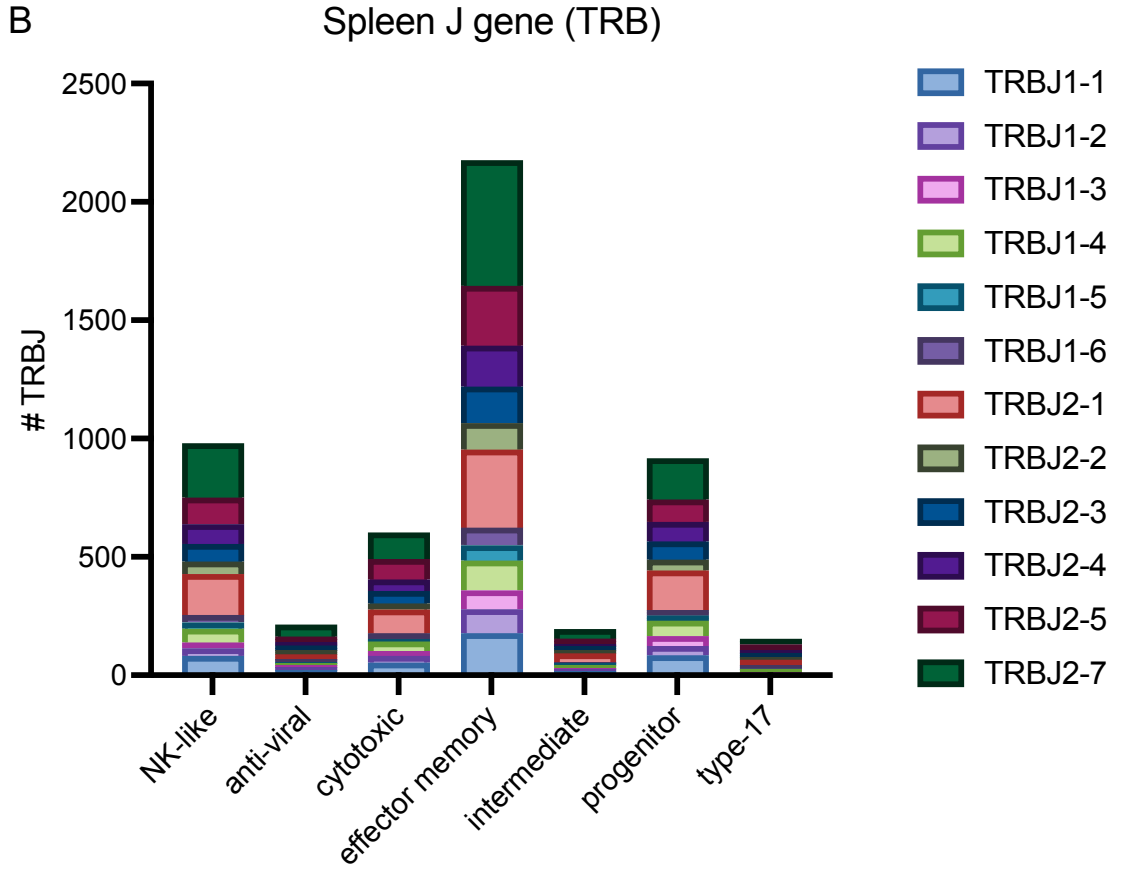
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TRBJ2-2
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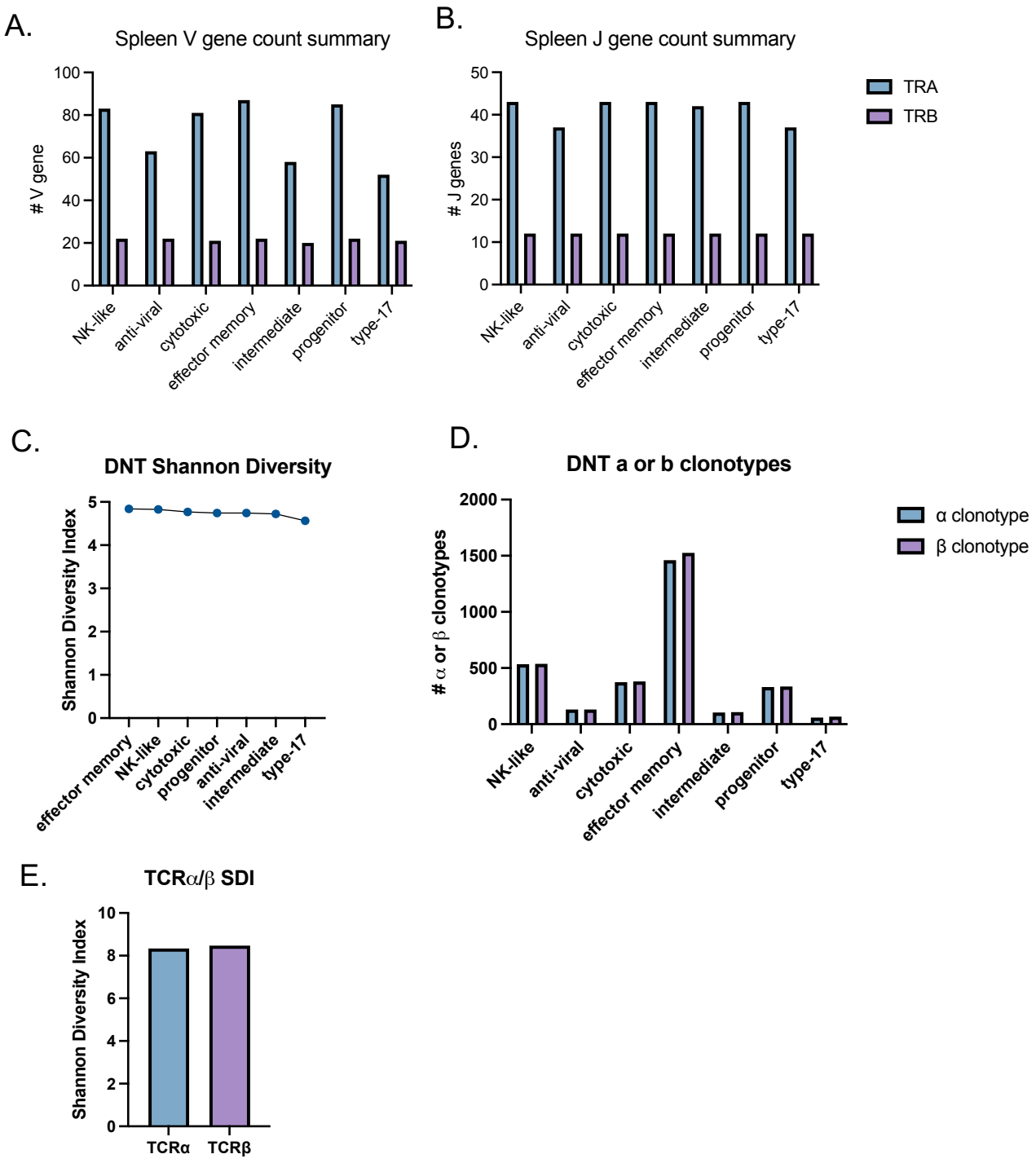
TRBJ2-5
TRBJ2-7

12 **Figure 3.5:** Diversity in J gene distribution in splenic DNT cells

sc-TCR-sequencing of splenic DNTs, clusters annotated post-Trbv removal. **(A)** J gene distribution in T cell receptor alpha. **(B)** J gene distribution in T cell receptor beta.



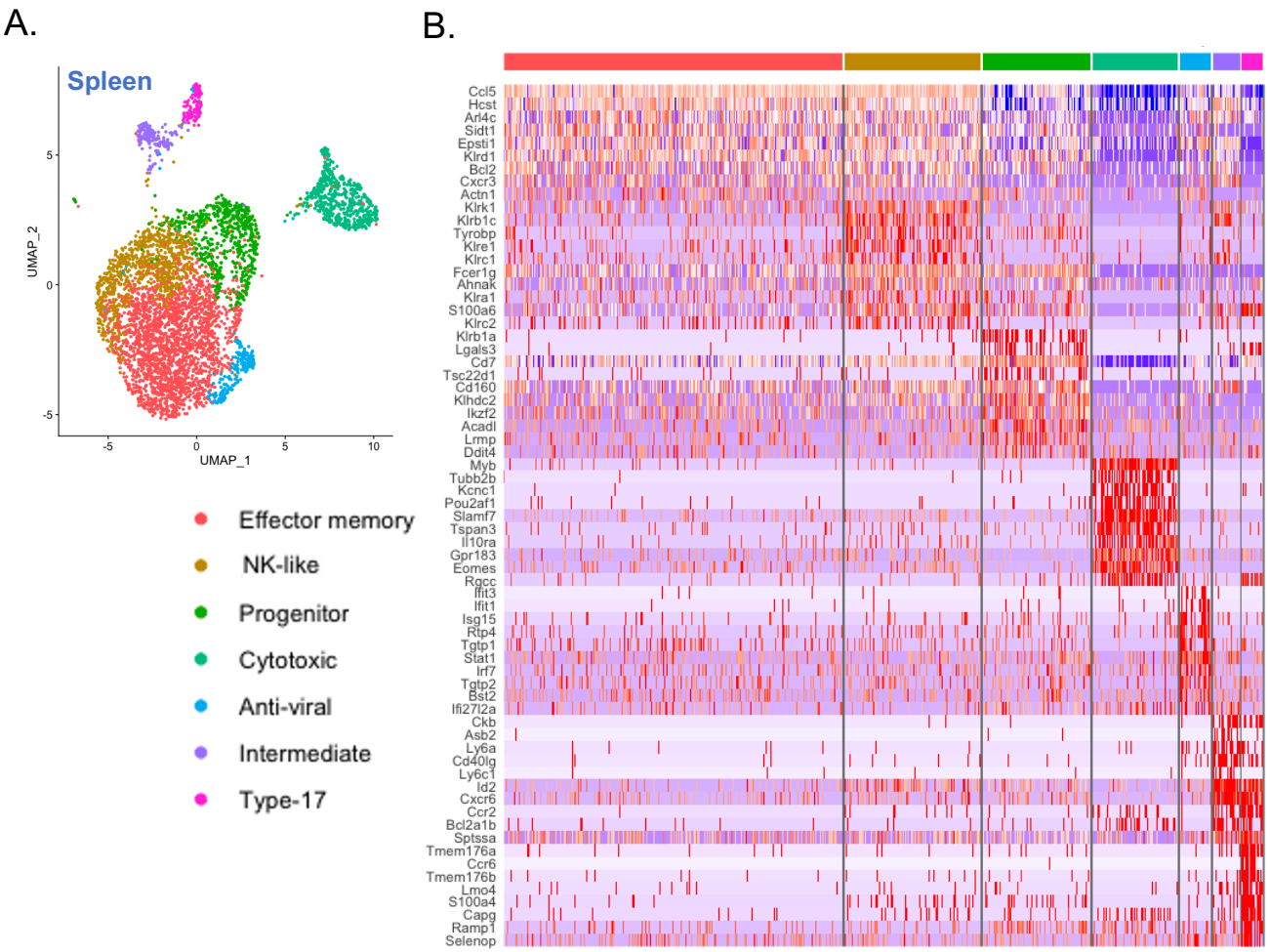
13 **Figure 3.6:** TCR clonotype diversity in spleen DNT cells

TCR-sequencing of splenic DNT cells. **(A)** Spleen V gene count summary **(B)** Spleen J gene count summary **(C)** Shannon diversity index of annotated effector types in splenic DNT (post-*Trbv* removal). **(D)** Count of α or β clonotypes with splenic DNT clusters. **(E)** Shannon diversity index of TCRα compared to TCRβ in splenic DNTs

3.4 DNTs are a heterogenous population with conserved transcriptional expression between spleen and adipose tissues

Following *Trbv* removal, unsupervised clustering and UMAP dimensionality reduction revealed seven transcriptionally distinct populations which we termed *Effector memory*, *Progenitor*, *Cytotoxic*, *NK-like*, *anti-viral*, *Intermediate*, and *Type-17* (**Figure 3.7.A**). Subsets were annotated based on the top differentially expressed genes (DEGs; **Figure 3.7.B**), and lineage-defining transcriptional profiling (**Figure 3.8**). To further classify the splenic DNT subsets, we ranked the top highly expressed genes and categorised them into plasma membrane-associated, transcription factor, and secreted effector groups based on gene ontology (GO) terms (**Figure 3.9**). A number of the DNT clusters expressed *Tbx21*, the encoding gene for T-bet, a critical transcription factor for regulating type-1 immunity[127,128](**Figure 3.9**). Within the Tbx21 expressers, we identified sub-clusters that align with various components of type-1 immunity, including an *anti-viral* population with high expression of Interferon Regulatory Factor (IRF) transcription factors (*Irf1, 9, 7*), and Signal Transducer and Activator of Transcription 1(*Stat1*), known to be pivotal in the immune response to infectious diseases[129]. Additionally, amongst the *Tbx21* population, we observed a cluster enriched in NK receptors, termed *NK-like*, including many Killer cell Lectin-like Receptors (Klr); *Klrb1a, Kcnj8, Klrc1, Klre1*, and the most highly expressed *Klrb1c* which encodes NK1.1. This indicates the type-1 arm of DNTs is linked to innate immunity, as is suggested by pathway analysis (**Figure 3.10**). Interestingly, we observed a distinct non-Tbx21 expressing *Cytotoxic* cluster that also possess hallmark features of type-1 immunity, namely expression of *Eomesodermin* (*Eomes)*, a t-box transcription factor and master regulator of cytotoxic function in cytotoxic T lymphocytes and NK cells[130,131]. Additionally, we observed a *Progenitor* subset high in Programmed cell death protein 1 (*Pdcd1)*, encoding PD-1 and Protooncogene c-KIT (*Kit*). Ikzf2 is expressed throughout all DNT populations, but highest in the progenitor cluster, indicating a potential role for this Ikaros family member in the differentiation and peripheral stability of DNTs, akin to its role in $CD4^+$ T-regs[132]. A separate *type-17* arm of the DNTs marked by high expression of *Rorc*, *IL23r*, and (C-X-C chemokine receptor type 6) *CXCR6* (**Figure 3.7 and 3.8 and 3.9**). The latter is associated with type-17 producers, both in contexts of trafficking and as a marker of type-17 cells with

pro-inflammatory effector function, as well as being involved in leukocyte homing to the liver [133,134]. At the transcriptional level, we also see a cluster, potentially connecting type-1 and type-17 populations, termed *Intermediate*, that has high expression of both *Klrb1c* and *CXCR6*, amongst other overlapping features of both clusters. Five of the seven splenic DNT clusters were also identified in the adipose DNT population, with the absence of the progenitor and anti-viral clusters (**Figure 3.11.A**). Spleen and adipose DNTs were merged and batch corrected, with subsequent UMAP showing a homogenous overlap of clusters, highlighting the conserved nature of DNTs of both lymphoid and non-lymphoid tissue origin (**Figure 3.11.B/C/D**). DEG analysis of splenic and gWAT DNTs shows ~35% of genes are unchanged between tissues, indicating a high degree of conservation and lineage sharing (**Figure 3.12.B**). Additionally, heatmap analysis (**Figure 3.12.A**) indicates the transcriptional differences between splenic and adipose DNTs are attributed to adaptations to tissue state and residency, including adipose upregulated genes *Anxa1, S100a4/a6, Cebpb, Nr4a1* and *Dusp5*[135-140]. Together, this data defines the polyclonal and diverse transcriptional landscape of DNTs, revealing multiple functionally specialized subsets within the type-1 and type-17 arms of immunity, whilst also displaying substantial overlap and cell-type continuity between spleen and adipose tissue.



14 **Figure 3.7:** Seven distinct clusters identified within splenic DNTs.

(A) UMAP of splenic DNTs and annotated clusters, post-removal of highly variable Trbv genes, and ribosomal genes. **(B)** Heatmap of top 10 differentially expressed genes of splenic DNT clusters. sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio.

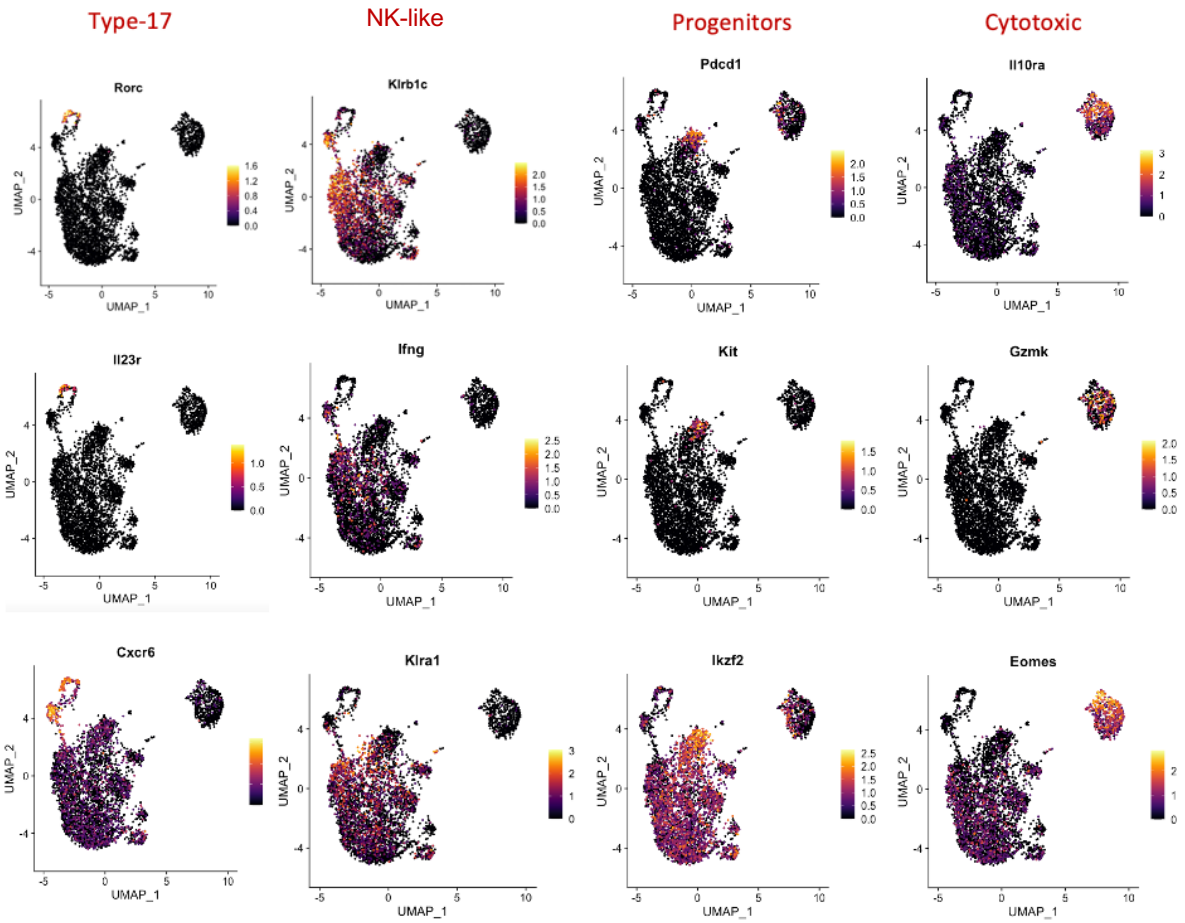
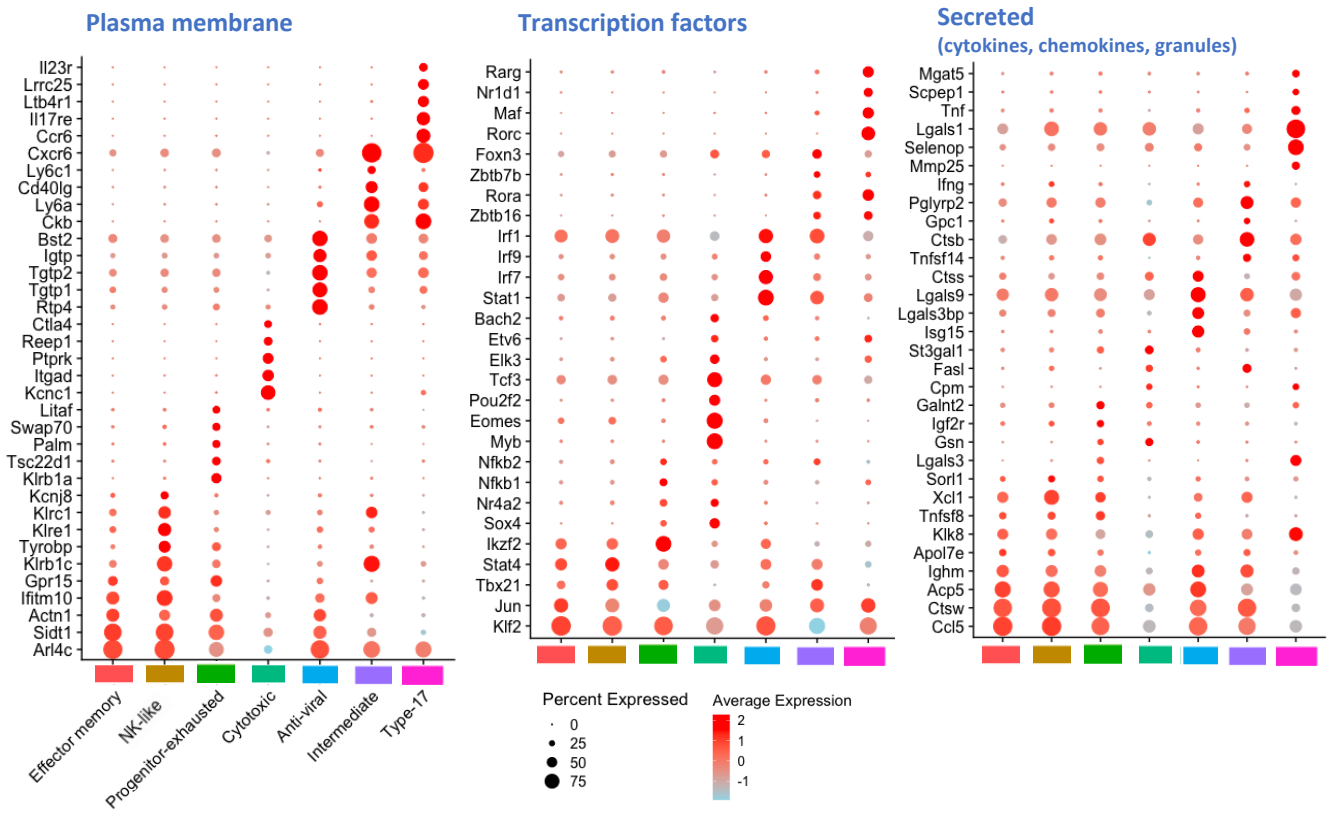


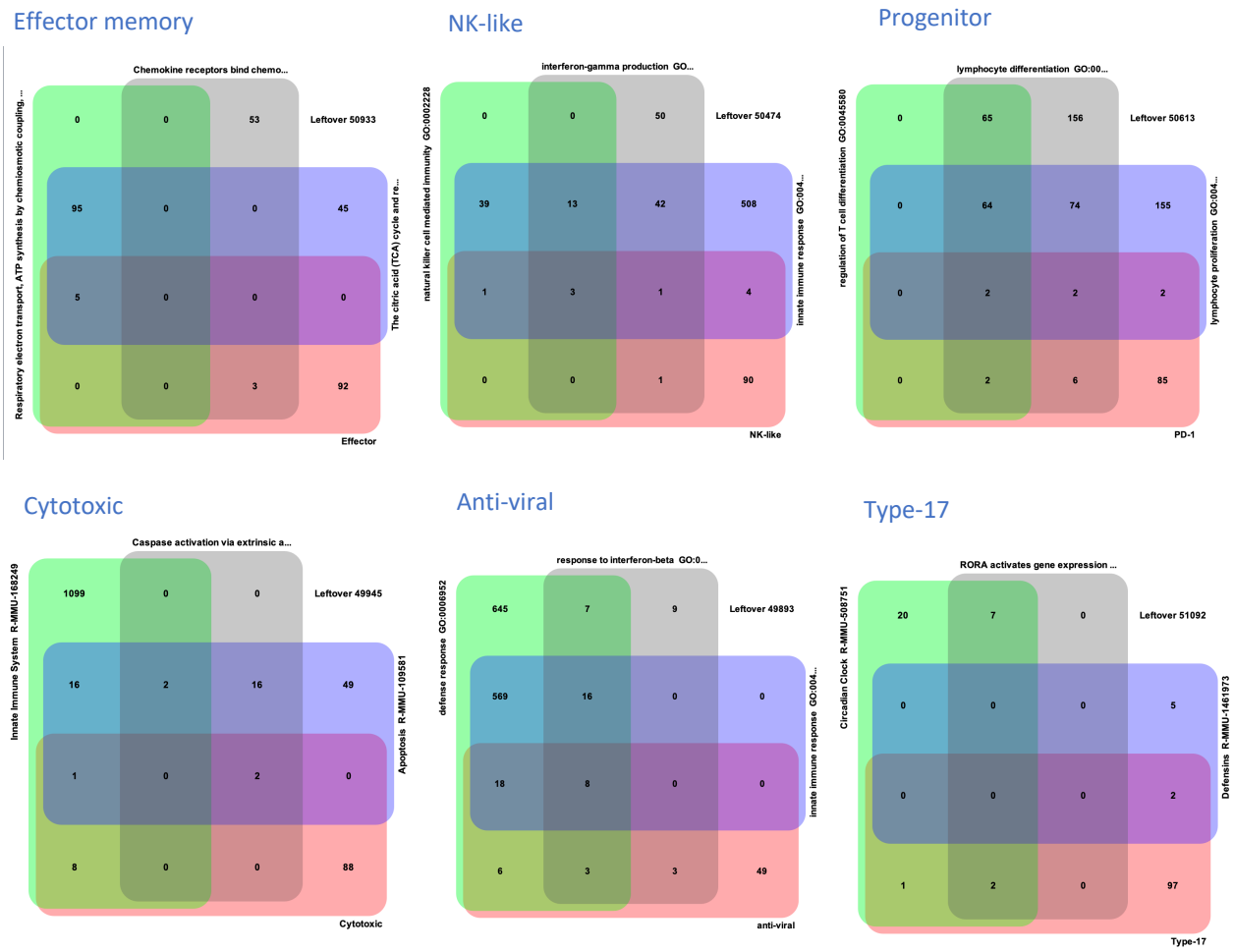
Figure 3.8: Cluster identifiers within splenic DNTs.

Feature plots of key subtype marker expression in spleen DNT clusters; Progenitor (*Pdcd1, Kit, Ikzf2*), NK-like (*Klrb1c, Ifng, Klra1*), Cytotoxic (*Il10ra, Gzmk, Eomes*), Type-17 (*Rorc, Il34r, Cxcr6*). sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio.



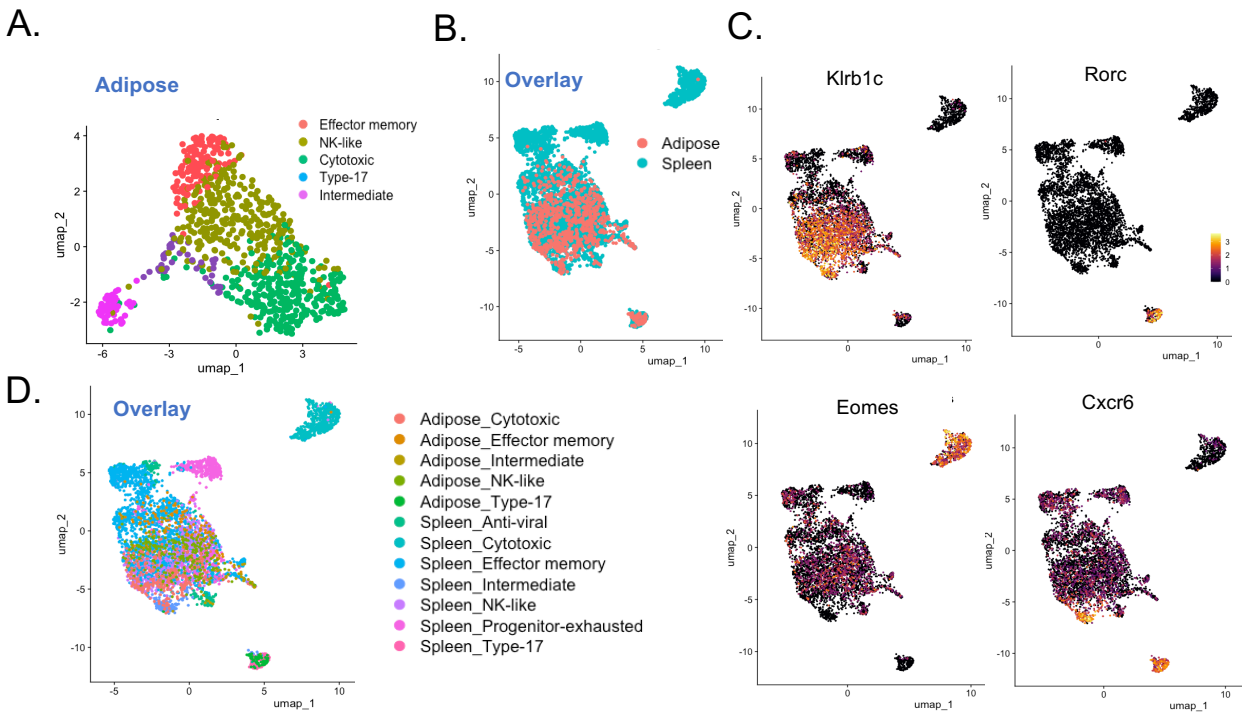
16 **Figure 3.9:** Highly expressed genes amongst splenic DNT clusters

Top HEGS of splenic DNTs organised into subcategories based on gene ontology (GO) terms; plasma membrane, transcription factors, secreted (cytokines, chemokines, granules). sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio.



17 **Figure 3.10*:*** Pathway enrichment of splenic DNT clusters

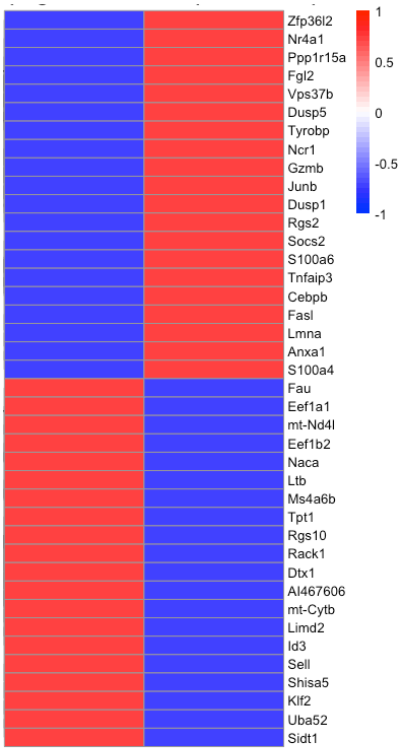
Overlay of number of genes within each cluster involved in enriched pathways identified by gene ontology (GO) terminology. Enriched pathways are listed on axis for each sub-cluster.



18 **Figure 3.11***:* Comparison of splenic and adipose DNT cells

(A) UMAP of adipose DNTs showing conservation of 5 of the 7 splenic clusters. **(B)** UMAP of merged and harmony batch corrected splenic and adipose DNTs. **(C)** Feature plots of key subtype markers on merged adipose and spleen DNT populations; *Klrb1c, Rorc, Eomes, Cxcr6* **(D)** UMAP of Harmony corrected, merged adipose and splenic DNTs, labelled by originating cluster. sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio.

A.



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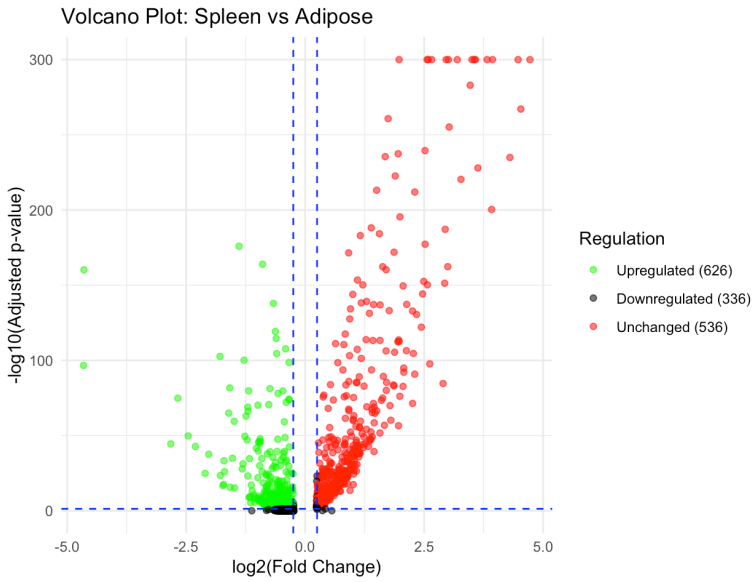


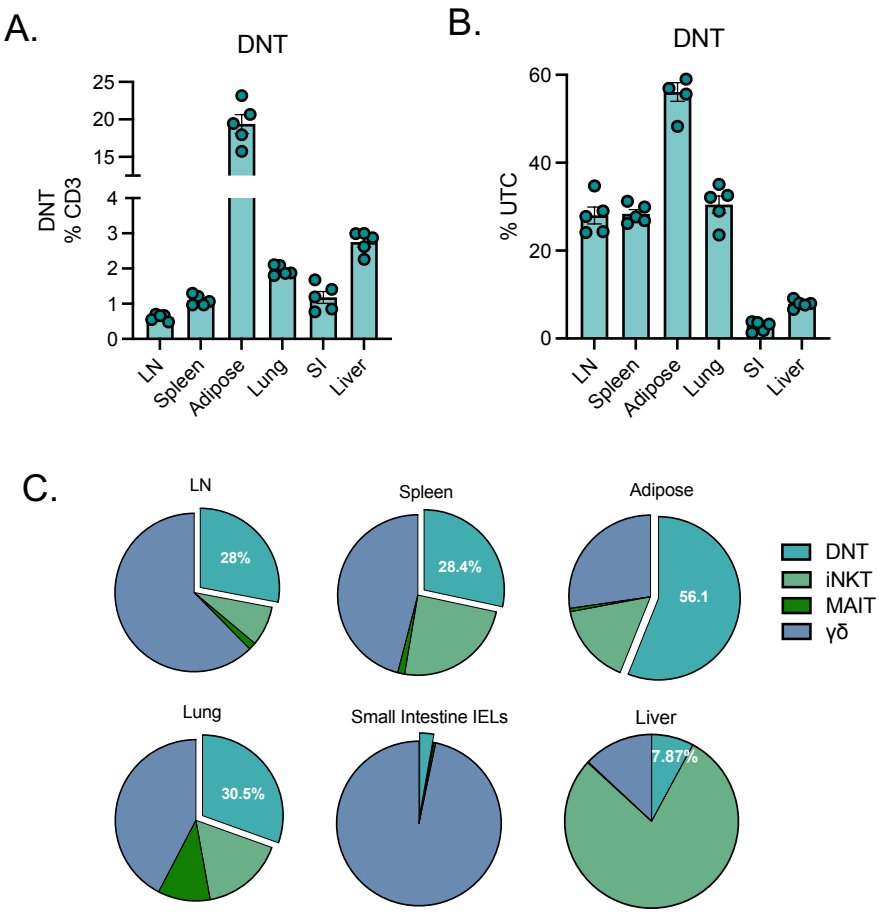
19 **Figure 3.12*:*** Differentially expressed genes amongst splenic and adipose DNT cells

(A) Heatmap of top DEGs between adipose and spleen DNT cells. **(B)** Volcano plot showing upregulated, downregulated & unchanged gene numbers between spleen and adipose DNT cells. sc-RNA/TCR sequencing pipeline, QC, clustering, DEG analysis and statistics were performed on Seurat package with r-studio.

3.5 DNTs have defined type-1 and type-17 effector phenotypes, with widespread tissue distribution

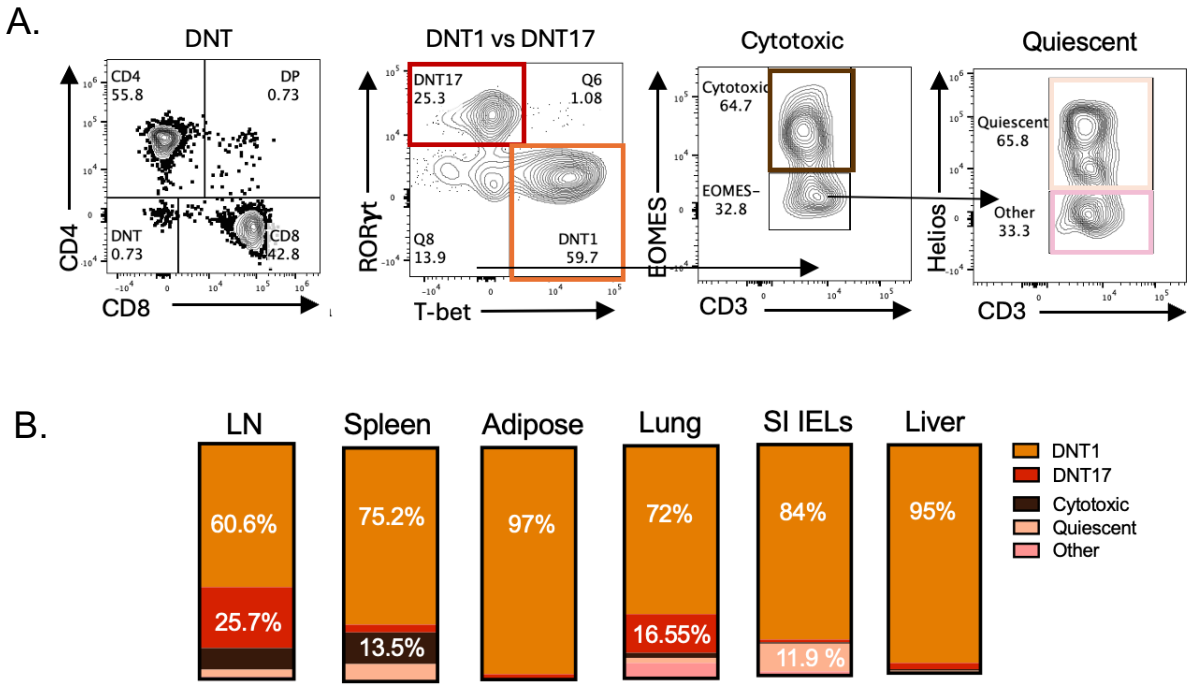
To confirm functional effector subtypes implied from sc-seq, and to investigate tissue-wide distribution of DNTs, we analysed the immune cell population of inguinal lymph node (iLN), spleen, gWAT, liver, lungs, and small intestine intraepithelial lymphocytes (SI IELs) via flow cytometry. We show that DNTs are most enriched amongst total CD3+ cells in the adipose tissue (**Figure 3.13.A**), and are a significant population within the total unconventional T cell pool across tissues (**Figure 3.13.B**). Notably, DNTs are highly enriched amongst unconventional T cells in gWAT (56.1%), while still forming a significant proportion in the spleen (28.4%), lung (30.5%) and LN (28%), with lower frequencies observed in liver (7.87%) and SI intraepithelial lymphocytes (IELs), where iNKT, and γδ T cells predominated respectively (**Figure 3.13.C**). Furthermore, we confirmed the presence of distinct DNT subpopulations, including DNT1 (T-bet$^+$), DNT17 (RORγt$^+$), cytotoxic (T-bet$^-$EOMES$^+$), quiescent (Helios$^+$), and a remaining minor subset negative for these markers (**Figure 3.14.A**). Further analysis of subset distribution within the DNT compartment revealed tissue-specific enrichment patterns (**Figure 3.14.B**). The DNT1 (T-bet$^+$) subset dominated in all examined organs, with the DNT pool being primarily DNT1 in both adipose (97%) and liver (95%). In contrast, DNT17 (RORγt$^+$) cells were prevalent in the iLN (25.7%) and the lungs (16.6%), suggesting tissue-intrinsic factors for type-17 polarisation or recruitment. A minor population exists that is Helios$^+$, but negative for effector-type transcription factors, possibly akin to the progenitor population identified by RNA seq and thus termed quiescent DNTs, notably present at near 12% in the SI IELs. Flow cytometric UMAPs of combined iLN-derived DNTs (**Figure 3.15.A**), revealed clear segregation of type-1 (DNT1), and type-17 (DNT17) populations, consistent with their transcriptomic profile (**Figure 3.15.B**). The NK cell surface marker, NK1.1 is highly expressed on DNT1, whereas the leukocyte trafficking receptor, CXCR6 clearly defines the DNT17 population. EOMES is highly expressed within the DNT1 subtype (**Figure 3.15.B and C**) but interestingly can also be found in the less frequent T-bet$^-$ population; Cytotoxic DNTs. Helios expression can be observed in all subtypes but separated into an intermediate and high expression in DNT17 and DNT1 cells, respectively. Conversely, PLZF is more strongly associated with DNT17 (**Figure**

3.15.B and C). Together, these findings confirm that DNTs are a heterogenous population, exhibiting clear subset compartmentalisation and functional specialisation across tissues.



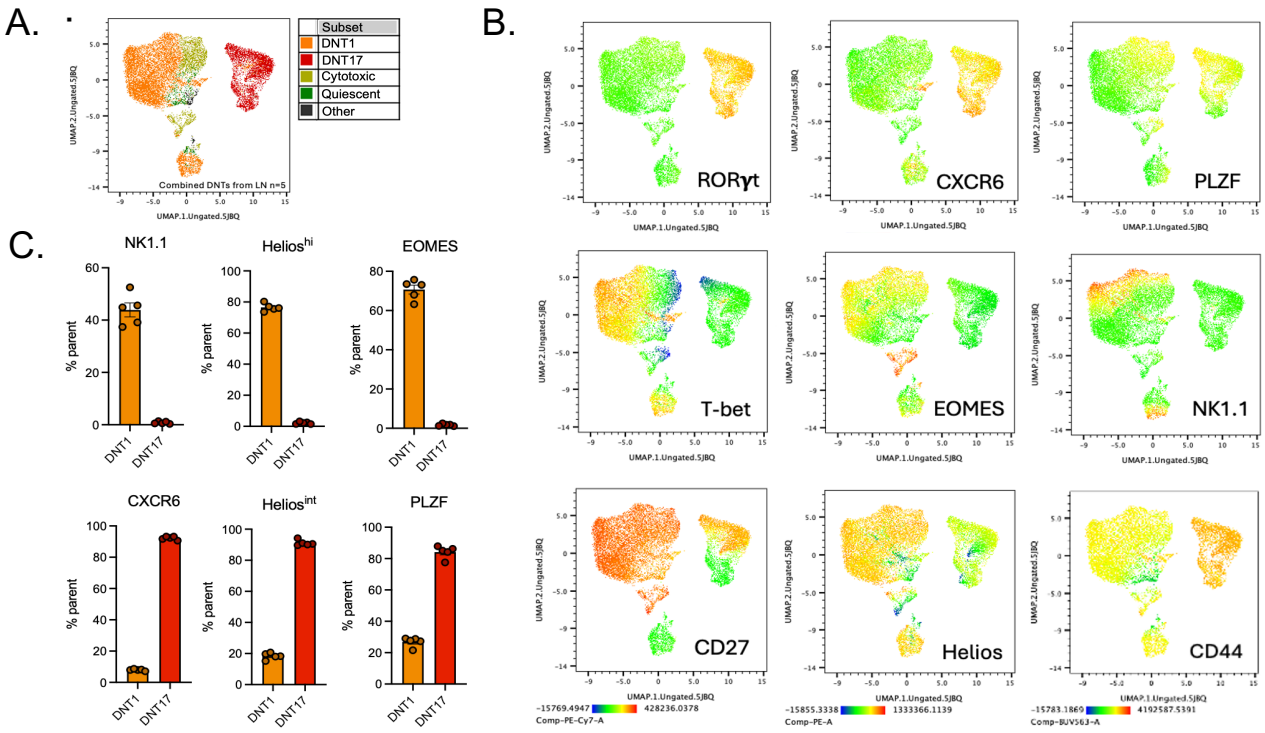
20 **Figure 3.13***:* Contribution of DNT cells to unconventional T cell (UTC) compartment

Frequency of DNT cells as a percentage of **(A)** total CD3 and **(B)** total UTC (MAIT, iNKT, γδ, DNT) across murine tissues; inguinal LN, spleen, adipose (gonadal), lung, small intestine intraepithelial lymphocytes, and liver, n=5 **(C)** Pie charts of mean proportion of DNT cells within the unconventional T cell compartment.



21 **Figure 3.14***:* DNTs have defined type-1 and type-17 subtypes

(A) Representative images of DNT subset gating in LN; DNT1 (T-bet^{+}), DNT17 (RORγt+), Cytotoxic (T-bet-, RORγt-, Eomes+), Quiescent (T-bet-, RORγt-, Eomes-, Helios+). **(B)** Vertical slices: DNT subtype frequencies across organs. n=15 LN, spleen, adipose, liver, n=5 lung, SI.



22 **Figure 3.15***:* DNT1 and DNT17 have distinct phenotypic markers

(A) Flow cytometric UMAP analysis of concatenated DNT cells from inguinal LN, with subtype gating used to identify clusters. **(B)** Flow cytometric UMAP analysis of concatenated DNTs from inguinal LN showing subset usage of RORyt, CXCR6, PLZF, T-bet, EOMES, NK1.1, CD27, Helios, CD44. **(C)** Frequency of subtype markers in DNT1 and DNT17 cells, shown as percentage of parent. n = 5, for concatenated flow analysis, with corresponding samples analysed to assess frequency distribution of markers within subtypes.

3.6 Discussion

This chapter provides a detailed characterisation of DNT cells across lymphoid and non-lymphoid tissues, revealing a highly polyclonal, transcriptionally heterogeneous population with conserved effector programs across tissues. Yang et al. (2021) provides a foundational single-cell transcriptomic atlas of NK1.1$^-$ DNT cells, classifying them broadly into helper, cytotoxic, and innate subsets[141]. The paired TCR-seq and flow cytometric validation shown here builds on this work, revealing DNT cells to be highly polyclonal, with no dominant clonotypes either spleen or adipose tissues, in contrast to the oligoclonality typical of other unconventional T cells. This expansive TCR repertoire suggests that DNTs are not constrained by a narrow set of ligands and may instead respond to a wider pool of antigens. Importantly, the lack of dominant V or J gene usage in either chain and the persistence of high Shannon diversity across transcriptional subtypes suggests lack of antigen-specific clonal expansion as a driver of their peripheral distribution. However, this does not rule out the possibility of clonal expansion within disease-specific contexts, as has been demonstrated for γδ T cell subsets in response to certain viral infections and cancer settings[142].

Through mapping DNT subset frequencies across mouse organs, we identified tissue-specific enrichment patterns with DNT1 dominance in adipose and liver, potentially pointing to roles in metabolic and inflammatory control, similar to that of iNKTs[45]. Additionally, DNT17 enrichment is observed in the LN and lungs, the latter indicating a role in barrier defence, typical of innate IL-17 producers[143]. This suggests that local microenvironmental cues, including cytokine gradients and presence of specific antigens, could shape the functional composition of the DNT population through tissue-intrinsic polarisation, recruitment or retention. Interestingly, at the transcriptional level, the detection of an “Intermediate” population co-expressing NK1.1 and CXCR6 suggests potential plasticity or transitional states between type-1 and type-17 effector arms, which requires further exploration. The progenitor-like population enriched for *cKit*, *Pdcd1* and *Ikzf2* expression, is notable given the known role of Ikzf2/Helios in maintaining regulatory and tissue-resident phenotypes in other T cell subsets[144]. Whilst *Ikzf2* is expressed widely amongst DNTs in the spleen and adipose, this spleen-specific progenitor population expresses high *Ikzf2* without presence of effector transcription factors. This is confirmed by flow, and may suggest a minor population

of quiescent, less differentiated cells that have the potential for peripheral development.

Overall, this chapter identifies DNTs as a polyclonal yet functionally coherent population with conserved effector phenotypes across lymphoid and non-lymphoid tissues. Given their abundance in the adipose and dominant type-1 signature, they may participate in metabolic inflammation, while their distribution across barrier sites suggests host defence and epithelial repair. Thus, further work is needed to define their specific antigenic responses, and contributions to protective versus pathological immunity in these tissue environments. Combining TCR repertoire analysis with functional assays in models of infection or tumour settings could prove useful to explore preferential TCR usage or clonal expansion in specific contexts.

Chapter 4:

DNT cells possess key features of innate T cell immunity and a distinct developmental lineage within the thymus

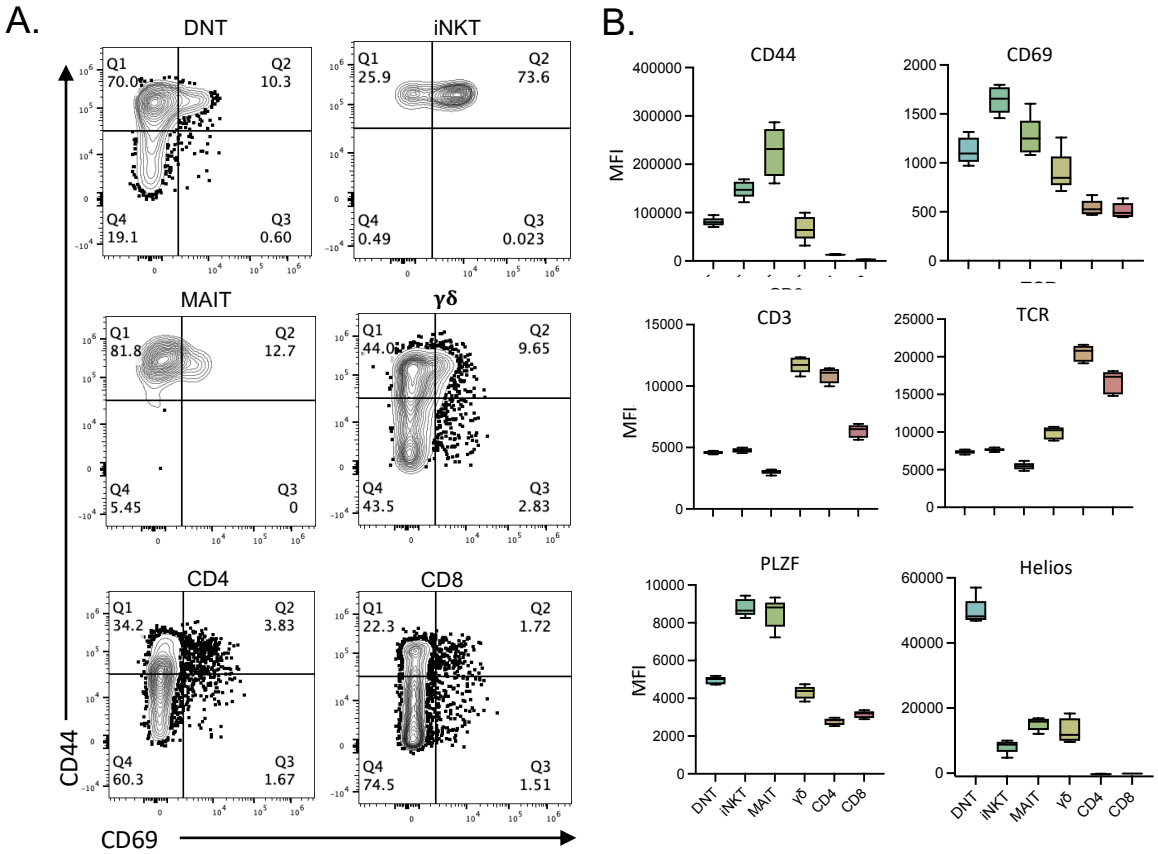
4.1 Introduction

Unconventional T cells undergo unique developmental programs in the thymus that are distinct from conventional T cells. As previously mentioned, iNKT cells originate from double-positive thymocytes and are selected by self-lipid antigens presented by CD1d molecules on neighbouring thymocytes; this agonistic selection is associated with the induction of key transcription factors such as PLZF that drive their innate-like effector program[145]. Similarly, MAIT cells develop in the thymus following a process of positive selection mediated by MR1 expressed on double-positive thymocytes and are influenced by microbial-derived riboflavin metabolites during early life[146]. In contrast, γδ T cells arise from double-negative thymocytes and are selected via strongly TCR-dependent signals that occur in overlapping developmental waves, resulting in a more TCR repertoire with preferential tissue homing patterns[147]. Helios, a transcription factor encoded by IKAROS Family Zinc Finger 2 (*Ikzf2*) has been more recently associated with innate T cells, routinely appearing on innateness gradients in both mice and humans[148,149]. These distinct thymic selection events, mediated by specialised selection molecules and associated transcriptional programming, imprint an innate phenotype on these T cell subsets[150].

Given we observe the presence of these transcriptional identifiers of innate immunity within DNT cells, we aimed to determine their innate functional capacity by investigating their ability to respond rapid to environmental stimuli. In this chapter, we provide clear evidence that DNT cells align with the other innate T cell family members in terms of a peripheral effector-memory-like phenotype. Additionally, we show DNTs can rapidly engage effector functions to produce type-1 and type-17 cytokine responses. Further, they have increased capacity to respond to IFNγ-inducing cytokines and show heightened production over other innate T cells. Despite, the TCR repertoire of DNT cells being more akin to that of adaptive T cells, we show evidence of agonist selection and non-classical TCR selection within DNT subtypes in the thymus.

4.2 DNTs present with pre-programmed effector memory-like phenotype in the periphery

Unconventional T cells are often defined by their innate reactivity, compared to conventional T cells. A hallmark of innate T cells is their “ready to go” effector-memory like phenotype, acquired during thymic development, allowing them to quickly engage effector functions within minutes of TCR and/or cytokine receptor stimulus[1]. To determine whether DNTs possess characteristics associated with innate T cells, we first assessed their steady-state phenotype and activation features. DNTs from naïve WT spleen had elevated surface expression of CD44 and CD69 compared to $CD4^+$ and $CD8^+$ T cells at both population expression and MFI intensity, consistent with a pre-activated or effector-memory–like state (**Figure 4.1.A and B**). This pattern was shared amongst iNKT, MAIT, and γδ T cells, which also have high CD44 and CD69 expression (**Figure 4.1.A and B**). DNTs expressed intermediate levels of CD3 and TCRαβ MFI compared to T-conv, aligning more with the expression profile observed in αβ+ iNKT and MAIT cells[47] (**Figure 4.1.B)**. Consistent with a shared innate transcriptional program, DNTs expressed higher PLZF than T-conv, while lower than that of MAITs and iNKTs. PLZF expression by DNTs is similar to the γδ population and is largely confined to the type-17 subsets of both DNTs and γδ T cells[34,151]. Helios expression was observed solely on the unconventional T cell populations in the naïve setting, with highest expression on the DNTs (**Figure 4.1.B**), indicating a potentially substantial role for transcriptional regulation of these cells by Helios in development and/or sustained proliferation in the periphery.

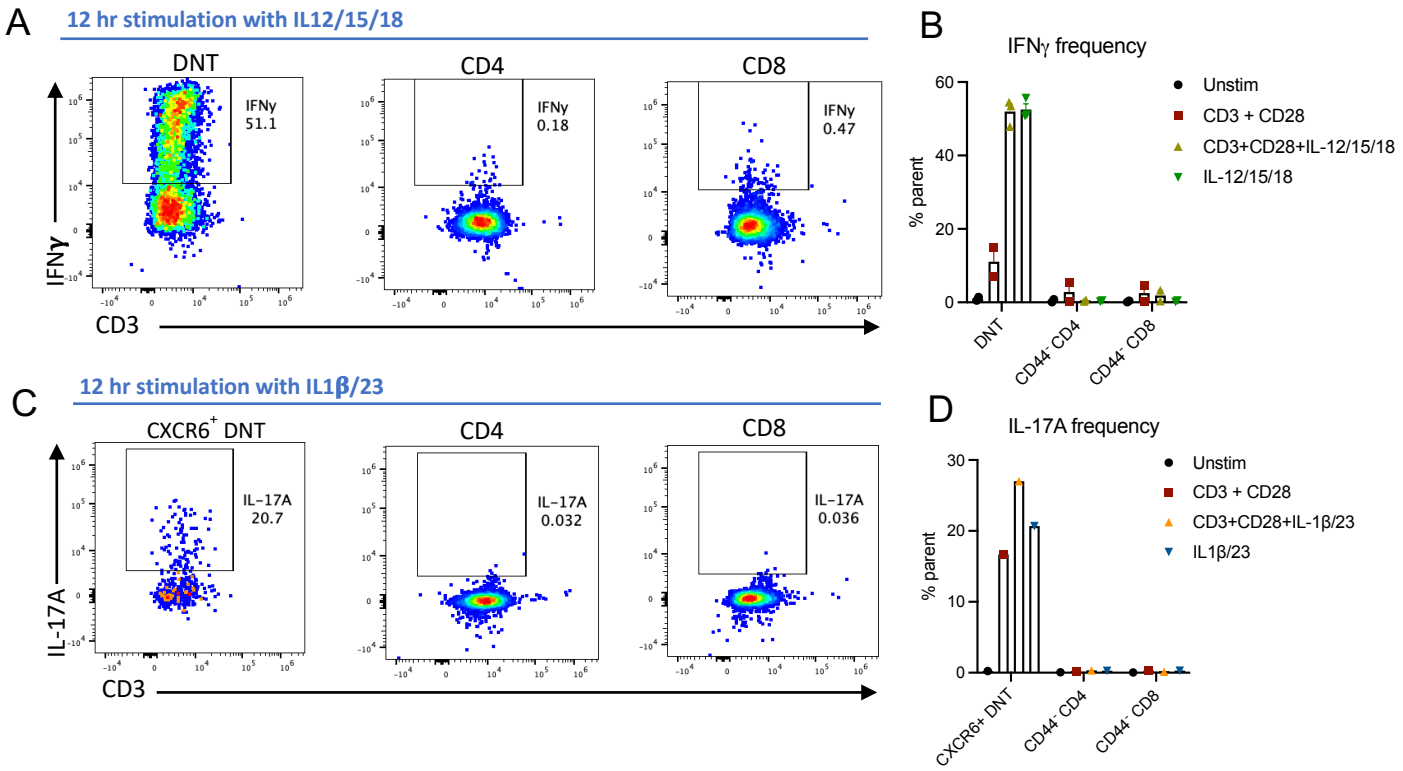


23 **Figure 4.1***:* DNTs possess conserved features of innate-like T cells

(A) Representative images of CD44 and CD69 expression in DNT, iNKT, MAIT, **γδ**, CD4 and CD8 T cells from the spleen. **(B)** MFI of CD3, TCR**α** or **γ**, CD44, CD69, PLZF, Helios across T cell populations (n=5-10).

4.3 DNTs respond to innate cytokine cues to instigate rapid effector responses

To functionally assess the innate responsiveness of DNTs, we stimulated FACS-purified naïve conventional T cells and DNTs from pooled spleens/iLN and cultured T cells overnight in various stimulation conditions. DNTs robustly produced IFNγ in response to IL-12/15/18 stimulation, with over 50% producing IFNγ (**Figure 4.2.A**). In contrast, $CD44^{-}$ $CD4^{+}$ and $CD8^{+}$ T cells failed to respond under the same conditions and limited activation timeframe, with minimal IFNγ expression (<1%). Although IFNγ production in DNTs can be induced by TCR stim (CD3 + CD28), they have a more robust cytokine-driven responsivity, highlighting their rapid response kinetics, characteristic of innate immunity (**Figure 4.2.B**). Similarly, CXCR6+ DNT17 cells produced IL-17A production in response to IL1β/23 stimulation, again not observed by the conventional T cell populations (**Figure 4.2.C**). Interestingly, DNT17 are more responsive to TCR stimulation than their type-1 counterparts, showing a synergistic enhancement of IL-17A production in the combined TCR and cytokine stimulation condition (**Figure 4.2.D**). Altogether, the data supports the idea that DNTs share core phenotypic and functional features with established innate-like T cells, resulting in the capacity to rapidly produce an effector response with or without TCR engagement, via innate cytokine cues.



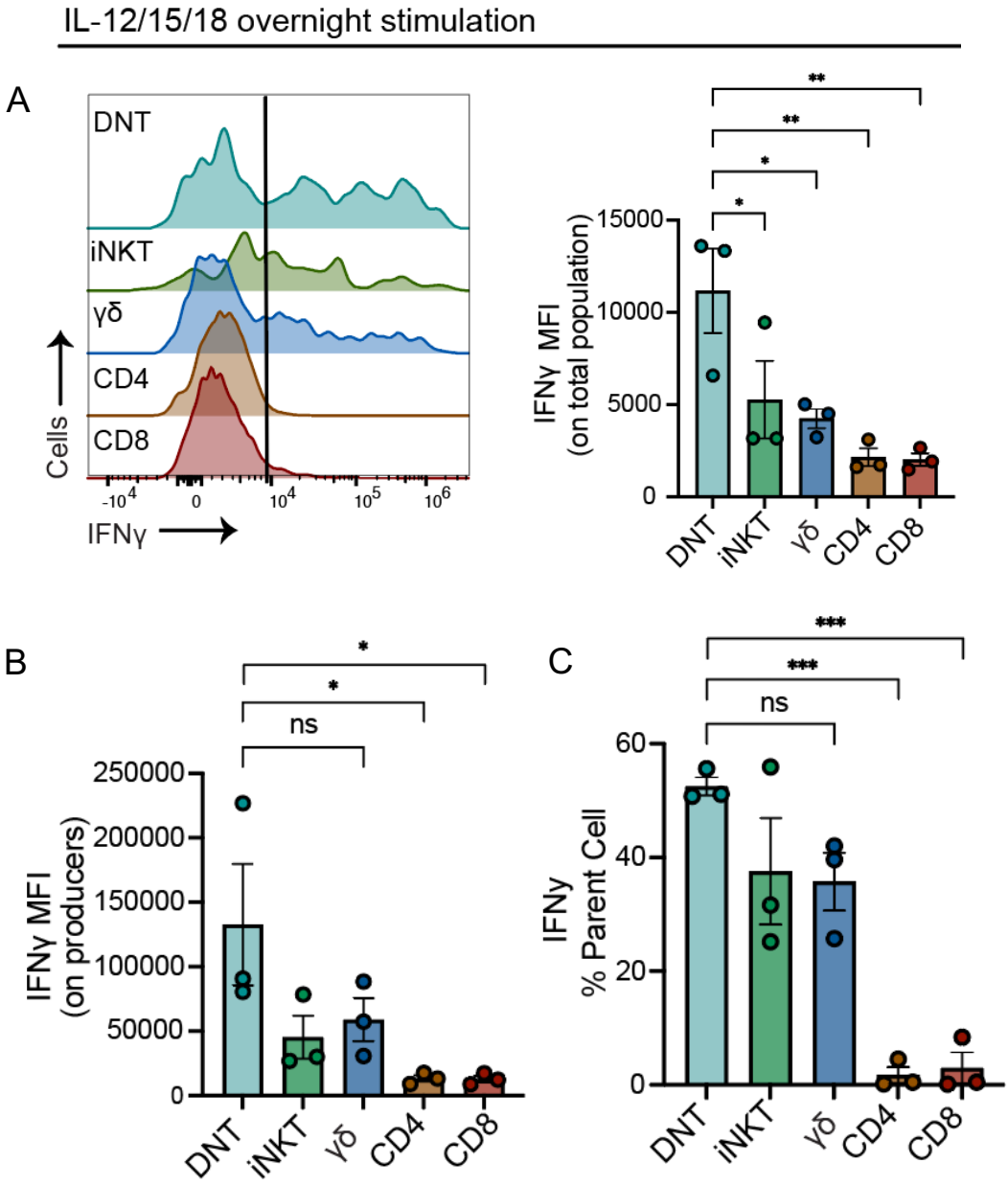
24 **Figure 4.2***:* DNTs possess rapid response effector-functions

Cells were isolated and FACS purified from spleen and cultured overnight in stated stimulation conditions. **(A)** Representative images of IFN**γ** expression on DNT, CD44$^-$ CD4, and CD44$^-$ CD8 T cells. **(B)** IFN**γ** as a percentage of parent cell; DNT, CD44$^-$ CD4 and CD8 T cells (n = 2 to 3, where one represents sorted cells from 10 pooled spleens). **(C)** Representative images of IL-17A expression on CXCR6$^+$ DNT, CD44$^-$ CD4, and naïve CD44$^-$ CD8 T cells. Cells were isolated and FACS purified from spleen and cultured overnight in stated stimulation conditions. **(D)** IL-17A as a percentage of parent cell; DNT, CD44$^-$ CD4 and CD8 T cells (n = 1, where one represents sorted cells from 10 pooled spleens).

4.4 DNT1 have elevated expression of IL-18R, facilitating increased cytokine-induced IFNy expression over other innate T cells

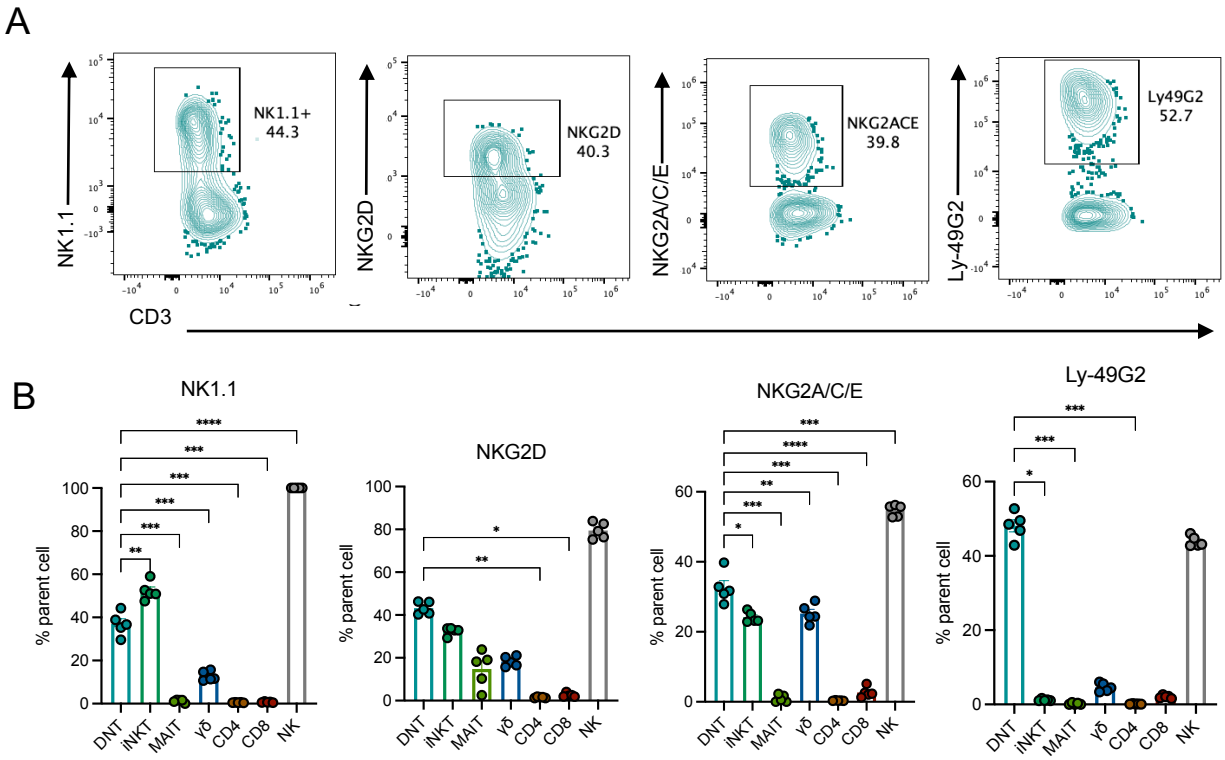
Next, we compared the level of IFNγ production by DNTs to that of iNKT, and γδ T cells under the same experimental conditions (overnight stimulation on FACS purified cells). DNTs exhibited the highest overall levels of IFNγ expression compared to all other populations (**Figure 4.3.A**) When analysis was restricted to Type 1 DNTs (IFNγ-producing) DNTs maintained elevated IFNγ MFI levels relative to iNKT, γδ, and conventional T cell subsets, although frequency was not significantly higher but trending so (**Figure 4.3.B and C**). Given the enhanced IFNγ production in response to cytokine stimulation, we hypothesized that DNTs may express a distinct repertoire of activating receptors. We next evaluated surface expression of canonical NK-associated receptors on splenic DNT cells at steady-state and found widespread expression of innate NK receptors; NKG2D (40.3%), NKG2A/C/E (39.8%), Ly49-G2 (52.7%), and NK1.1 (44.3%) by the DNT1 subset. Indeed, DNTs expressed significantly higher levels of all four NK-associated receptors compared to $CD4^+$ and $CD8^+$ T cells (**Figure 4.4.A and B**). While iNKT and γδ T cells also expressed NK1.1 and NKG2D, DNTs remained the only subset with broad expression of NK1.1, NKG2D, NKG2A/C/E, and Ly-49G2. Although Ly49-G2 is an inhibitory receptor, high expression on NK cells is associated with an enhanced cytolytic phenotype[152].

Since IL-18 synergises with IL-12 and IL-15 to induce robust IFNγ production[153], we next assessed IL-18 receptor (IL-18R) expression, and found that 82.3% of DNTs expressed IL-18R, substantially higher than frequencies observed in iNKT (58.6%), MAIT (43.9%), and γδ T cells (51.5%) (**Figure 4.5.A and B**) and $CD4^+$ and $CD8^+$ T cells, which expressed low to negligible IL18R (**Figure 4.5.B**). $NK1.1^+$ DNTs expressed a significantly higher proportion of $IL\text{-}18R^+$ cells compared to $NK1.1^+$ iNKT or γδ T cells (**Figure 4.5.C**), suggesting that elevated IL-18 responsiveness is higher amongst DNT cells with the same subsetting as iNKT and γδ T cells. Together, these findings support the conclusion that DNT1 cells have innate-responsive cytokine-induced IFNγ production, distinguishing them from other innate-like T cell subsets.



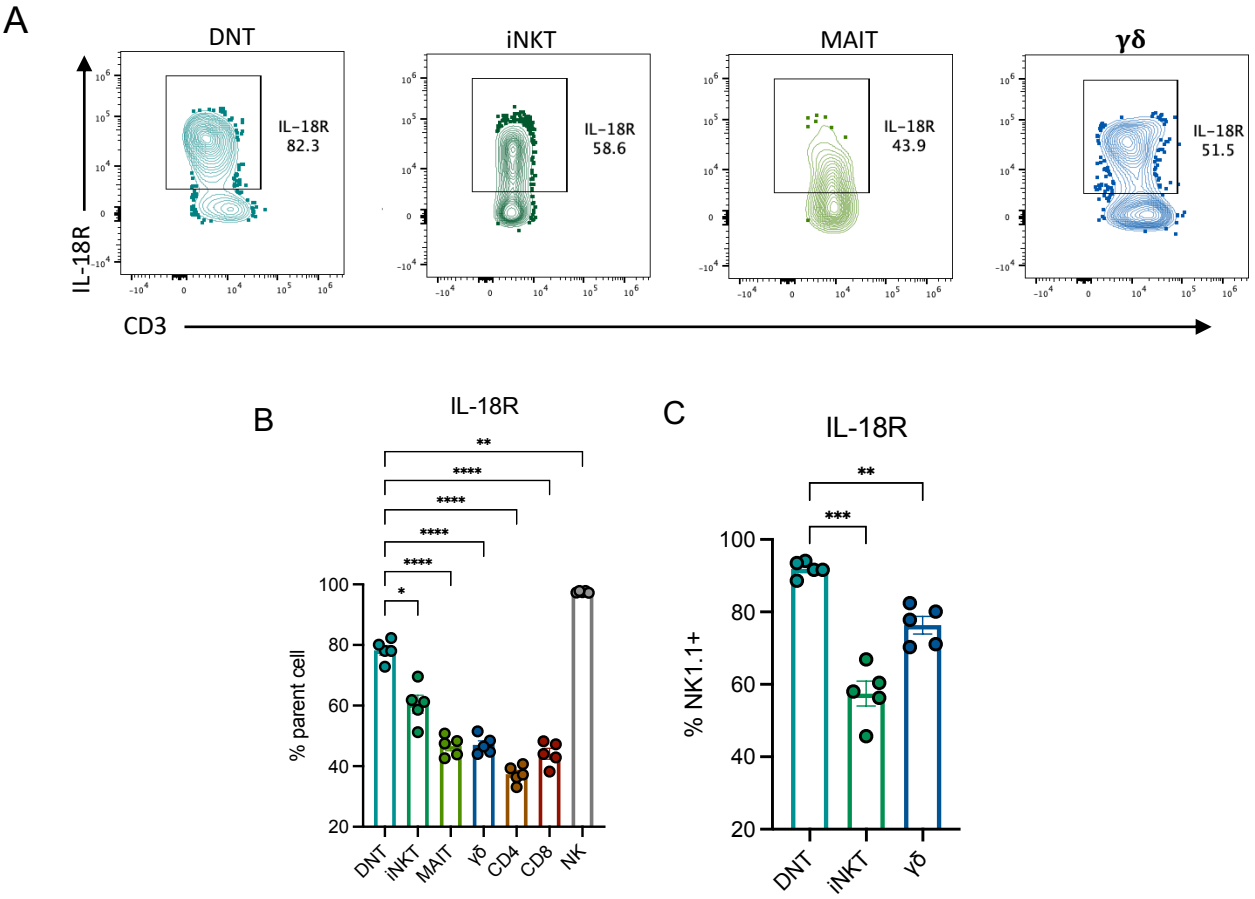
25 **Figure 4.3***:* DNT1 have increased cytokine-induced IFNγ expression over other innate T cells

In-vitro activated DNT, iNKT, **γδ**, CD4 and CD8 T cells, cultured separately overnight with IL-12/15/18. N=3 experiments with 10 pooled mice spleens/iLN per experiment. Data for DNT IL-12/15/18 stimulation shown here are also presented in Figure 3D for comparison across stimulation conditions. **(A)** Representative images of total IFN**γ** MFI, corresponding bar plot shows MFI of IFN**γ** production on total population. **(B)** IFN**γ** MFI pre-gated on IFN**γ** producers (to show extent of production within subtypes that produce IFN**γ**) **(C)** IFN**γ** frequency shown as a percentage of parent cell. Data are shown as mean ± SEM. Significance calculated using one-way anova. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, ****p < 0.001



26 **Figure 4.4***:* DNTs have widespread basal expression of NK-associated receptors

Ex-vivo splenic lymphocytes at steady-state unstimulated and stained for lymphocyte surface markers and NK-associated receptors (N = 1 experiment, n = 5 mice) **(A)** Representative flow staining of NK receptors on ex-vivo splenic DNTs. **(B)** Bar plots show frequency of NK receptors (NK1.1, NKG2D, NKG2A/C/E and Ly-49G2), shown as a percentage of parent cell (total population). Data are shown as mean ± SEM. Significance calculated using one-way anova. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, ****p < 0.001

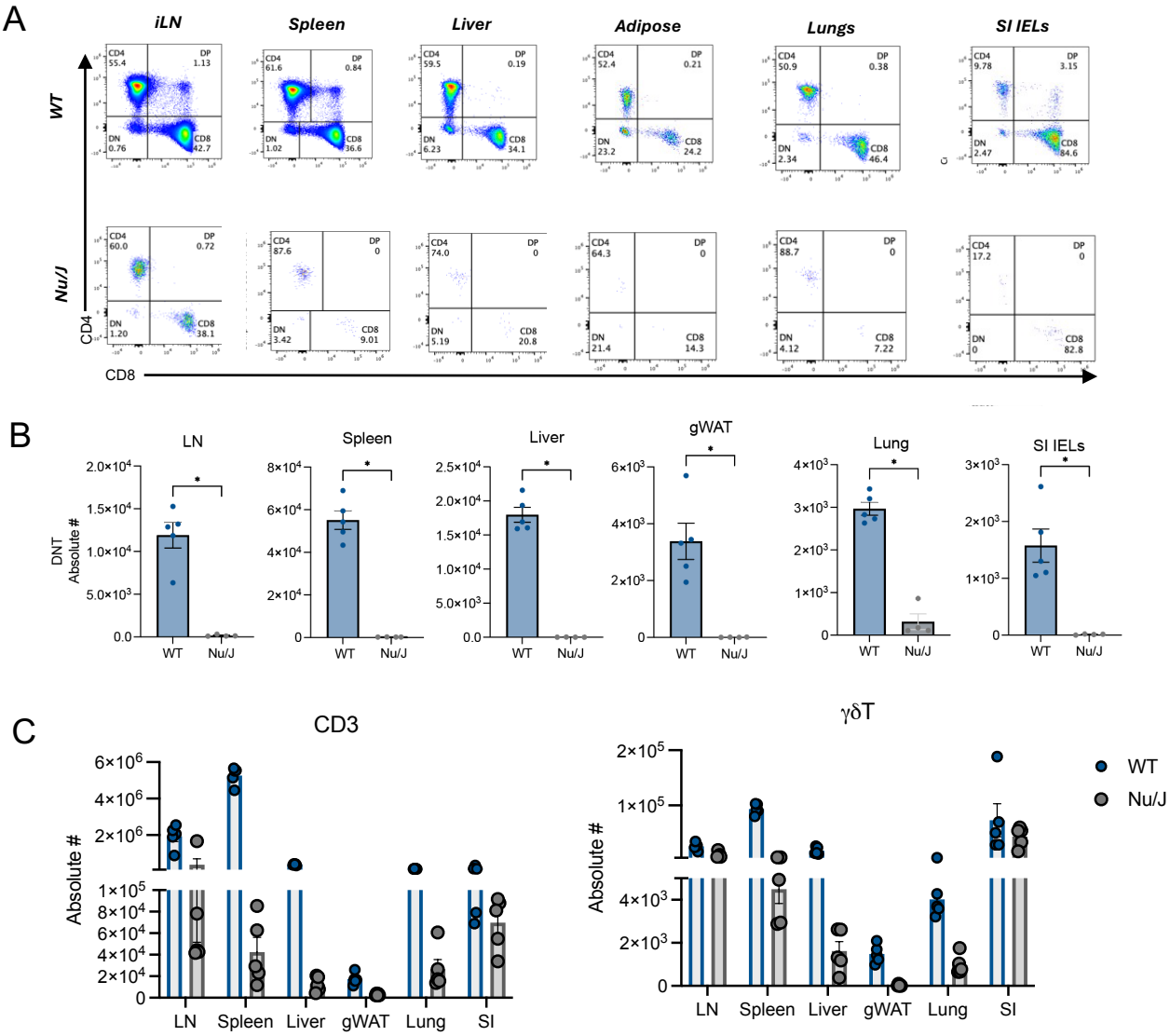


27 **Figure 4.5***:* DNTs have elevated expression of IL-18R

Ex-vivo splenic lymphocytes at steady-state unstimulated and stained for IL-18R expression (N = 1 experiment, n = 5 mice) **(A)** Representative images of IL-18R expression on innate T cells; DNT, iNKT, MAIT, γδ **(B)** Frequency of IL-18R expression shown as a percentage of parent cell. **(C)** Frequency of IL-18R on NK1.1^{+} DNT, iNKT, & γδ T cell subtypes (MAITs not shown due to low NK1.1 expression). Groups were compared using one-way ANOVA with Dunn's or Dunnet's multiple comparison depending on gaussian distribution of data, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, ****p < 0.001

4.5 DNTs development is linked to the thymus

Innate T cells have a unique selection process within the thymus, often mediated by strong agonist selection and antigen presentation by non-classical MHC-like molecules, which in turn initiates innate transcriptional programming and effector differentiation[2]. Given we observe the presence of PLZF, T-bet and RORγt in DNT subsets, we aimed to assess TCR selection requirements of the DNTs to identify classical or non-classical TCR restriction. We employed a series of knockout models that target various components essential to T cell development and probed the impact of this on DNT frequency and number across tissues. We first asked if DNT development is thymic-dependent, or if there is a possibility for alternative extrathymic development as is reported for γδ T cells[154-156]. Nu/J athymic mice show a near complete loss of DNTs from every organ we examined, compared to wildtype C57BL6/J (WT) mice, highlighting DNT dependence on the thymus (**Figure 4.6.A and. 4.6.B**). Additionally, we confirmed a severe loss of $CD3^+$ cells and persistence of γδ T cells in athymic mice (**Figure 4.6.C**)

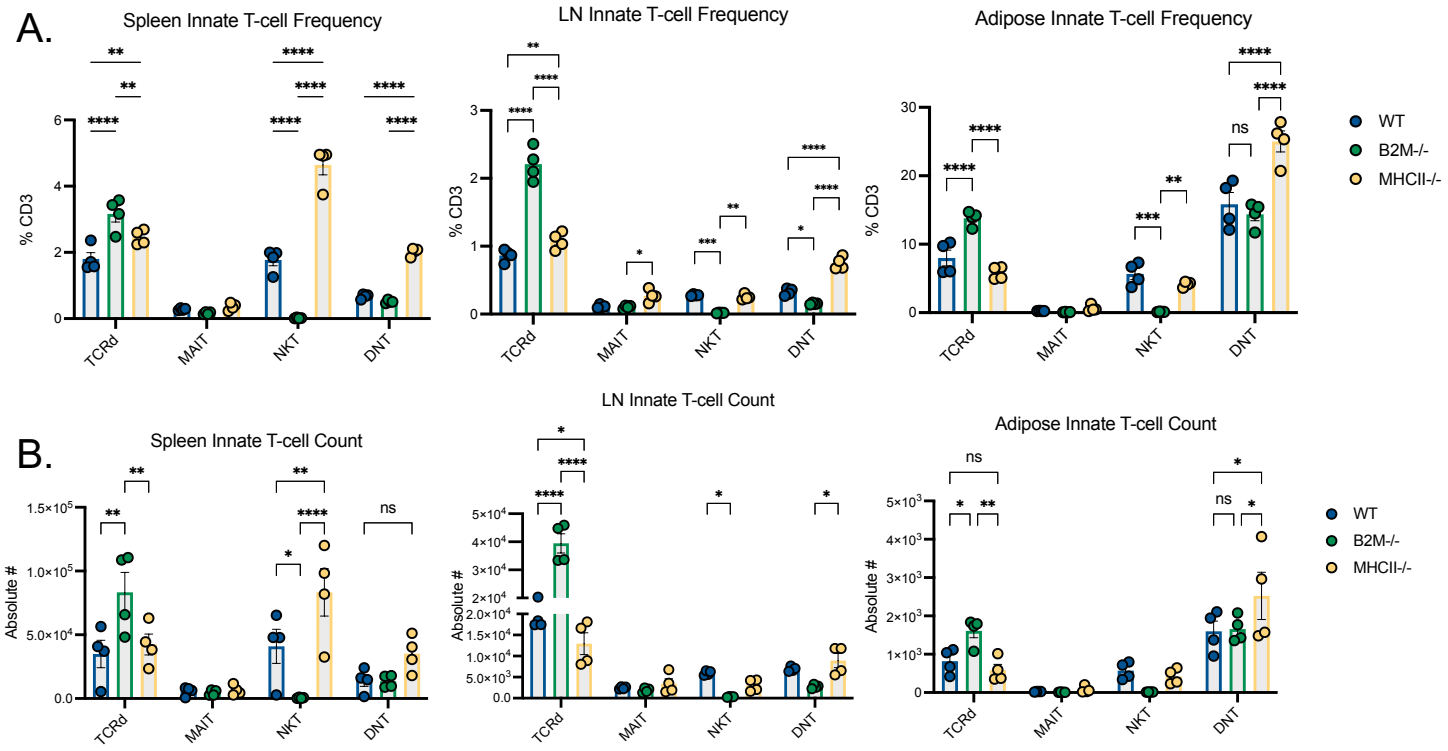


28 **Figure 4.6***:* DNTs are absent in *Nu/J* athymic mice

Cells were isolated from iLN, spleen, gWAT, lung, or SI IELs from C57BL6/J (WT) or *Nu/J* athymic mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Representative images of DNT cells in WT and *Nu/j* mice across tissues. **(B)** Absolute number of DNTs in WT or Nu/J athymic mice across tissue compartments. **(C)** Total CD3 and γδ T cell numbers across tissues. Groups were compared using one-way anova or the unpaired Student's t test and error bars in graphs represent mean ± SEM. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.005, n = 5, one experiment.

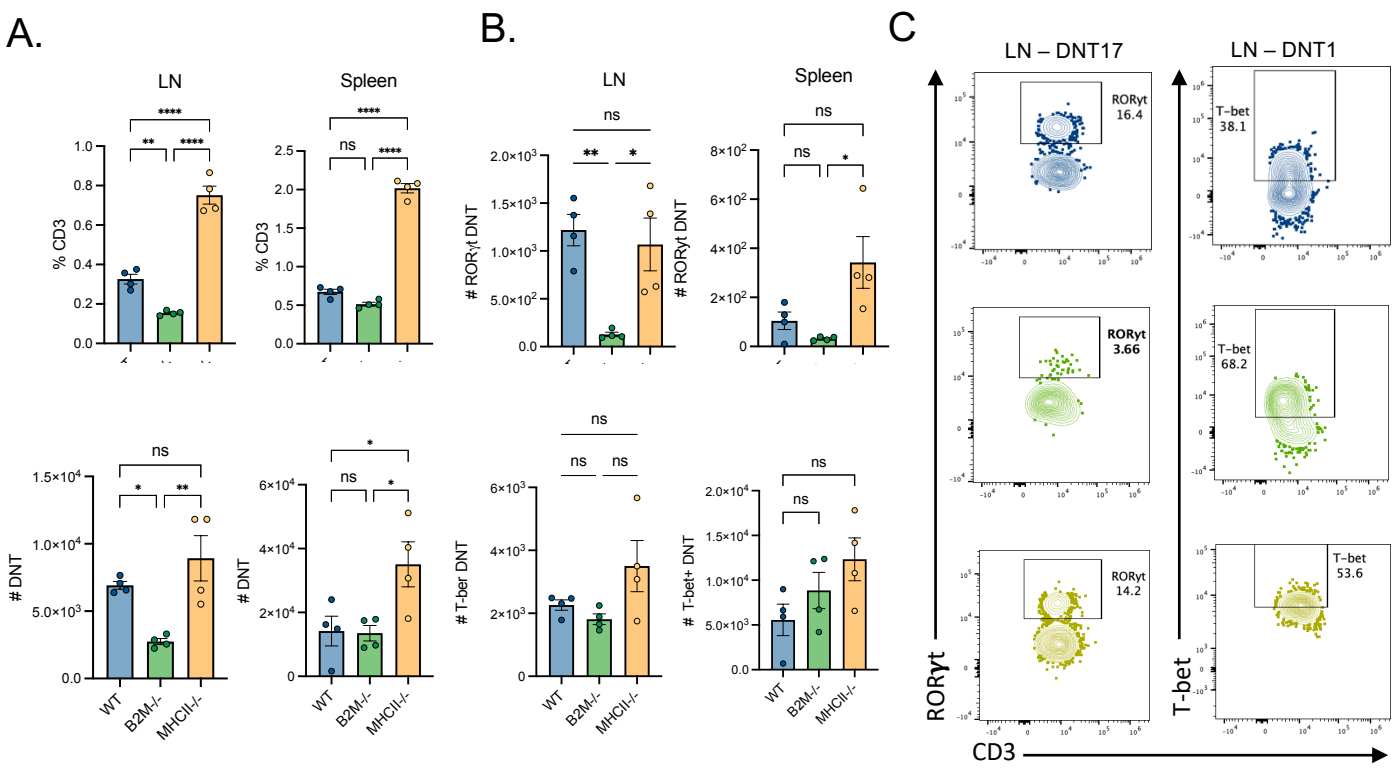
4.6 DNTs are MHC-II independent but have subtype-specific dependence on B2M-mediated selection

Conventional $CD4^+$ and $CD8^+$ T cells recognise a diverse range of peptides via presentation by MHC-I and MHC-II respectively[2]. β2M is essential for the MHC-I complex, mediating MHC-I surface expression and thus antigen presentation to CD8s[157]. We therefore examined the DNT compartment in *MHC-II-/-* and *B2M-/-* mice. Loss of a major T cell population can cause a tissue-dependant compensation by one or more of the remaining T cell subsets to fill the T cell compartment or "niche" (**Figure 4.7A and 4.7B**). We found that DNTs are expanded in LN, spleen and adipose in the absence of $CD4^+$ T cells in MHC-II-/- mice (**Figure 4.7A and 4.7B**), possibly filling the niche but also indicating that their development is independent of MHC-II antigen presentation. Conversely, we observed a significant decrease in DNTs in the LN compartment of β2M-/- mice, that is not apparent in splenic and adipose DNTs **(Figure 4.8A and 4.7B)**. Given the distribution of DNT1 and DNT17 differs between tissues, we examined subset specific differences within the restriction molecule knockouts. Surprisingly, we found a near complete loss of DNT17 in β*2M-/-,* whereas DNT-1 are unaffected (**Figure 4.8B and 4.8C**). To rule out the possibility of direct or indirect CD8 interactions with DNTs that could result in the loss of this subtype in β*2M-/-* which lack CD8 T cells, we assessed the DNT population in *CD8-/-* mice, with normal MHC-I and B2M expression. There were no differences between WT and *CD8-/-* in either the total DNT population or the type-17 subtype across tissues and thus CD8 T cells are not required for DNT cells (**Figure 4.9**). The presence of DNT cells in the absence of CD4 and CD8 T cells also suggests that DNTs are not 'ex' CD4 or CD8 T cells.



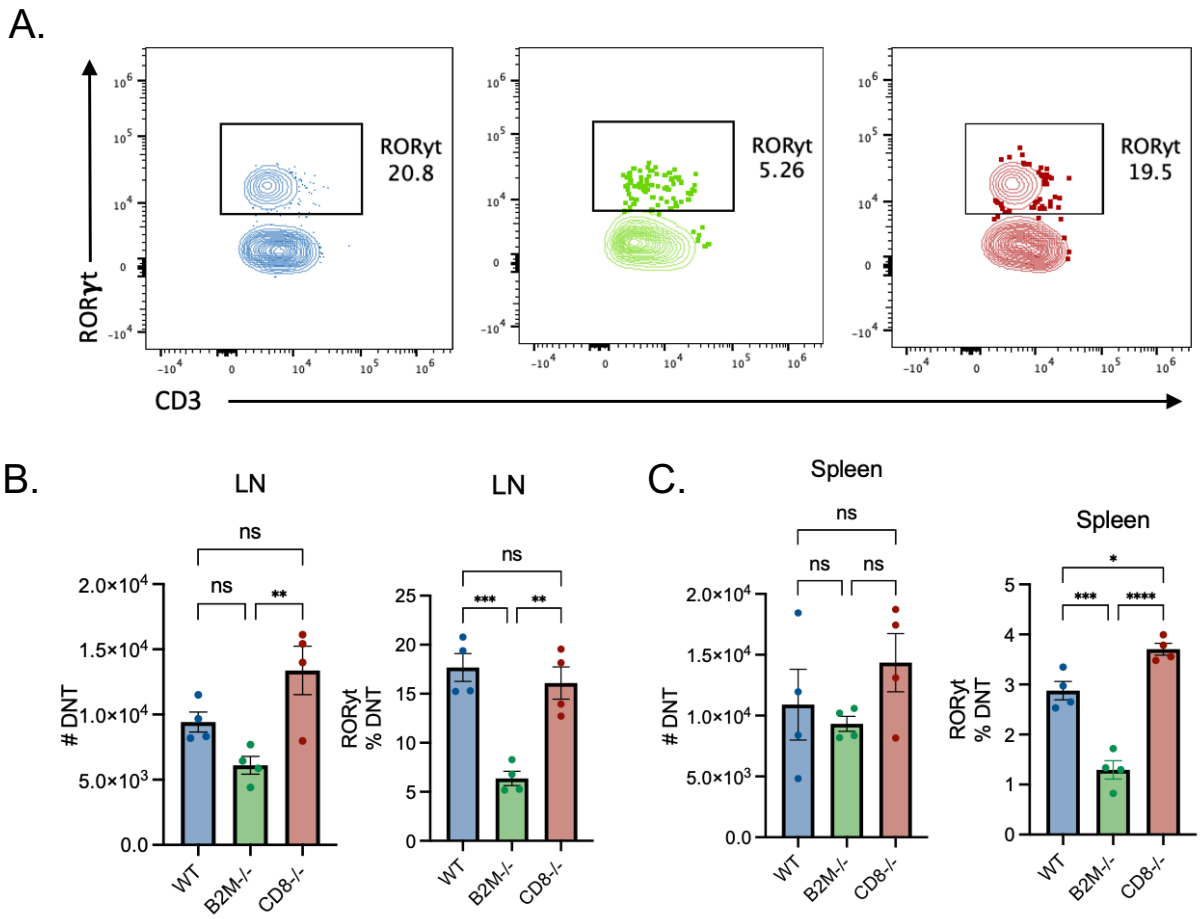
29 **Figure 4.7*:*** DNTs assist in T cell niche filling across tissue compartments in *MHC-II-/-* mice

Cells were isolated from iLN, spleen or gWAT from C57BL6/J (WT) or β2*M or MHC-II-/-* mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Unconventional T cell subsets shown as a percentage of total $CD3^+$ cells in spleen, iLN and gWAT of WT, β2*M or MHC-II-/-* mice. **(B)** Absolute number of unconventional T cell subsets shown as a percentage of total $CD3^+$ cells in across tissue compartments in WT, β2*M or MHC-II-/-* mice. Groups were compared using two-way anova and error bars in graphs represent mean ± SEM. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.005, ∗∗∗p < 0.0001, n = 4, one experiment.



30 **Figure 4.8***:* DNT17 are reduced in *β*2M-deficient mice

Cells were isolated from iLN or spleen from C57BL6/J (WT) or β*2M or MHC-II-/-* mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Frequency of DNT out of total CD3+ cells (top), and absolute number of DNTs (bottom) from iLN (left) and spleen (right), in WT, *B2M-/-,* and *MHC-II-/-.* **(B)** Absolute number of RORγt$^+$ DNT17 RORγt$^+$ (top), and T-bet$^+$ DNT1 (bottom) from iLN (left) and spleen (right), in WT, B2M-/-, and *MHC-II-/-* **(C)** Representative images of RORγt$^+$ and T-bet$^+$ frequency on iLN DNTs in WT, *B2M-/-, and MHC-II-/-*. Groups were compared using one-way anova and error bars in graphs represent mean ± SEM. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.005, n = 4, one experiment.



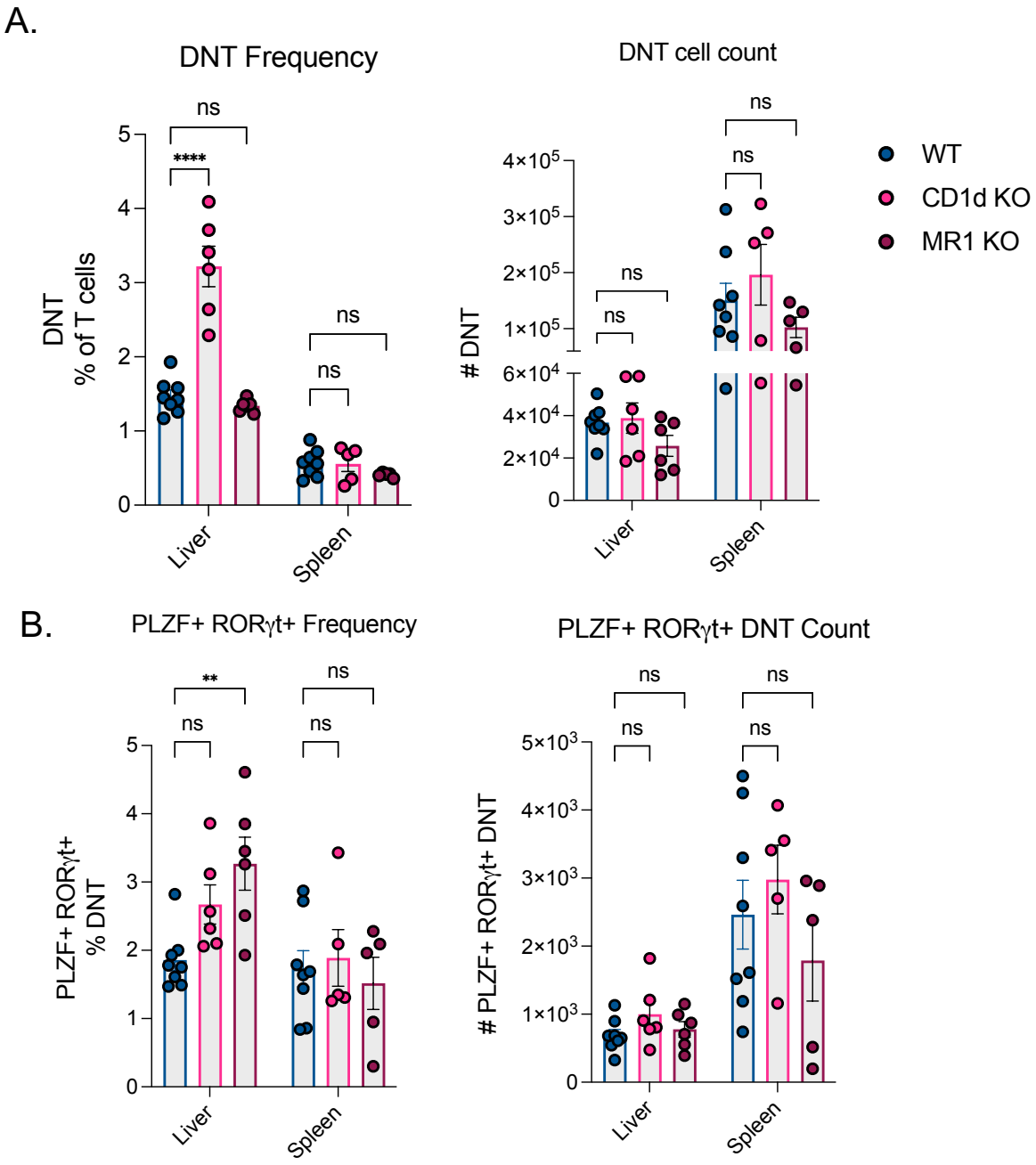
31 **Figure 4.9***:* DNT development and presence in periphery is not impacted by CD8

Cells were isolated from iLN or spleen from C57BL6/J (WT) or β*2M or CD8-/-* mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Representative images of RORγt$^+$ frequency on iLN DNTs in WT, *B2M-/-, and MHC-II-/-*. **(B)** Absolute number of RORγt$^+$ DNT17 RORγt$^+$ (left), and frequency of RORγt$^+$ as a percetntage of DNT (right) from iLN in WT, B2M-/-, and *MHC-II-/-* **(C)** Absolute number of RORγt$^+$ DNT17 RORγt$^+$ (left), and frequency of RORγt$^+$ as a percetntage of DNT (right) from spleen in WT, B2M-/-, and *MHC-II-/-.* Groups were compared using one-way anova and error bars in graphs represent mean ± SEM. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.005, n = 4, one experiment.

4.7 DNT-17 require B2M but are Tap1-independent

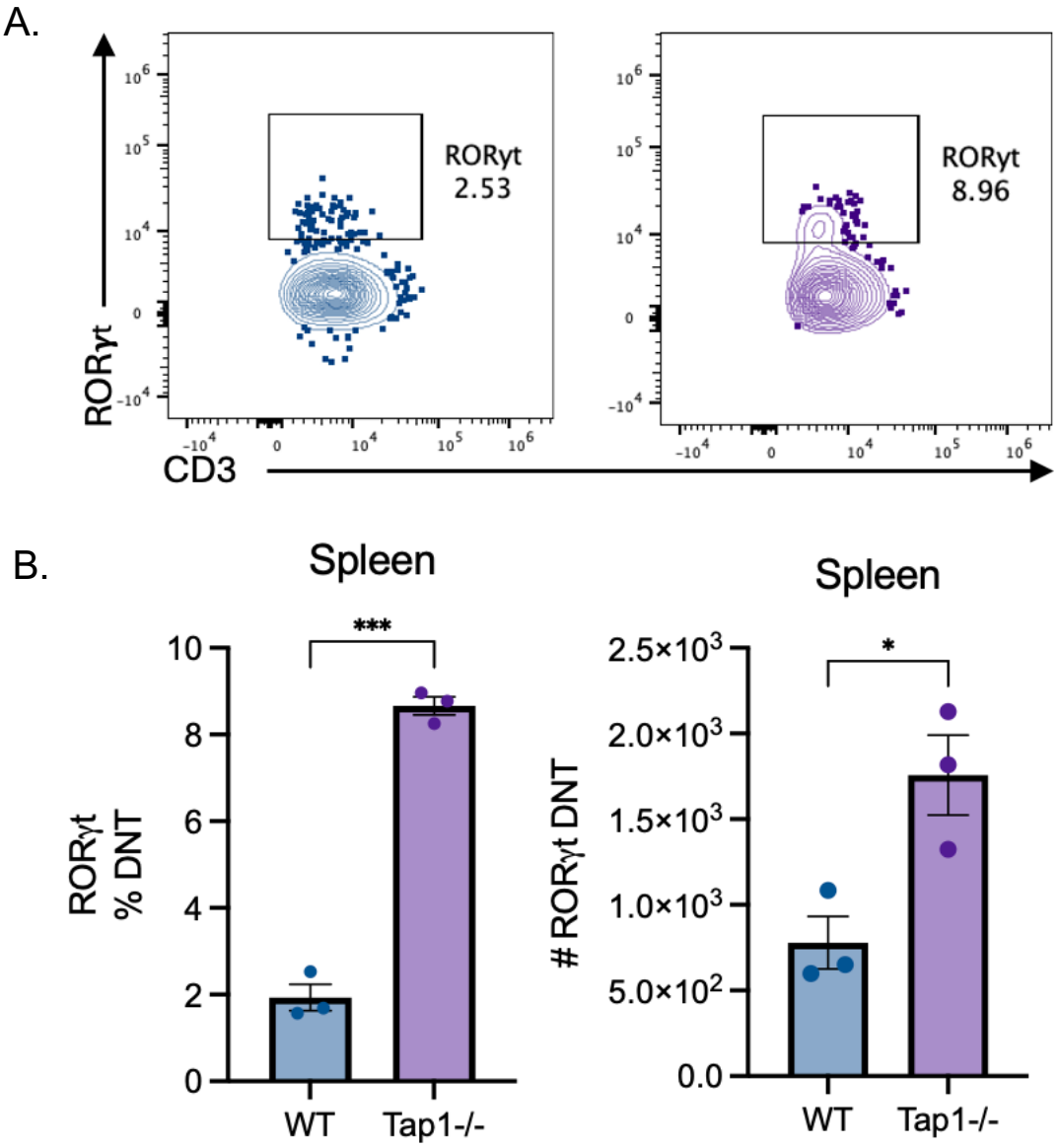
β2M can also act as an adaptor molecule to other non-classical MHC-1b complexes, including CD1d and MR1, which present antigens to iNKT and MAIT cells respectively[2,158]. Accordingly, we examined DNT presence in *CD1d-/-* and *MR1-/-,* to rule out DNT selection by these molecules via non-typical antigen presentation. As previously published by Koay et al[159]., we found no differences in total DNT number or frequency, or fluctuations in the type-17 population specifically (**Figure 4.10A and 4.10B**). Additionally, DNTs again show ability to compensate for the loss of other T cell populations, with an expansion in the liver in the absence of CD1d and iNKT cells. Whilst DNT numbers are not impacted by loss of CD1d in knockout models, it is worth acknowledging that CD1d-restricted non-invariant NKT cells (type 2 NKT) are not comprehensively excluded by invariant CD1d-tetramer gating alone. Therefore, it is a caveat of this study that in WT mice, a minor contribution of DNT cells may be type 2 NKT and cannot be formally ruled out without additional approaches such as broader CD1d-lipid tetramer panels or functional lipid-antigen responsiveness. This technical caveat does not contradict the observation that the overall DNT compartment persists in CD1d deficiency.

Since b2M stabilises both classical and non-classical MHC-I molecules, and helps them fold for antigen presentation, we examined Transporter Associated with antigen Processing 1 (Tap1) knockout mice to identify whether type-17 DNTs depend specifically on classical MHC-I presentation via cytosolic peptide processing by Tap1[160,161]. iNKTs and MAITs, although relying on b2M, do not require Tap1 due to their unique antigen recognition of non-peptide lipid and metabolite antigens respectively, both of which are processed within the endosome, bypassing the need for Tap1 peptide presentation[22,162,163]. Interestingly, DNT17 expanded in the spleen of Tap1-/- mice in comparison to WT controls, indicating their independence from classical MHC-I presentation and selection (**Figure 4.11A and 4.11B**). Taken together, we present distinct developmental requirements for DNT1 (MHC-I/II-independent) and DNT17 (B2M-dependent), both linked to the thymus and in line with an unconventional innate lineage, but differing in their dependence on selection molecules (**Figure 4.12**).

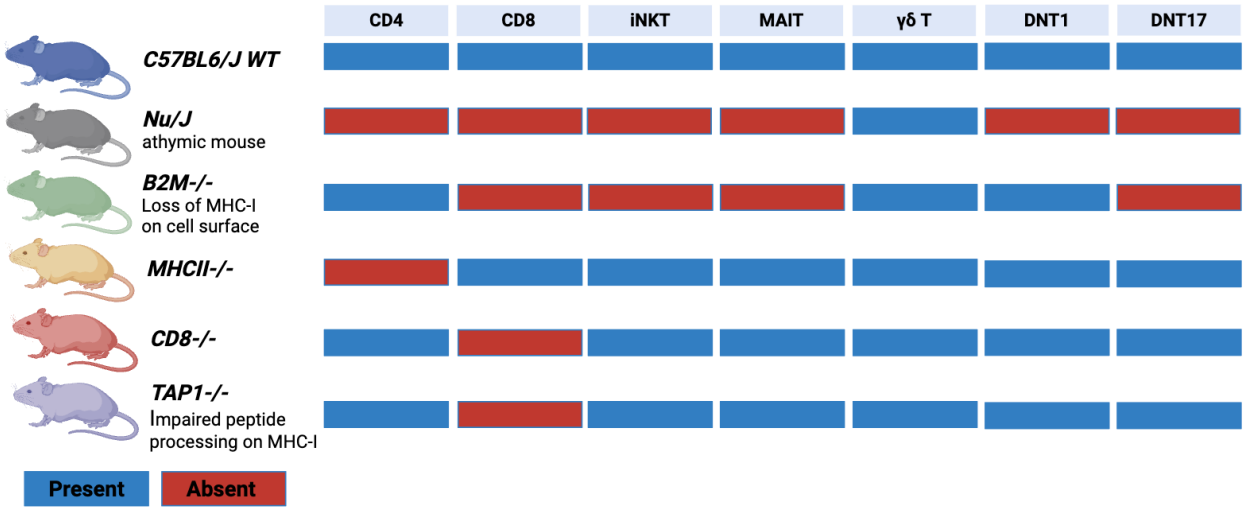


32 **Figure 4.10***:* DNT do not require CD1d- or MR1-mediated presentation for selection

Cells were isolated from liver or spleen from C57BL6/J (WT) or *CD1d or MR1-/-* mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Frequency of DNT out of total T cells (left), and absolute number of DNTs (right) from the liver or spleen of *WT, CD1d or MR1-/-* **(B)** Frequency of PLZF+ / RORγt^+ (DNT17) as a percentage of total DNTs (left), and absolute number (right) from the liver or spleen of *WT, CD1d or MR1.* Groups were compared using two-way anova and error bars in graphs represent mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, n = 5 to 8, two to three experiments. *Data was provided by collaborators; Dr. Fern Koay and Dr. Calvin Xu from the University of Melbourne.*



33 **Figure 4.11***:* Tap1-presentation is not required for DNT-17 development Cells were isolated from liver or spleen from C57BL6/J (WT) or *Tap1-/-* mice as indicated. Cells were stained *ex vivo* and analysed by flow cytometry. **(A)** Representative gating of RORγt$^+$ expression on DNTs from WT (left) and *Tap1-/-* (right). **(B)** Frequency of RORγt$^+$ (DNT17) as a percentage of total DNTs (left), and absolute number (right) from spleen of *WT, CD1d or MR1.* Groups were compared using unpaired student's t-test and error bars in graphs represent mean ± SEM. ∗p < 0.05, ∗∗∗p < 0.005, n = 3, one experiment.



34 **Figure 4.12***:* Summary table of TCR selection requirements for conventional and unconventional T cell subsets, including DNT1 and DNT17 cells

4.8 Discussion

This chapter demonstrates that despite polyclonality, DNT cells constitute a unique unconventional T cell lineage with innate-like features, defined by a pre-programmed effector-memory phenotype, heightened cytokine responsiveness, and distinct developmental requirements. This polyclonality coupled with innate “readiness” could indicate the ability of the DNT TCR to recognise and mount a response towards a broader range of antigens, within a timelier manner than conventional T cells due to their thymus-selected effector state, further bridging the gap between innate and adaptive immunity.

DNTs display elevated expression of CD44 and CD69 in the steady state, consistent with an effector-memory-like phenotype normally associated with antigen-experienced conventional T cells or agonist selected innate T cells[1]. Importantly, this phenotype was evident in naïve mice and likely not dependent on prior antigen exposure. Their intermediate to low CD3/TCRαβ expression, coupled with PLZF and Helios expression, supports their alignment to known innate T cells. However, the enrichment of Helios in DNTs, is beyond levels seen in γδ, iNKT and MAIT cells, suggesting a potentially more pronounced role for Helios in DNT development and/or peripheral persistence, particularly in the PLZF-low DNT1 population where Helios expression is highest. Helios, is an Ikaros-family transcription factor best described in regulatory T cells (Tregs), where it proves to be essential to maintain their suppressive ability. Helios-deficient Tregs show diminished IL-2R/STAT5 signalling, loss of Foxp3 stability, acquisition of effector cytokines (IFNγ, IL-17A), and impaired suppressive function[144,164]. Initially proposed as a marker to distinguish thymic-derived from peripherally induced Tregs, Helios expression is now known to alternate depending on activation status and is not a lineage-specific marker[165,166]. Beyond Tregs, Helios is known to be expressed across innate T cell subsets but very few studies indicate potential function. MAIT cells frequently express Helios, and human IKZF2 loss-of-function causes combined immunodeficiency with a marked reduction of circulating MAIT cells, indicating potential importance for Helios in MAIT cell development and/or peripheral homeostasis[167]. In human γδ T cells, Helios marks specific subsets with about one-third of circulating Vδ2 cells expressing Helios and upregulating it with co-

stimulation, while intestinal intraepithelial γδ T cells show prominent Helios expression[168]. While Helios expression on innate T cells are known, it's role or potential requirement for development and function of innate T cells have not been identified. Considering Helios expression is highest amongst T-bet+ IFNγ producing cytotoxic DNTs, it seems less likely that Helios is also involved in maintaining stable IL-2R signalling, which plays important roles in differentiation and control across T cell subsets[169]. Given, instability or targeted deletion of Helios in T-reg populations, leads to a conversion from regulatory to more effector-like T cells[164], it's possible Helios acts as an internal control mechanism for innate T cells, that prevents chronic activation and mediates a more "resting" state until recognition of ligand, cytokine or stress signals. However, further work is warranted to determine precise roles for Helios in transcriptional control of innate T cell subsets, particularly in DNT1 cells where it's role could be more pronounced in the absence of MHC-I and MHC-II selection.

Both DNT subtypes responded rapidly to cytokine stimulation, with DNT17 cells being more responsive to combined TCR and cytokine signals than DNT1 cells, which may relate to the more restricted TCR of DNT17, which could imbue stronger TCR-specific responses. Additionally, DNT1 cells were particularly potent producers of IFNγ in response to IL-12/15/18, surpassing that of iNKT and γδ T cells. Mechanistically, this was associated with high expression of IL-18R, the critical receptor for IFNγ production[170]. Given that IL-18 synergises with IL-12 and IL-15 to amplify IFNγ responses[153], the elevated IL-18R expression seen here likely contributes to their heightened cytokine responsivity. The diverse basal expression of NK receptors (NK1.1, NKG2D, NKG2A/C/E, Ly49-G2) further distinguishes DNTs from iNKT and γδ T cells, which express some but not all of the examined receptors. Of note, DNT expressed comparable levels of Ly49-G2 to that of NK cells, where as it was near absent in γδ, iNKT, and MAIT cells. Although Ly49-G2 is an inhibitory receptor, high expression on NK cells is associated with an enhanced cytolytic phenotype[152]. This broad NK receptor expression may enable DNTs to respond to stress ligands and inflammatory cues in a more similar capacity to that of NK cells, potentially relying more heavily on this means of effector response due to their MHC-I/II-independent TCR.

Unlike conventional T cells, DNTs do not rely on MHC-II, and total DNT numbers are either preserved or expanded in MHC-II deficient mice, depending on the tissue. This signals the importance of tissue environment, cellular composition and cytokine milieus in regulating DNT populations, and their ability to respond to such cues, with the adipose tissue clearly favouring DNT expansion in MHC-II knockout mice. Interestingly, DNT1 cells developed independently of both MHC-I and II, while DNT17 cells were critically dependent on β2-microglobulin, strongly suggesting two-separate unconventional T cell lineages within the DNT pool. Importantly, the requirement for β2M was not attributable to CD8 T cell absence, as CD8-deficient mice maintained normal DNT frequencies. Instead, β2M dependence may reflect selection by non-classical MHC-I molecules. Especially given Tap1 deficiency not only spared but expanded DNT17 cells, indicating they may bypass classical MHC-I peptide presentation and instead rely on a β2M-associated non-classical molecules that do not require Tap1-mediated peptide loading.

Taken together, this data suggests DNTs are functionally positioned to act as early responders in immunity, with the ability to sense both cytokine and stress ligands in a manner overlapping but distinct from γδ T cells, MAITs, and iNKTs. Their enhanced IFNγ responses through increased IL-18R expression may be of particular relevance in infections where IL-18 is abundant. Future work should aim to define the precise non-classical ligands and selecting elements required for DNT17 development, the functional significance of Helios expression in maintaining their lineage identity, and the contexts in which DNT1-driven IFNγ production are of importance in immune outcomes.

Chapter 5:

DNTs infiltrate multiple syngeneic tumour models and confer anti-tumour immunity in the MC38 model

5.1 Introduction

Immunotherapy has revolutionised cancer treatment, with much of its success attributed to the sustained activation of antigen-specific $CD8^+$ T cells, achieved through immune checkpoint blockade (ICB)[171]. Additionally, autologous chimeric antigen receptor (CAR)-T cell therapies have proven to be of benefit in clinical settings, but cost per treatment and scale-up of manufacturing, as well as risks for off-target toxicity and GvHD continue to be roadblocks to regulatory approvals and widespread use. As a result, there is growing interest in adapting innate unconventional T cell populations for use in CAR-based therapies[172]. Several characteristics of innate T cells, make them advantageous targets for immunotherapy, including their multiple tumour targeting mechanisms, efficient tumour infiltration and their ability to enhance the adaptive response through early inflammatory activation[173]. A 2022 study by Vasic et al., demonstrated that a mixed population of $CD3^+CD4^-CD8^-$anti-CD19-CAR (CAR19)-DNT had comparable efficacy CAR19-CD8 T cells in mouse models of blood and lung cancer. However, unlike conventional CAR19 cells, CAR19-DNT did not cause alloreactivity or xenogeneic GvHD, highlighting their usefulness as potential "of-the-shelf" therapies[174]. More recently, Tin et al., performed a sc-transcriptomic analysis of in-vitro expanded human DNTs for adoptive therapy. Although, a mixed population of cells, they show $TCR\gamma\delta^-$ DNT cells demonstrate greater persistence characteristics and cytotoxic potential[175]. Whilst beneficial studies, more work is needed to tease apart the contributions of specific unconventional subsets within these settings. For example, Zhou et al., recently showed that CAR-NKT cells displayed superior anti-tumor activity over traditional CAR-T cells, due to their ability to eliminate M2-like macrophages and promote epitope spreading and sustained activation of CD8 T cells in the tumour[176]. While significant differences exist between mouse and human innate T cell populations, murine models provide indispensable mechanistic insights that can inform translational studies.

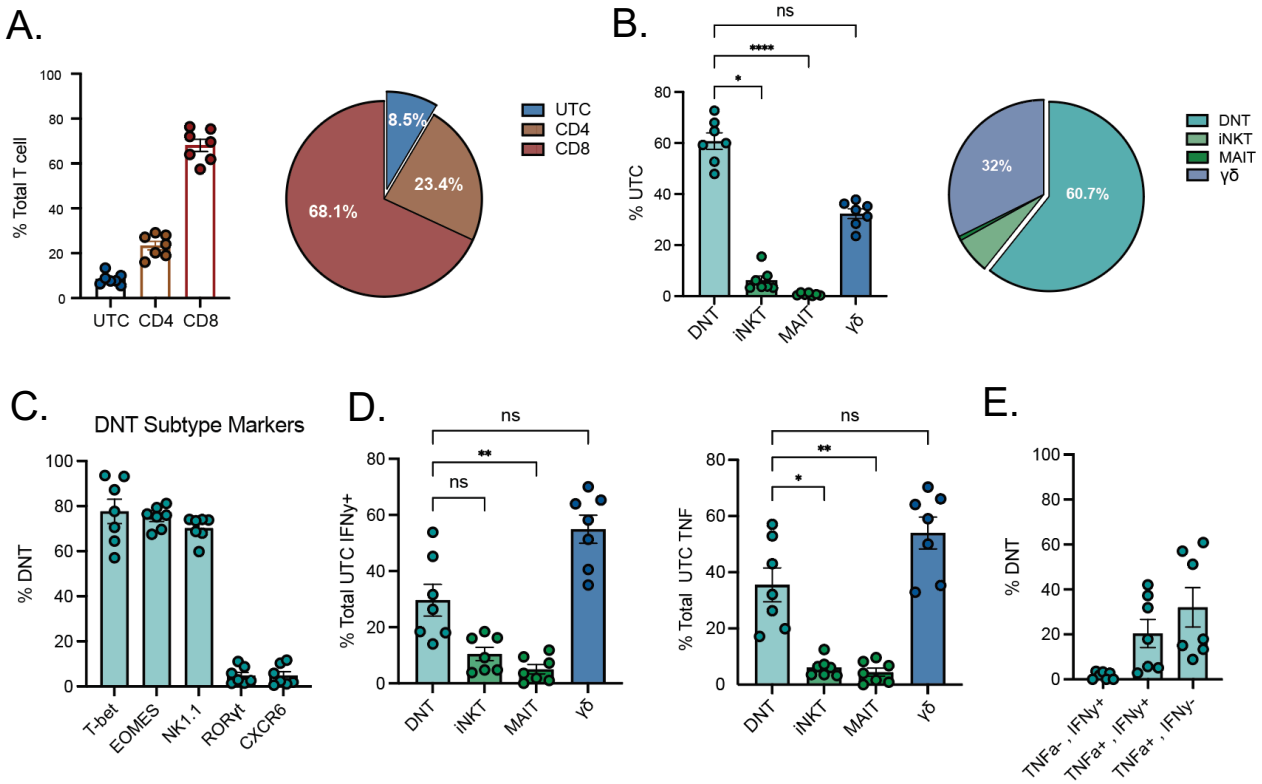
In chapter 5, we set out to understand the role of DNT cells in the TME, whether they represent a substantial population of tumour-infiltrating T cells (TILs) and how they modulate the TME and impact tumour progression. Given the widespread tissue distribution of DNT1 and their potent IFNγ production compared to other T cell

populations, we hypothesised DNT1 would be beneficial contributors to anti-tumour immunity. To assess their potential role in this setting, we first profiled DNTs across multiple syngeneic tumour models and assessed their role in the tumour following ex-vivo expansion of DNT1 and adoptive transfer to the MC38 colorectal tumour model. In doing so, we identified DNT cells as one of the primary unconventional T cells infiltrating E0771, Yumm1.7 and MC38 tumor models, contributing significant IFNγ and TNFα within the TME. Further, we observed a marked reduction in MC38 tumor progression upon adoptive transfer of DNT cells, that was associated with an increased immune cell milieu within the tumour. This data confirms the anti-tumour capacity of DNT1 cells and signals their potential translational utility in future immunotherapies.

5.2 DNT1 infiltrate multiple syngeneic tumour models and significantly contribute to cytotoxic protein production in the tumour

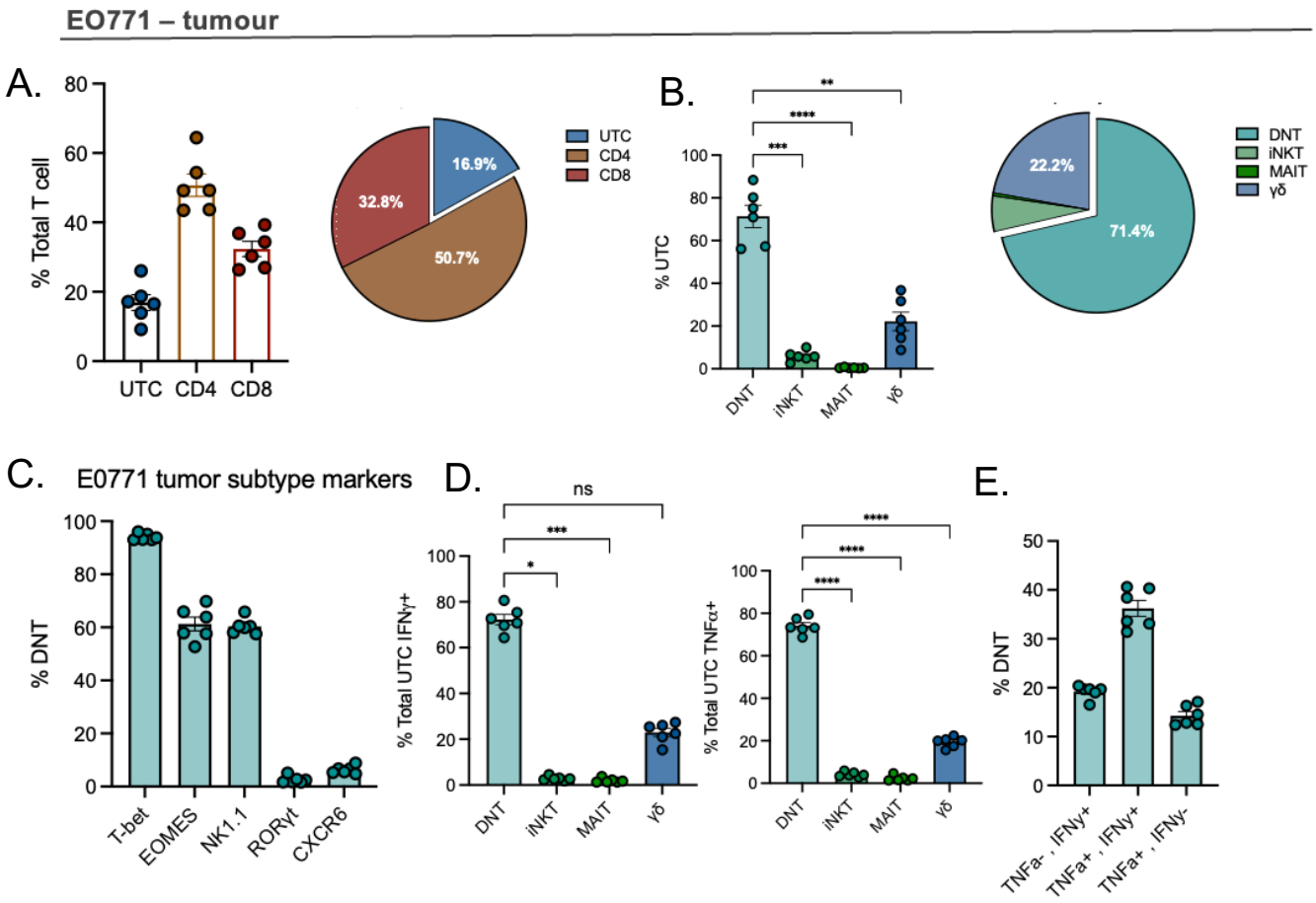
Innate-like T cells are important players in tumour immunosurveillance as they have distinct mechanisms of tumour recognition and killing, mediated via direct cytotoxicity, non-peptide antigen recognition, cytokine production, and modulation of the tumour microenvironment[177]. Given the NK-like DNT1 cells are the most abundant DNT subtype throughout tissues, and have superior cytokine induced IFNγ production, we examined if they might infiltrate tumours and have anti-tumour effects in the TME. We examined three syngeneic solid tumour models; EO771 (breast), Yumm1.7 (melanoma), and MC38 (colorectal). Across all three models, unconventional T cells represented a small fraction of the total T cell infiltrate, ranging from 8.5% in MC38 to 16.9% in E0771 (**Figure 5.1A, 5.2A, and 5.3A**). Within the unconventional T cell compartment, DNTs surprisingly accounted for the majority of cells. Of the unconventional T cell compartment, DNTs comprised 71.4% in E0771, 51.3% in Yumm1.7 and 60.7%, in MC38 tumours (**Figure 5.1B, 5.2B, and 5.3B**). In contrast, MAIT and iNKT cells were minor contributors across all models, each comprising <10% of unconventional T cells. Phenotypic profiling revealed that the DNT population was highly enriched for NK1.1$^+$ T-bet$^+$ EOMES$^+$ cells consistent with a DNT1 phenotype. This subset accounted for nearly all DNTs within each tumour model, while expression of RORγt and CXCR6, associated with type 17 programs, were low to absent (**Figure 5.1C, 5.2C, and 5.3C**). Thus, intratumoural DNTs are phenotypically skewed toward a type 1 effector profile across tumour types. We next assessed cytokine production within the unconventional T cell populations with PMA stimulation. In line, with their increased frequency in the tumour, DNTs were the primary source of both IFNγ and TNFα within the unconventional T cell compartment E0771 and Yumm1.7 tumour models, although not significantly higher than γδ (**Figure 5.1D, 5.2D, and 5.3D**). Furthermore, we examined the co-expression of IFNγ and TNFα within DNTs, to find the majority of IFNγ production by DNT cells in the tumour, is co-produced alongside TNFα, suggesting robust synergetic anti-tumour responses[178,179] (**Figure 5.1E, 5.2E, and 5.3E)**.

While in MC38 tumours, the majority were either IFNγ$^+$TNFα$^+$ or TNFα$^+$ only, with fewer IFNγ single producers. Considering cytokine stimulation induced a robust IFNγ response by DNTs at steady state (**Figure 4.3A**), we compared PMA/ionomycin stimulation with IL-12/15/18 stimulation on the DNTs and other TILs from MC38 tumours. As expected, cytokine stimulation increased the frequency of DNTs producing IFNγ from ~10 – 50%, compared to the PMA/ionomycin stimulation (**Figure 5.4A**). Furthermore, the MFI of IFNγ producers was significantly higher amongst the DNTs, compared to γδ, CD4 and CD8 T cells, and comparable to IFNγ production by NK cells (**Figure 5.4B**). Whilst cytokine stimulation alone is typically not sufficient to induce IFNγ production by naïve conventional CD8 T cells, at least a subset of tumour-isolated CD8 T will likely have acquired antigen experience in the TME and be in a primed activation state, upregulating IL-12 and IL-18 receptors, allowing responsivity to cytokine stimuli[180-183]. These findings demonstrate that intratumoural DNTs not only predominate within the unconventional T cell compartment but also represent a functionally robust population capable of potent innate IFNγ/TNFα production, suggesting a potentially important role for NK1.1$^+$ DNT1s in shaping the TME.



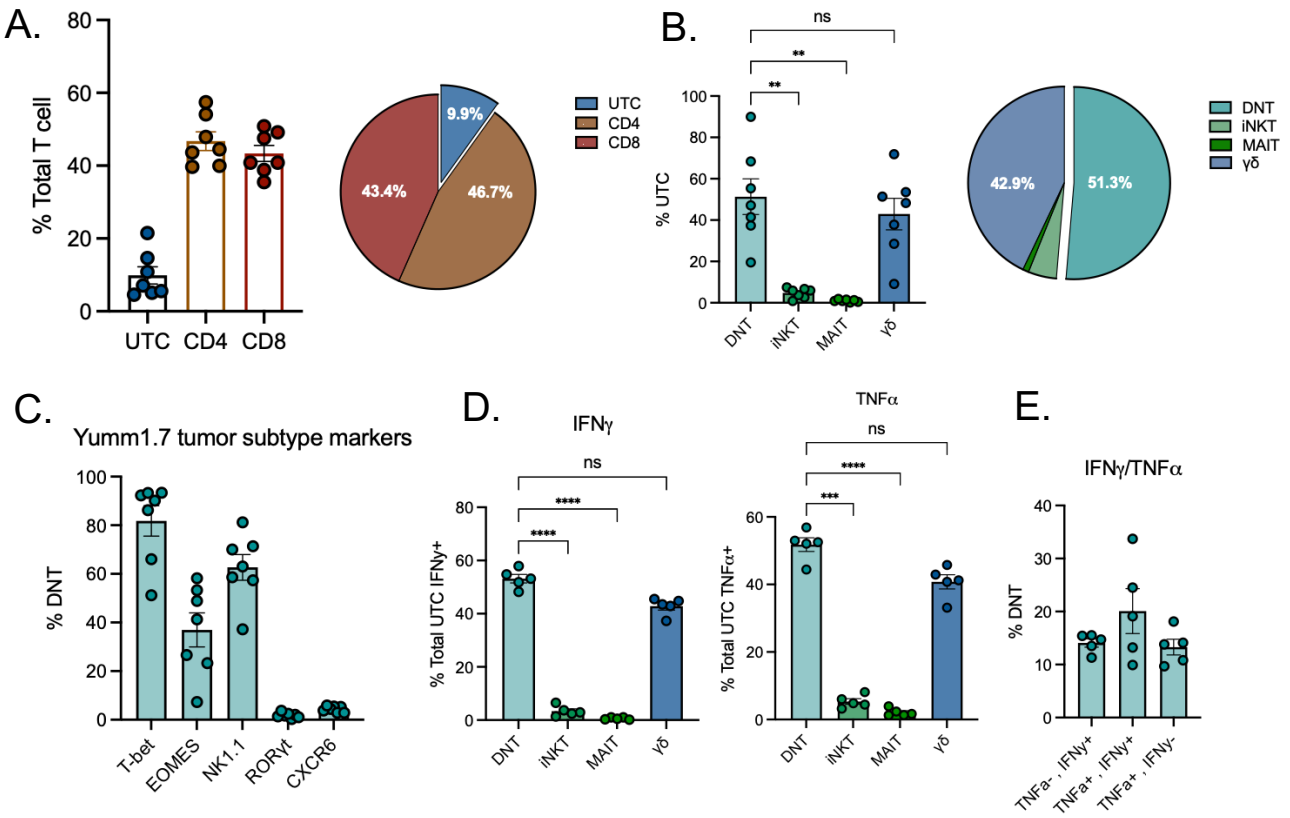
35 **Figure 5.1***:* NK1.1+ DNT1 are infiltrate MC38 tumours and contribute to cytotoxic production in the TME

Flow cytometric analysis of tumor infiltrate from MC38 (colorectal) model. MC38, intradermal injection, day 11 **(A)** Frequency of UTC (combined DNT, MAIT, iNKT, **γδ**), CD4 & CD8 T cells shown as a percentage of combined T cell counts and corresponding mean proportions shown in pie chart. **(B)** Frequency of UTCs as a percentage of combined UTC counts, and corresponding mean proportions. **(C)** DNT subtype markers shown as a percentage of total DNT in the tumor. **(D)** Frequency of UTCs as a percentage of total UTC IFN**γ** or TNF**α** producers. **(E)** Frequency of IFN**γ** and TNF**α** on tumor infiltrating DNTs following 4-hour stimulation with PMA/ionomycin. Groups were compared using one-way or two-way ANOVA with Dunn's or Dunnet's multiple comparison depending on gaussian distribution of data, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=6, two experiments



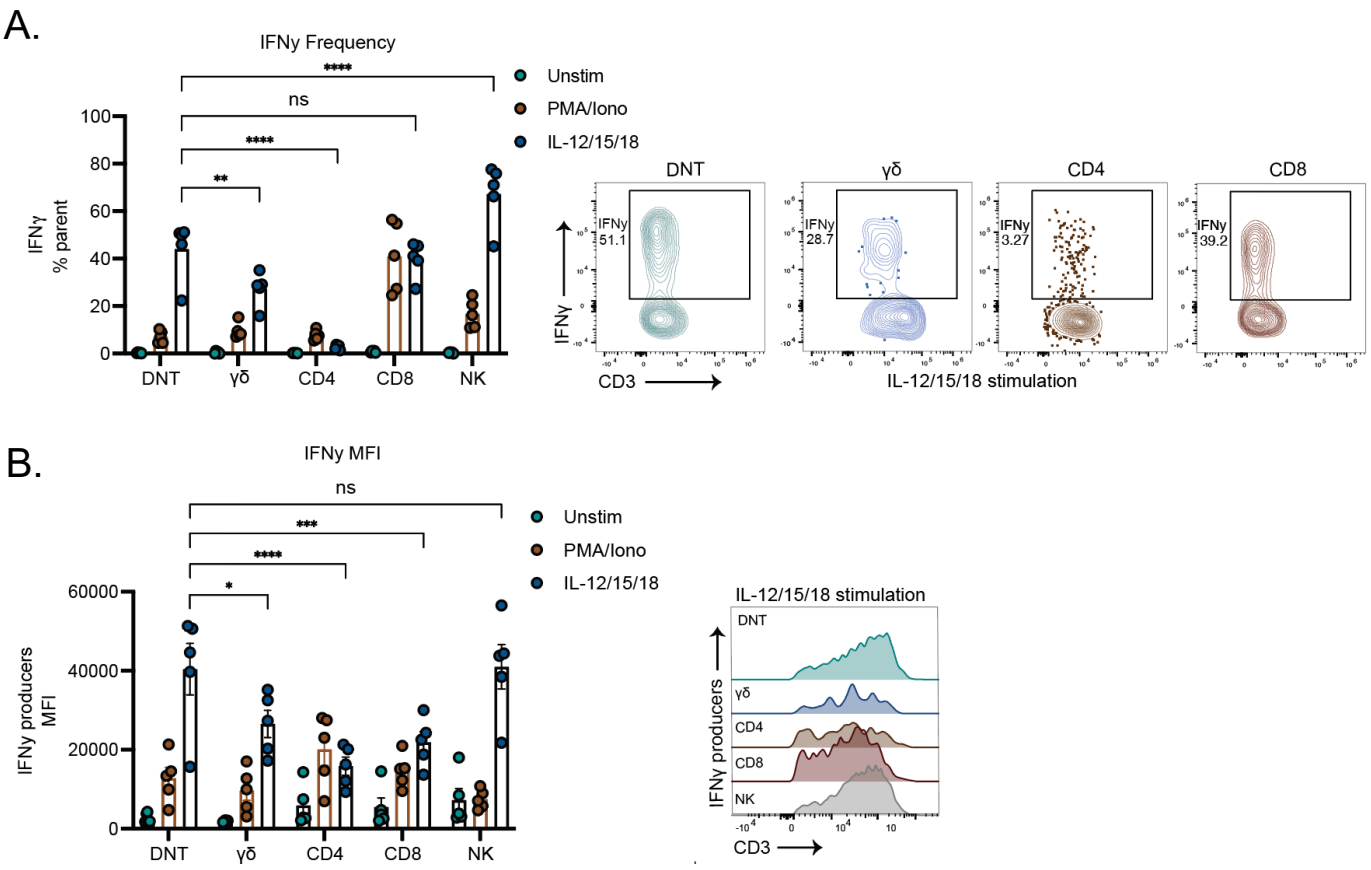
36 **Figure 5.2***:* DNT infiltrate E0771 tumours

Flow cytometric analysis of tumor infiltrate from E0771 (breast cancer) model. E0771, injection into mammary fat pad, day 21 **(A)** Frequency of UTC (combined DNT, MAIT, iNKT, **γδ**), CD4 & CD8 T cells shown as a percentage of combined T cell counts and corresponding mean proportions shown in pie chart. **(B)** Frequency of UTCs as a percentage of combined UTC counts, and corresponding mean proportions. **(C)** DNT subtype markers shown as a percentage of total DNT in the tumor. **(D)** Frequency of UTCs as a percentage of total UTC IFN**γ** or TNF**α** producers. **(E)** Frequency of IFN**γ** and TNF**α** on tumor infiltrating DNTs following 4-hour stimulation with PMA/ionomycin. Groups were compared using one-way or two-way ANOVA with Dunn's or Dunnet's multiple comparison depending on gaussian distribution of data, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=6, one experiment



37 **Figure 5.3***:* DNT infiltrate Yumm1.7 tumours

Flow cytometric analysis of tumor infiltrate from Yumm1.7 (melanoma cancer) model. Yumm1.7, subcutaneous injection, flank, day 15 (**A)** Frequency of UTC (combined DNT, MAIT, iNKT, **γδ**), CD4 & CD8 T cells shown as a percentage of combined T cell counts and corresponding mean proportions shown in pie chart. **(B)** Frequency of UTCs as a percentage of combined UTC counts, and corresponding mean proportions. **(C)** DNT subtype markers shown as a percentage of total DNT in the tumor. **(D)** Frequency of UTCs as a percentage of total UTC IFN**γ** or TNF**α** producers. **(E)** Frequency of IFN**γ** and TNF**α** on tumor infiltrating DNTs following 4-hour stimulation with PMA/ionomycin. Groups were compared using one-way or two-way ANOVA with Dunn's or Dunnet's multiple comparison depending on gaussian distribution of data, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=7, two experiment

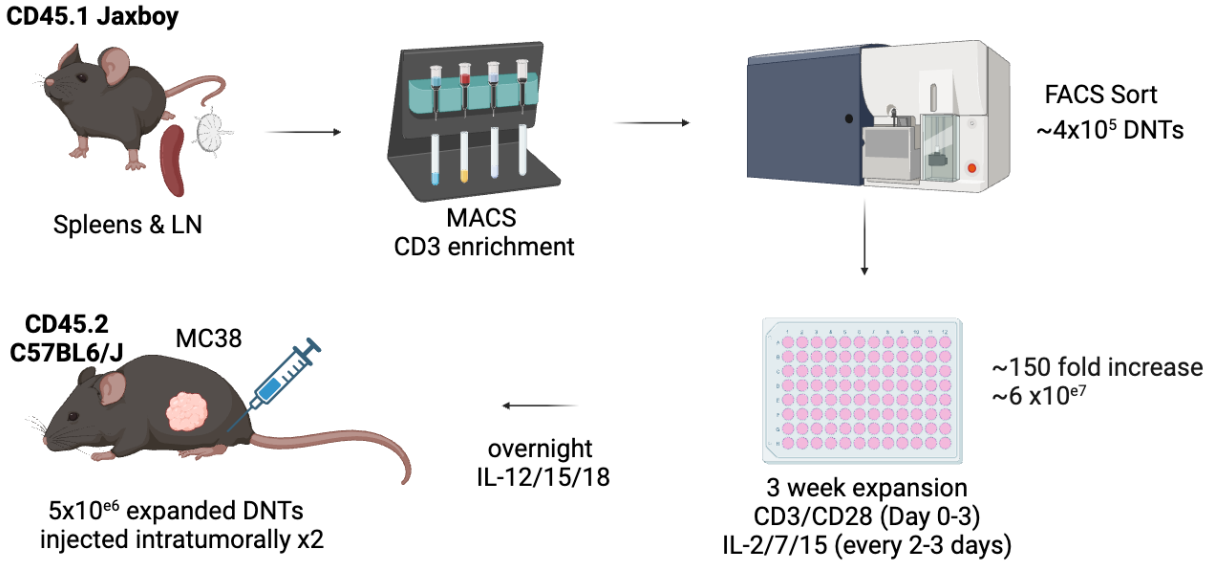


38 **Figure 5.4***:* DNT cells in tumour respond more to IL-12/15/18 stimulation compared to other T cell subsets

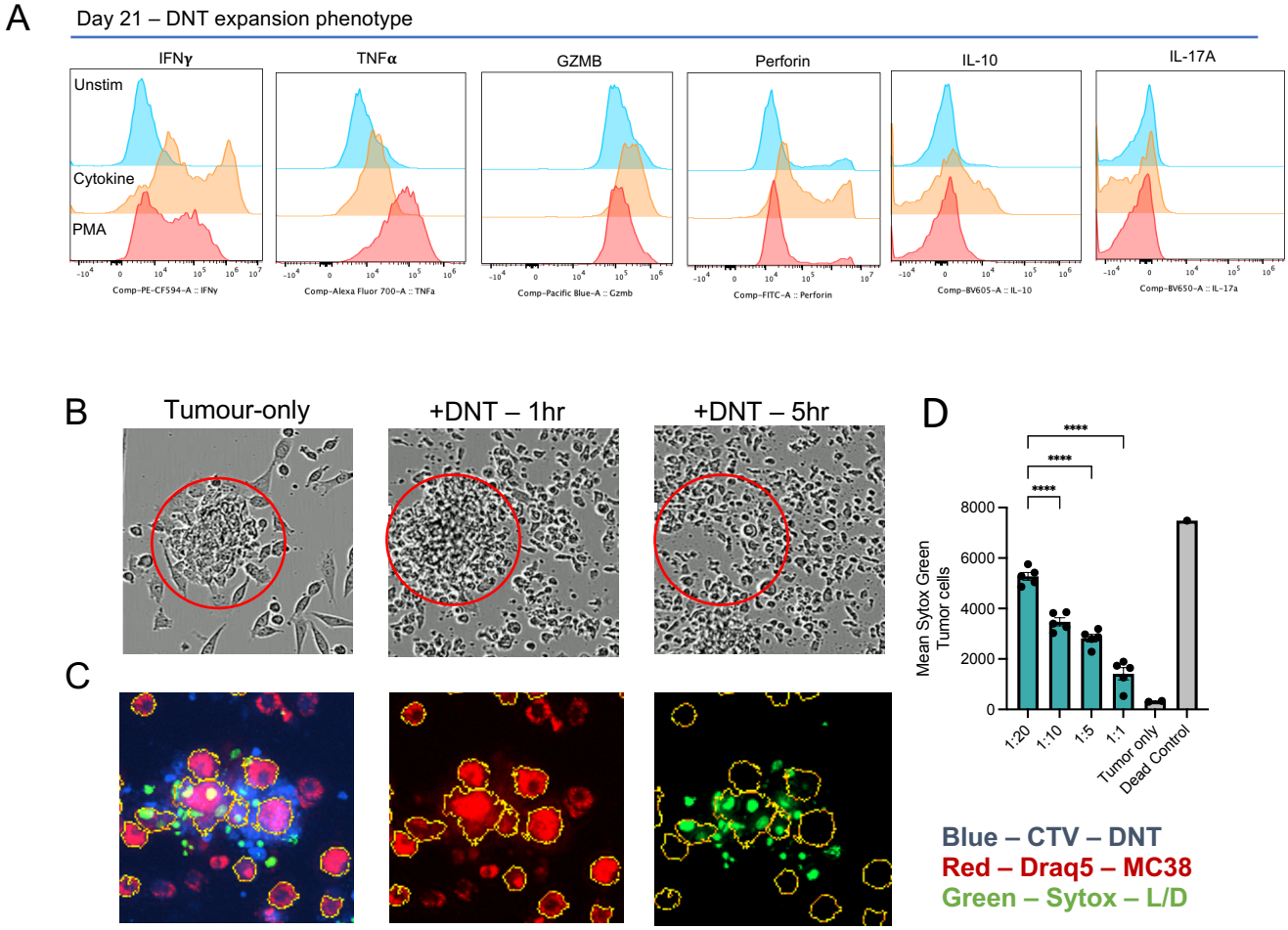
Flow cytometric analysis of tumor infiltrate from MC38 (colorectal) model. MC38, intradermal injection, day 11 **(A)** Frequency of IFN**γ** across cell subsets in MC38 tumor, under unstimulated, PMA/ionomycin or IL-12/15/18 stimulation conditions. **(B)** MFI of IFN**γ** producers across cell subsets, under stated stimulation conditions, and corresponding histogram overlay from IL-12/15/18 stimulation. Groups were compared using one-way or two-way ANOVA with Dunn's or Dunnet's multiple comparison depending on gaussian distribution of data, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=5, one experiment

5.3 Ex-vivo expanded DNT1 highly express cytotoxic proteins and can directly kill tumour cells in-vitro

To evaluate the functional relevance of DNT1 cells on modulating tumour progression, we evaluated their ability to control tumour growth following expansion of spleen-derived DNT cells from non-tumour mice and subsequent adoptive transfer via intratumoural injection to MC38 mouse models. We first optimised an ex-vivo expansion of DNT cells and polarisation to type-1 IFNγ-producing DNT1 (**Methods 2.2.7 and Figure 5.5**). The polarised DNTs were potent producers of IFNγ, TNFα, GZMB and perforin, with modest induction of IL-10 and absence of IL-17A expression (**Figure 5.6A**). To assess direct killing of tumour cells by DNTs, expanded DNT1 were co-cultured with MC38 tumour cells and live imaged. Visually, over the course of 5 hours, type-1 polarised DNTs could be observed targeting and clearing tumour cells (**Figure 5.6B**). By lionheart analysis of Sytox viability stain on MC38 tumours, we saw a concentration dependent induction of cell death on tumour cells when incubated with DNT cells, highlighting their ability to directly target and eliminate MC38 cells (**Figure 5.6C and Figure 5.6D**). We performed adoptive transfer of type-1 polarized DNT into MC38 tumour–bearing mice. To track adoptively transferred DNTs, we expanded cells from CD45.1+ mice before intratumoural injection of $5x10^6$ DNTs into CD45.2 mice once tumours were palpable, and again two days later (**Figure 5.5**).



39 **Figure 5.5***:* Schematic of expansion protocol for DNT1 and adoptive transfer to MC38

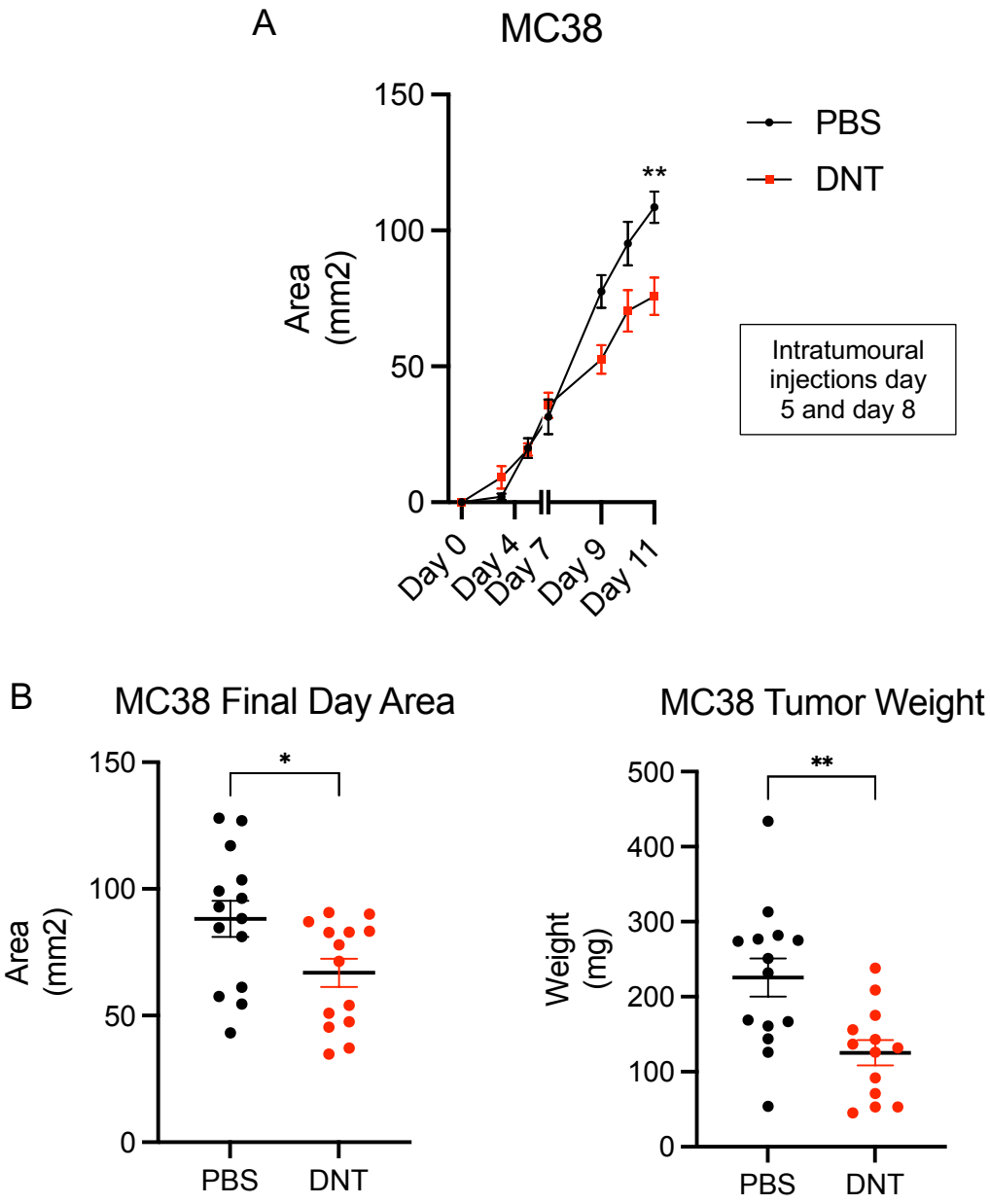


40 **Figure 5.6***:* Expanded DNT1 are potent producers of cytotoxic proteins and can directly kill tumours in-vitro

DNT cells were flow sorted and expanded over a three-week period and polarised to DNT1 with IL-2/15/7 stimulation. DNT1s were restimulated with IL-12/15/18 before flow confirmation or functional assays. **(A)** Flow cytometric images of cytokine and cytotoxic protein expression by Day 21 expanded DNTs. **(B)** Incucyte images of MC38 tumour cells cultured on their own (left), with DNT1s (middle) and 5 hours post-DNT addition (right). **(C)** Lionheart image of tumour:DNT at 1:5 concentration, blue dye; DNT cells, red dye; tumour cells, green dye; viability. **(D)** Quantification of mean fluorescence of Sytox green cell death dye within tumour cells in culture with various concentrations of DNT cells. Groups were compared using one-way ANOVA, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=5, where each sample is a different well with 5000 tumour cells, one experiment

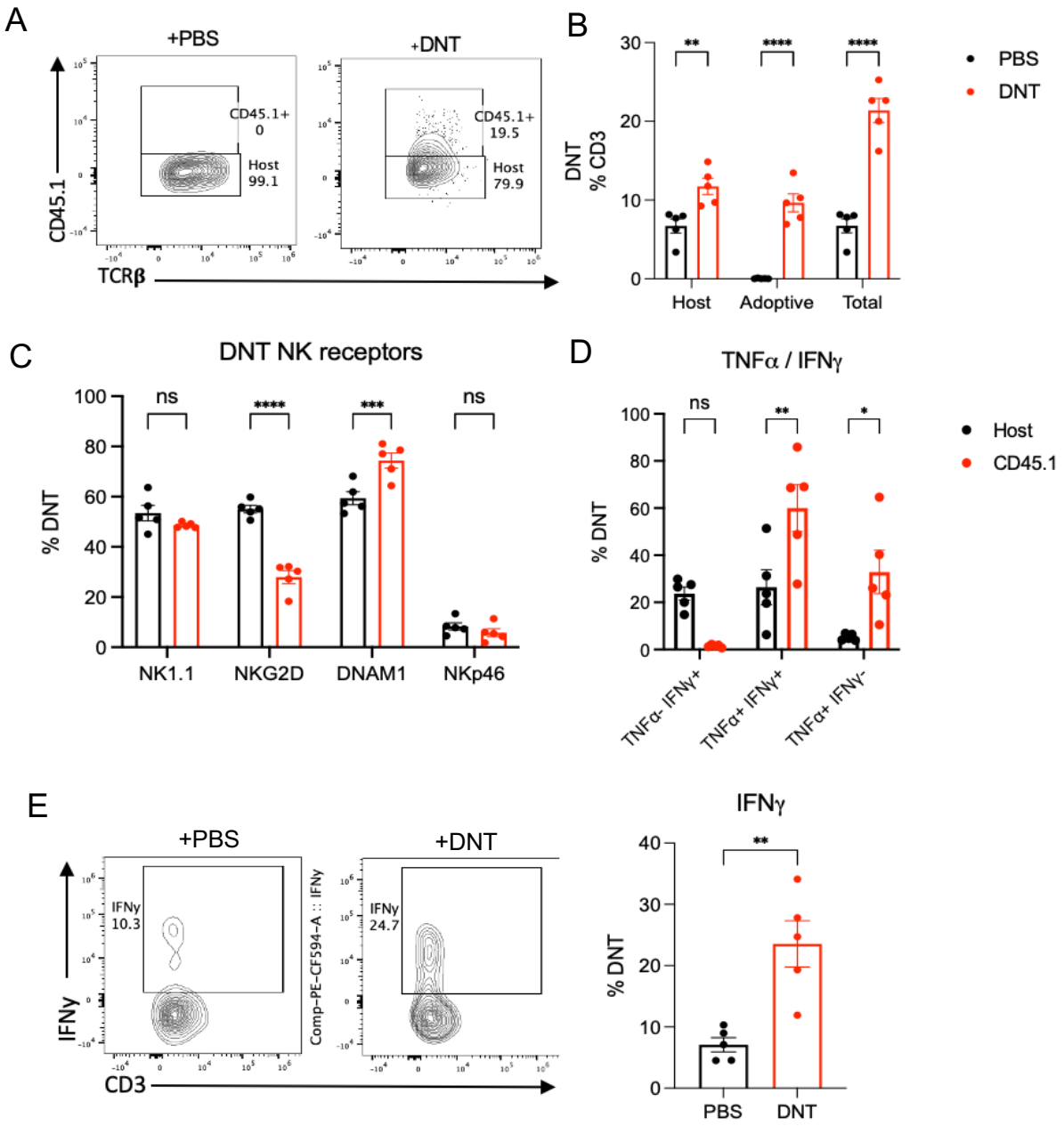
5.4 Adoptively transferred DNT1 significantly reduce MC38 tumour growth

Tumour-bearing mice receiving DNT1 adoptive therapy showed significantly reduced tumour growth compared to PBS controls (**Figure 5.7A**), with a marked reduction in both final tumour weight and area at endpoint (**Figure 5.7B**). Transferred DNTs ($CD45.1^+$) were detectable in the tumour 3 days post transfer (**Figure 5.8A**). In the mice that received DNT cells, we saw a significant increase of DNTs in the tumour, increasing total DNT frequency to 20% of total $CD3^+$ cells within the tumour (**Figure 5.8B**). Interestingly, we also observed a significant increase in host DNT cells following adoptive transfer, potentially indicating increased proliferation or recruitment of DNT cells to the tumour (**Figure 5.8B**). Both host and CD45.1 DNTs highly expressed activating NK receptors; NKG2D and DNAM1 in the TME, indicating their ability to respond to stress ligands such as PVR and RAE-1, which are known to be present in MC38 tumours[184] (**Figure 5.8C**). Host DNT cells expressed higher NKG2D compared to $CD45.1^+$ DNT which expressed higher DNAM1, potentially impacted by the in-vitro expansion conditions, these differences could offer enhanced recognition capacity to the total DNT pool within the tumour towards available stress ligands (**Figure 5.8C**). Adoptively transferred DNT cells also had significantly higher double-positive production of IFNγ and TNFα compared to host DNT cells (**Figure 5.8D**), as well as a significant increase in the frequency of IFNγ expression by total DNT cells in the tumour following a PMA/ionomycin stimulation (**Figure 5.8E).**



41 **Figure 5.7***:* Adoptively transferred DNT cells reduce tumour growth in MC38 model

Expanded DNTs were intratumourally injected into MC38 tumours, once tumours palpable, 2 injections, 2 days apart **(A)** MC38 tumor growth, shown as area (mm^2) in PBS and DNT treated mice, representative of 1 experiment. **(B)** Final day area and weight of MC38 tumors. Groups were compared using student's t-test, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=14, three experiment



42 **Figure 5.8***:* Adoptively transferred DNT cells reduce tumour growth in MC38 model

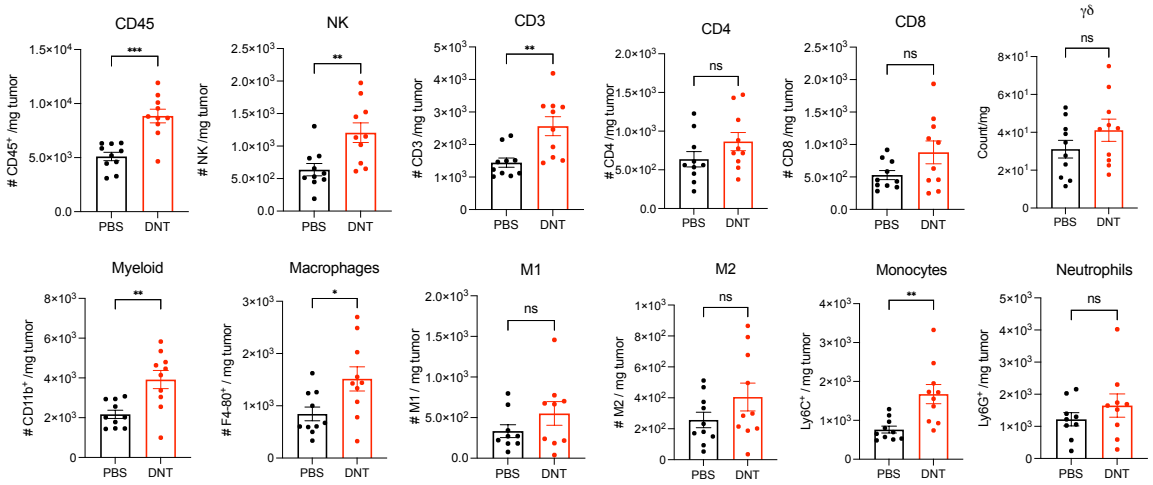
Identification of transferred DNT cells 3 days after last intratumoural injection **(A)** Representative flow images of adoptively transferred DNTs marked by CD45.1 expression in tumor. **(B)** DNTs as a frequency of CD3s shown as host, adoptively transferred or total DNTs within the tumor. **(C)** Flow cytometric analysis of activating NK receptor expression, shown as a frequency of host vs adoptive DNTs in MC38 treated tumors. **(C)** TNFa/IFNy production by host and adoptive DNT in the tumour. (**D**) IFNy production by total DNT in the tumour. Groups were compared using student's t-test, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. n=5, representative of 3 experiments.

5.5 DNT1 induce increased immunogenicity within the tumour via enhanced chemokine production

Interestingly, analysis of the whole TME revealed significant increases in total $CD45^+$, $CD11b^+$ myeloid, $F4/80^+$ macrophage, $Ly6C^+$ monocyte, NK cells and $CD3^+$ T cell numbers in DNT-treated mice (representative gating; **Figure 2.2**), indicating enhanced immunogenicity and a "immunologically hotter" TME after DNT transfer (**Figure 5.9**). Of note, MHC-II^+ $CD86^+$ 'M1' and $CD206^+$ 'M2' macrophage populations were both trending upwards with no bias towards either subset. Similarly, trending increases were observed across CD4, CD8 and γδ T cell subsets, with no particular bias (**Figure 5.9**). Additionally, $CD45.1^+$ DNT cells could be observed in the draining LN (dLN), indicating their ability to traffic from the injection site within the tumour to nearby lymphoid sites (**Figure 5.10A**). Within the dLN, DNT-treated mice exhibited trending higher absolute numbers of total dendritic cells (DCs), including $CCR7^+$ and X-C motif chemokine receptor 1 $(XCR1)^+$ subsets (**Figure 5.10B**), as well as higher $CD45^+$ and NK cell counts (**Figure 5.10C**). Although trending, this increase in cross-presenting DCs in the dLN, could contribute to sustained activation and anti-tumour responses, with $XCR1^+$ DCs in particular being shown to be crucial in tumour antigen delivery and T cell cross-priming[185].

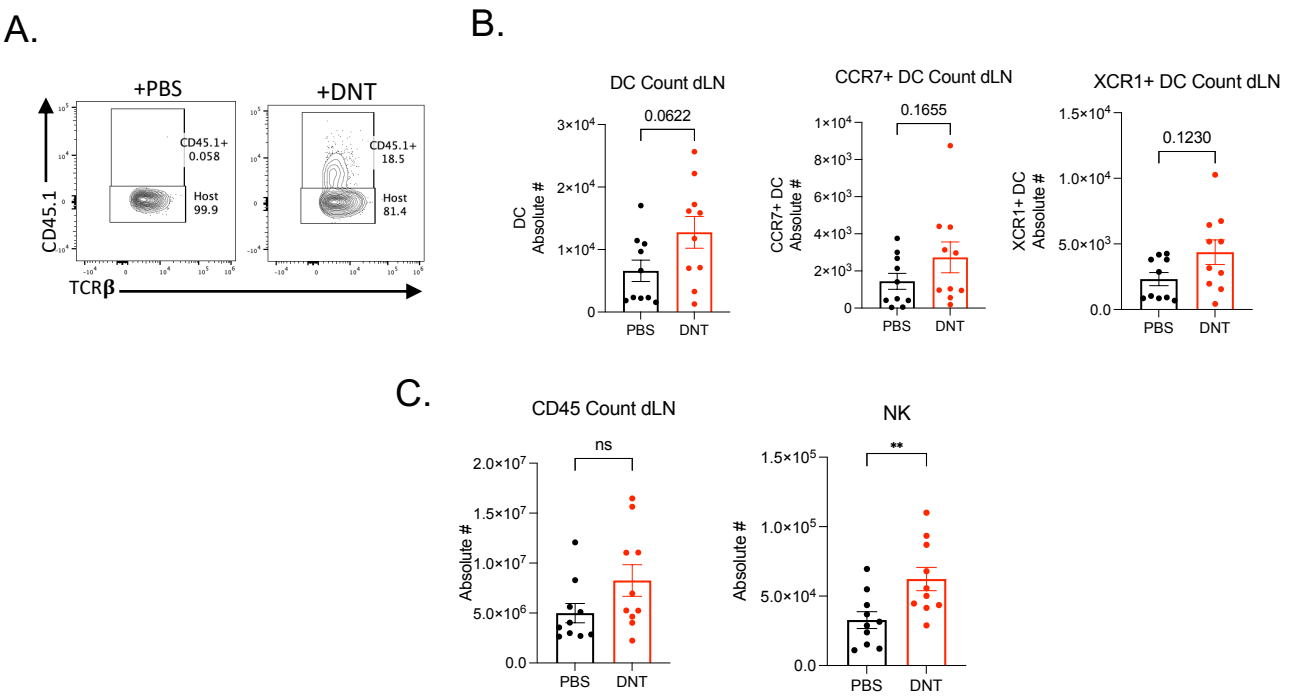
To assess how the DNTs mediate increased immune cell infiltrate within the tumour, we performed Luminex analysis of tumour interstitial fluid (TIF) from PBS and DNT-treated MC38 tumours, with an extensive panel of cytokines and chemokines (**Figure 5.11A**). Importantly, IFNγ concentration was significantly increased in the DNT treated tumours, in addition to the chemokines CCL4 and CCL5, which bind the CCR5 receptor (**Figure 5.11B**). A number of other chemokines were also observed to be trending up in the DNT treated tumours (**Figure 5.11B).** CCL5 can be produced by monocytes/macrophages by indirect means through STAT1 activation by IFNγ, particularly when in combination with TNFα[186]. T cells themselves can also produce CLL5 in response to TCR or cytokine stimulation[187]. Flow and TIF Luminex data support increased levels of CCL5 in DNT treated tumours, possibly due to the enhanced IFNγ response mediated by the adoptively transferred cells. In accordance to this, we see increased CCR5 expressing cells in both the lymphocyte and myeloid compartment in DNT treated tumours (**Figure 5.12**), indicating enhanced recruitment

or proliferation of these cells within the TME. Additionally, CXCL9 is significantly increased in the DNT-treated tumours, and is the present at the highest concentration compared to other chemokines in the tumour (**Figure 5.11B**). CXCL9 binds the CXCR3 receptor and plays a crucial role in anti-tumour immunity for recruitment of both effector T cells and NK cells[188,189]. CXCL10, a separate chemokine contributing to this process is also trending higher in DNT-treated tumours (**Figure 5.11A).** IFNγ has previously been shown to enhance CXCL9/10 production within the TME, and potentially mediates increased effector cell numbers within the tumour post-DNT transfer[190]. Despite previous reports of innate T cells and particularly IFNγ producing subsets inducing enhanced IFNγ responses on other effector cells within the tumour[57], we do not observe this phenomenon across NK cell and T cell subsets (**Figure 5.13**). However, given the enhanced IFNγ observed within the total tumour TIF, the combination of transferred DNT cells and increased immune cell infiltrate likely contributes to higher overall expression of IFNγ within the TME. Overall, the findings of this chapter demonstrate that DNT1 can enhance immunogenicity within the tumour leading to a “hotter” environment and suppressed tumour growth.



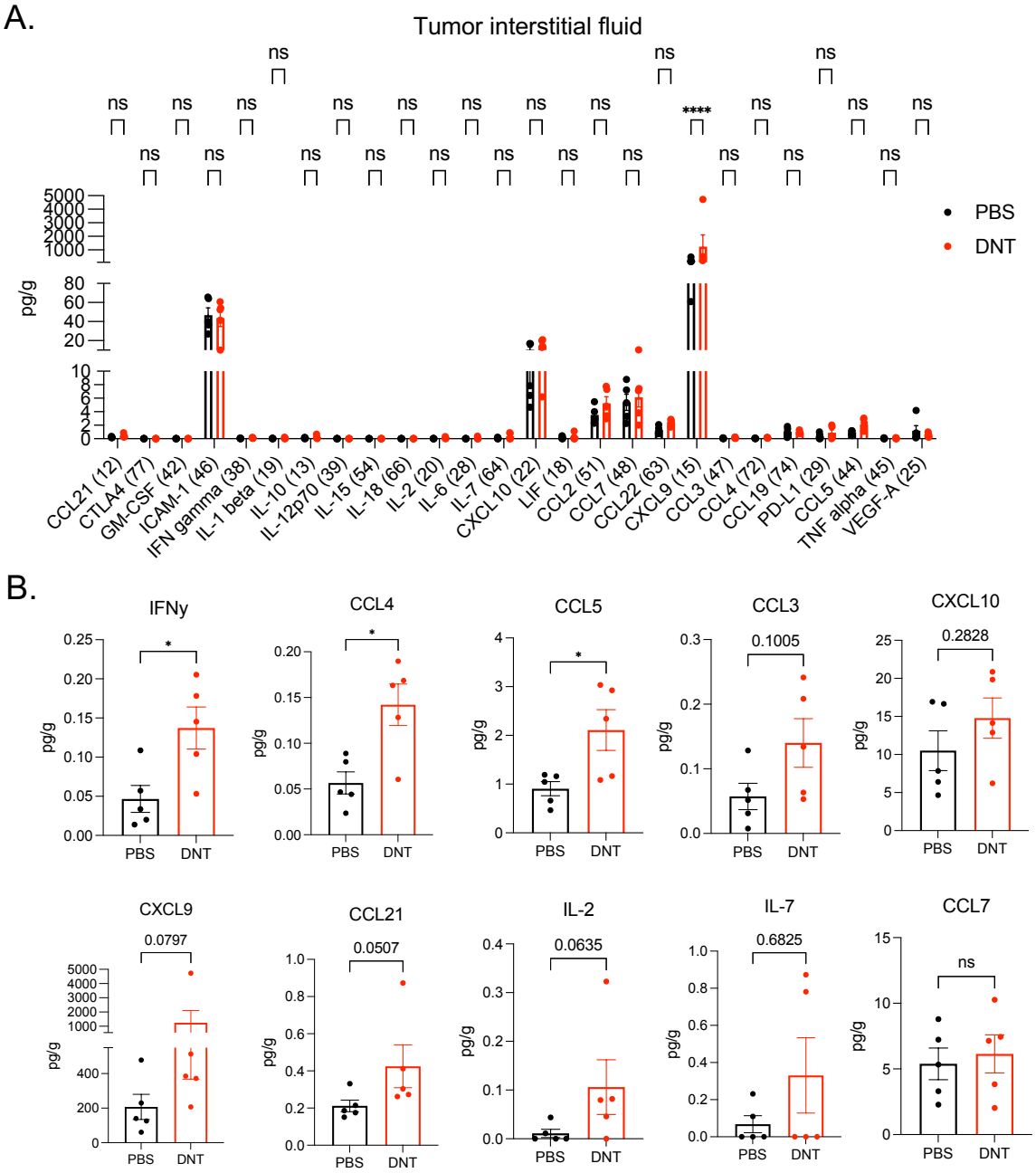
43 **Figure 5.9*:*** DNT adoptive transfer increases immune cell recruitment to the tumour

Flow cytometric analysis of Day 11-12 immune cell tumor infiltrate measured as count/mg of tumor in PBS or DNT treated MC38. (n=10, 2 experiments). See materials and methods for representative gating strategy. Groups were compared using student's t-test, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, **** p < 0.001 n=10, 2 experiments.



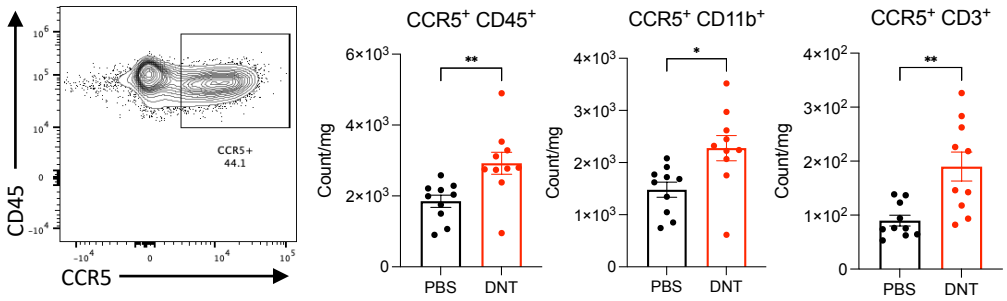
44 **Figure 5.10*:*** DNT infiltrate tumour draining LN and increase DC and NK cell populations

Flow cytometric analysis of day 11-12 immune cell populations within the draining LN, shown as absolute number in PBS or DNT treated MC38. See materials and methods for representative gating strategy. (A) Representative flow images of adoptively transferred DNTs marked by CD45.1 expression in draining LN. (B) Absolute number of DC subsets in draining LN. (C) Absolute number of CD45, NK cells and T cells in tumor draining LN of MC38. Groups were compared using student's t-test, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, **** $p < 0.001$ n=10, 2 experiments.



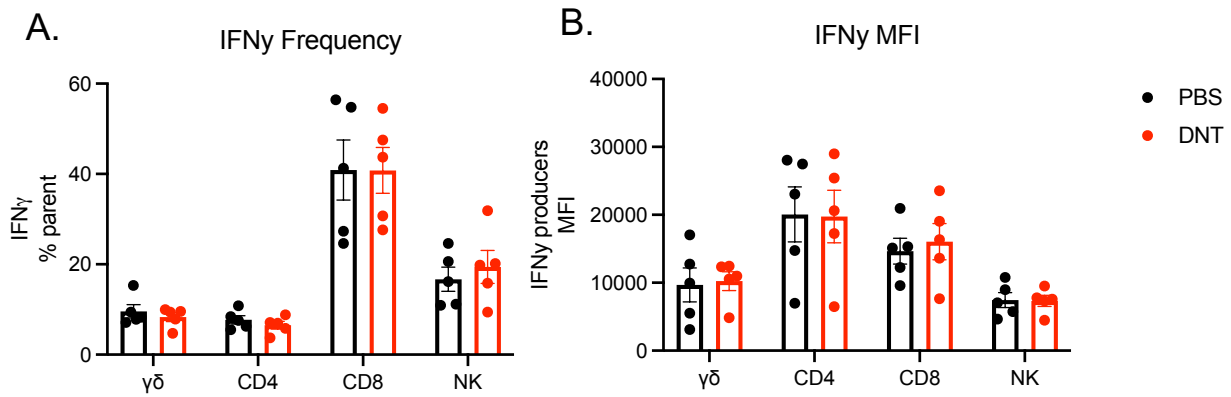
45 **Figure 5.11***:* DNT promote increased chemokine production in the TME

Luminex analysis of day 12 tumour interstitial fluid (TIF) from PBS and DNT treated MC38, normalised to pg/g of tumour. (A). Full panel of chemokines and cytokines. (B) Select analytes from tumour Luminex analysis of TIF; IFNy, CCL4, CCL5, CCL3, CXCL10, CXCL9, CCL21, IL-2, IL-7, CCL7. Groups were compared using student's t-test, or one-way ANOVA, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, **** p < 0.001 n=5, 1 experiments.



46 **Figure 5.12*:*** CCR5+ immune cells increased within DNT-treated tumours

Flow cytometric analysis of Day 11-12 CCR5 expression on immune cell tumor infiltrate measured as count/mg of tumor in PBS or DNT treated MC38. Groups were compared using student's t-test, error bars in graphs represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, **** p < 0.001 n=10, 2 experiments.



47 **Figure 5.13***:* DNTs do not induce enhanced IFNy responses on other T cells or NK cells

Flow cytometric analysis of Day 11-12 IFNy production on immune cell tumor infiltrate measured as frequency of parent cell (A) or IFNy MFI (B). n=5, representative of two experiments.

5.6 Discussion

Our findings in this chapter reveal DNT1 cells as the primary unconventional T cell subset infiltrating multiple syngeneic tumours and that they play a significant role in shaping the TME. Across three independent tumour models; E0771, Yumm1.7, and MC38, DNT1s not only represented a significant proportion of the unconventional T cell compartment but also demonstrated robust production of IFNγ and TNFα. Upon cytokine stimulation, IFNγ production by DNT1s in the tumour exceeded production by γδ, $CD4^+$ and $CD8^+$ T cells and matched that of NK cells. This indicates endogenous DNTs are well positioned to drive anti-tumour immunity, outperforming other effector cells in response to innate signals that can be available in the tumour. The cytokine profile of the ex-vivo expanded DNTs, with high IFNγ and TNFα production and limited IL-17A or IL-10, aligns with known mechanisms of tumour control through direct cytotoxicity, activation of myeloid subsets, and recruitment of additional effector cells[191-194]. Accordingly, ex-vivo expansion and adoptive transfer of polarised DNT1s into tumour-bearing mice resulted in significant suppression of tumour growth.

Importantly, DNT1 transfer led to broad increases in total immune cell infiltrates, spanning both lymphoid and myeloid compartments. This indicates that DNT1 cells may act as amplifiers of overall immunogenicity rather than selectively recruiting specific immune cells. Mechanistically, elevated IFNγ and chemokines such as CCL4/CCL5 in the TME likely contribute to this effect, consistent with established roles of IFNγ-induced STAT1 signalling in driving CCR5-ligand production[186]. The recruitment of $CCR5^+$ immune cells suggest DNT1-produced cytokines enhance immune cell influx and create a “hotter” tumour environment. It also aligns with previous work by Miller et al., that showed $NK1.1^-$ DNT cells increased CCR5+ anti-tumour macrophages in a model of pancreatic cancer, indicating the phenotype is robust amongst DNTs and potentially not dependent on the expression of NK1.1, at least for macrophage recruitment[115]. However, there are likely other mechanistic actions at play, a side from IFNγ-induced immunogenicity and chemokine induction, that still need to be explored.

Although anti-tumour mediators such as T and NK cells are amongst the increased tumour infiltrate in the DNT-treated tumours, there is notable increased presence of

immune cells more associated with pro-tumour roles, including monocytes and macrophages. Whilst we observe no preference towards anti-tumour, M1 or pro-tumour, M2 macrophage polarisation or infiltration, based on CD86 (M1), and CD206 (M2) expression, it does suggest that the transferred DNT1 may induce mixed effects in terms of tumour immunity. Although TAMs can have detrimental effects on tumour progression, they can also act as organising factors in anti-tumour immunity by producing CXCL9/10, which can attract NK and CD8 T cells to the TME. Accordingly, CXCL9 expressing TAMs have been shown to be important in the response to ICB treatment in both mouse and human tumours[195-197]. Therefore, the increased CXCL9 detected in MC38 TIF in our studies could in part originate from the increased macrophage presence in the tumour and contribute to recruitment of additional T and NK cells within the tumour. A comprehensive phenotyping of macrophage polarisation and function within DNT-treated tumours is necessary to determine if the increased macrophage numbers seen here are contributing more to anti- or pro-tumour immune responses. Additionally, understanding the source of chemokine production within the tumour could provide further information on which cells are being acted on by DNT/IFNγ to generate the associated immune recruitment.

This data positions DNT1s as unconventional innate T cells with dual effector and immunomodulatory functions. They share cytotoxic properties with $CD8^+$ T cells[198], but are distinguished by their potent innate-like responsiveness to cytokine stimulation and high expression of NK receptors such as NKG2D and DNAM1, likely enabling recognition of stress ligands independent of classical antigen presentation[41]. This is particularly relevant in tumours that downregulate MHC molecules, a common mechanism of immune evasion, as DNT1s may remain capable of effector activity in these contexts, as has been demonstrated by other innate T cells[199]. In the adoptive transfer setting, it is also important to consider that transferred DNT1 cells may enter an inhibitory checkpoint axis within the TME. Although DNT1 are characterised here as highly IFN-γ–competent effectors, activated tumour-infiltrating T cells commonly upregulate inhibitory receptors such as PD-1, TIGIT, LAG-3, TIM-3 which can inhibit a cytokine production and a sustained anti-tumor response[200,201]. Characterisation of immune checkpoint expression and activation within the endogenous and adoptively transferred DNTs is thus required for future studies to understand the potential utility of ICB in boosting and/or sustaining the effect of DNT-mediated immunotherapy.

Although, RORγt^{+} DNT17 are at very low frequencies within the examined tumours, understanding the effect of these cells in other cancer contexts could be beneficial, given the known pro-tumour effect of IL-17 producing cells in promoting angiogenesis and tumour metastasis[202].

Overall, we identify DNT1 cells as a dominant and functionally distinct unconventional T cell subset in tumours, capable of both direct cytotoxicity and reshaping the TME through chemokine-driven immune recruitment. However, the precise developmental origin of DNT1s in the tumour setting, their antigenic specificity, and their long-term persistence after adoptive transfer remain undefined. Expanded human DNT cells, although not separated from other unconventional populations, have been shown to be potently cytotoxic, exhibit donor-unrestricted recognition, and have proved positive in early clinical testing for adoptive therapy in acute myeloid leukaemia and other cancers[203,204]. The data here suggests potential benefit of DNT1-specific adoptive therapy if the enhanced cytotoxic capacity of DNT1 is also observed in humans. Future work should focus on identifying the potential for clonal expansion and/or specific tumour antigens recognised by the DNTs in this setting. Additionally, assessing synergy of DNT adoptive transfer with checkpoint blockade could prove advantageous, given their role in driving a more immunogenic tumour.

Chapter 6:

Overall discussion

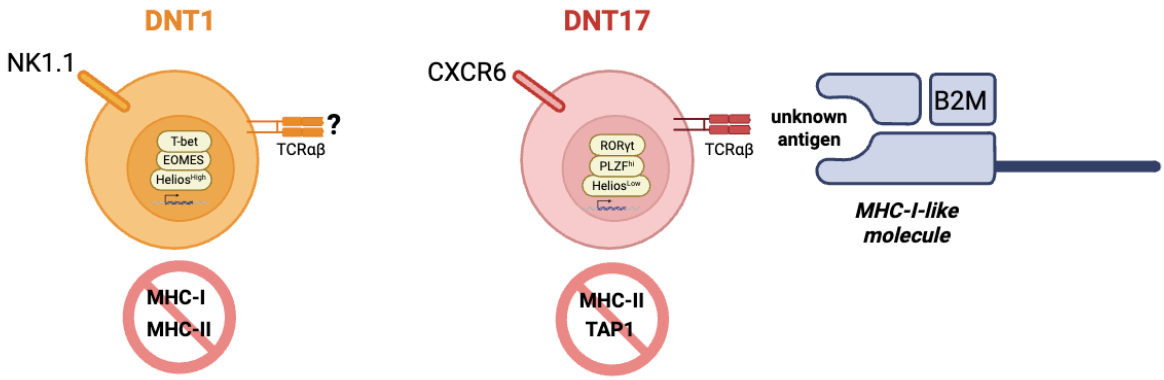
6.1 Introduction

Thus far, DNTs have not received the same level of recognition as other unconventional T cells but have been demonstrated to play important regulatory and inflammatory roles in certain settings. However, based on the lack of a cell type specific marker to identify DNTs, many of these studies gated TCR$\alpha\beta$+ CD4$^-$, CD8$^-$ negative cells and may have neglected to remove MAITs and iNKTs from the analysis. A primary goal of this research project was to define gating strategies and deeply characterise DNT cells in the mouse to better understand the functional classification of these cells, which separates them from other known unconventional subsets, and to provide a framework from which future studies can be derived. Sc-RNA sequencing identified seven distinct DNT clusters in the spleen, with five overlapping in the adipose. Previous research by Yang et al., provides a thorough transcriptomic overview of naïve and activated splenic DNTs, highlighting similarly high expression of *Ikzf2* and innate/cytotoxic subsets that parallel our findings, whilst lacking the *Klrb1c* (NK1.1$^+$) population[141]. Our study has provided additional functional confirmation of DNT transcriptional identifiers and subsetted the DNTs across mouse organs, identifying a fluid expression of various type-1 and type-17 markers that could collectively be described as DNT-1 and DNT-17. Functionally, the DNTs also exhibit innate-like rapid responsiveness to cytokine stimuli, unlike their conventional counterparts.

6.2 Divergence from other T cell lineages

Despite the innate features of DNT cells, they paradoxically have diverse TCR usage with no dominant clonotype in spleen or adipose, more akin with that of conventional T cells. This challenges the assumption that innate-like programming is necessarily coupled to limited TCR diversity and suggests that broad receptor diversity can coexist with stable effector bias which could be advantageous in environments where both antigen specificity and rapid responsiveness are required. How such agonist selection could be conferred within the thymus to mediate both effector readiness and TCR diversity is yet to be seen. MHC-independent $\alpha\beta$ and $\gamma\delta$ T cells have been reported before[205], but subtype specific dependence on β2M-mediated selection on DNTs is a

novel insight and signifies alternative lineages within the thymus for the DNT pool. Despite, the lack of apparent MHC restriction of DNT1 cells in steady-state, there are some reports of MHC-II restricted DNTs in disease. Mou et al., report that MHC-II restricted DNTs extensively proliferate in the parasitic induced disease, Leishmania major[107]. Potentially indicating CD4-derived DNTs in certain contexts that promote co-receptor downregulation. However, previous research has also reported MHC-independent TCR$\alpha\beta^+$ T cells possessing antibody-like recognition specificities, being able to directly recognise peptides via their TCR without the need of an intermediary antigen presenting molecule[206,207]. Similar observations have been made for $\gamma\delta$ T cells, whereby their TCR can sense conformational changes on protein receptors such as Skint and butryphillin molecules to mount a more targeted effector response[4]. Therefore, similar mechanisms of direct TCR recognition could be adapted by the MHC-I/II independent type-1 DNTs. Additionally, DNT1 highly express a number of NK cell receptors, so may also be more dependent on NK receptor-mediated MHC licencing[208], and responsivity to stress-induced ligands as an alternative activation means.



48 **Figure 6.1***:* DNT1 are MHC-I/II-independent while DNT-17 require a B2M-supported restriction molecule for thymic selection

Conversely, DNT17 require selection and presentation by a β2M-supported molecule, likely of the MHC-I family. The redundance of Tap1, and thus MHC-I protein processing machinery, in their development indicates the likelihood of DNT17 specificity towards a non-peptide antigen presented by an MHC-I-like molecule. Furthermore, the population is unchanged in *MR1-/-* and *CD1d-/-* mice, which poses an interesting question of which presentation molecule is directing the selection of

DNT17 in the thymus. Other known MHC-Ib molecules such as H2-M3/5 and QA-1/2 are more so associated with type 1 responders such as NK cells, and unconventional CD8 T cells and NK cells (**Table 6.1).** Moreover, they all share partial or complete reliance on Tap1 mediated peptide packaging and transport to the cell surface and therefore are unlikely candidates for DNT17 presentation[158,209-211]. However, there are other proteins which possess β2M-binding grooves but do not have clearly defined antigen presenting roles[212-214]. This suggests the need to explore both these molecules and the wider proteome for other potential restriction elements that necessitate β2M for surface expression that may play a role in selecting for DNT-17 cells in the thymus.

Molecule	Species	Antigen	Classified as	Tap1-dependant	B2M-dependant	Notes
MR1	Mo/Hu	Microbial metabolites	MHC-I-like	**NO**	YES	ER & endosomal trafficking
CD1d	Mo/Hu	glycolipids	MHC-I-like	**NO**	YES	Endolysosomal loading
CD1a-c	Hu	lipids	MHC-I-like	**NO**	YES	Presents to lipid-reactive T cells
CD1e	Hu	Lipid processing chaperone	MHC-I-like	**NO**	YES	Non-surface, helps process lipids for loading
H2-M3	Mo	N-formyl bacterial peptides	MHC-1b	YES (partial)	YES	Tap helps transport n-formyl peptides presents to CD8 in bacterial infection
H2-M5	Mo	N-formyl bacterial peptides	MHC-1b	Likely	YES	Less studied than H2-M3
Qa-1/HLA-E	Mo/Hu	MHC-I signal peptides	MHC-1b	YES (partial)	YES	Presents signal peptides to NK/CD8s (NKG2 receptors and/or TCR)
Qa-2 / Qa-3	Mo	Diverse less described peptides	MHC-1b	Likely	YES	Probably NK-interacting
T10/T22	Mo	Unknown - not a peptide	MHC-1b	**NO**	YES	Recognised by ydTs, non-polymorphic
TL	Mo	Thymus leukaemia antigen	MHC-1b	**NO**	YES	Expressed in thymus & gut epithelium – interacts with ydTs
HLA-G / HLA-5	Hu	Stress ligands	MHC-1b	YES	YES	NK interacting
ZAG (zinc-α2-glycoprotein)	Mo	No known role in antigen presenting	MHC-1-fold	**NO**	**NO**	Secreted, role in lipid metabolism, no binding groove for B2M
HFE	Mo	No clear role in antigen presenting	MHC-1 fold	**NO**	YES	Iron metabolism regulator, interacts with transferrin receptor

Table 6.1: Unconventional restriction molecules for antigen presentation & selection

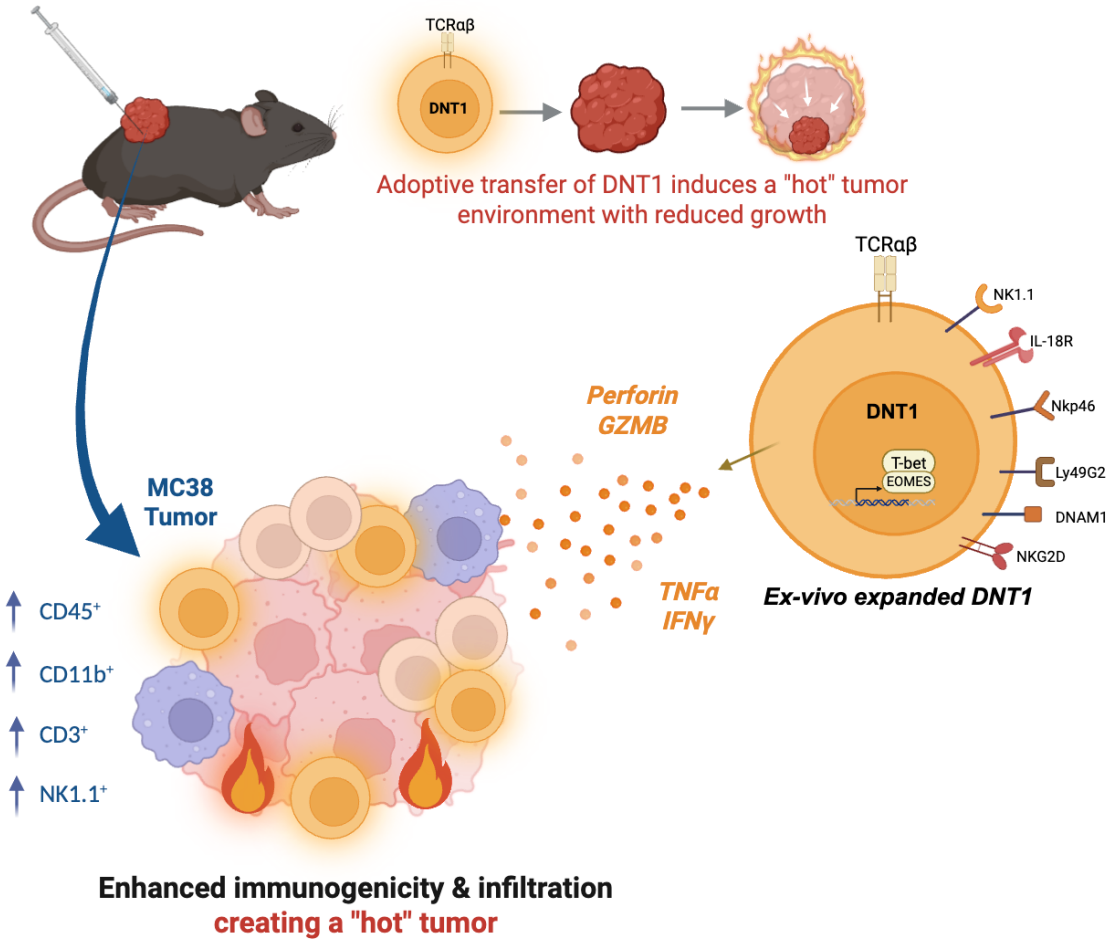
Thus, to advance research efforts into understanding DNT selection and produce a means of positively selecting the population from heterogenous T cell pools, identification of their restriction molecule and ligands are paramount. Conferring murine DNT phenotypes to human relevance becomes complicated as human conventional T cells can downregulate co-receptor expression upon activation, as demonstrated in-vitro by Tsokos et al[80]. Therefore, a better understanding of their TCR-restriction, will also be critical to facilitating a smoother translation of DNTs to human studies and examining potential presence or enrichment of the population across species and organs.

6.3 Type-1 DNT cells as mediators of anti-tumour immunity

In cancer immunity, unconventional T cells are increasingly recognised for their roles in shaping immune surveillance and tumour control. MAIT, iNKT, and γδ T cells have been implicated in early immune responses against transformed cells, particularly in mucosal and barrier tissues where innate surveillance is critical. These cells can rapidly produce IFNγ, TNFα, or IL-17, to modulate the cellular environment in the tumour, and in some cases exert direct cytotoxicity against tumour cells. Their capacity for TCR-independent activation makes them attractive for therapeutic manipulation in immunologically "cold" tumours that lack strong antigen-specific responses[50]. As such, innate T cells have been shown to synergise with checkpoint blockade, enhance vaccine responses, and mediate anti-tumour effects through adoptive transfer[172]. However, the clinical translation of these insights remains in early stages, and a broader understanding of the unconventional T cell landscape is essential to fully leverage their potential.

Here we show that DNT1 cells are capable tumour infiltrators and make up a significant proportion of the innate T cell pool in several solid tumours from breast, melanoma and colorectal syngeneic lines. Both host and adoptively transferred DNTs are strong double-producers of TNFα and IFNγ. Both cytokines are known to cooperatively promote an immunogenic environment that enables immune cells to better recognise and kill tumour cells[179]. TNFα mediates this in part via induction of apoptotic signalling, and a remodelling of the tumour vasculature to promote immune cell entry, whilst IFNγ induces a proinflammatory environment that supports cytotoxic lymphocyte and NK cell recruitment[215-217]. TIF analysis suggests that DNT-produced IFNγ/TNFα are modulating the MC38 TME in a similar capacity, as evidenced by increased chemokine concentration within the tumour. This inflammatory cytokine/chemokine storm in turn likely recruits an unbiased and mixed population of leukocytes. However, the increased immune cell populations within the tumour may also be attributed to increased proliferation of tumour infiltrating leukocytes, which requires further investigation. IFNγ has been shown to enhance T cell proliferation indirectly through induction of IL-15 and IL-12 by antigen-presenting cells, thereby sustaining effector and memory T-cell pools. TNFα, via TNFR2 signalling, can directly drive proliferation

of activated T cells, supporting their expansion during immune response[218-220]. Additionally, modest changes to the DC landscape in the dLN, skew the population towards XCR1+ cDC1s, which are known to promote cross-presentation to CTLs and are critical to a sustained anti-tumour response. Although, there have been previous reports of regulatory DNT cells in the past, we do not observe that in this setting. It's possible DNT1 could provide regulatory functions in certain settings given they can produce IL-10, but here we polarise them towards IFNγ production in the tumour model. It's also possible the less abundant EOMEs^{+} T-bet^{-} DNT population could possess a more regulatory phenotype and be expanded in specific contexts. Overall, in our work, DNT1s possess multi-modal means of adjusting the TME, however more work is needed to fully elucidate the mechanisms by which they confer an anti-tumour response.



49 **Figure 6.2:** Adoptive transfer of DNT1 results in a more immunogenic "hot" tumour microenvironment

6.4 Study limitations and future work

Due to the lack of a positive identification marker for DNTs to separate them from other T cell populations, we are unable to target DNTs directly in-vivo to delineate DNT-specific roles via depletion or knockout models. We instead approached this through ex-vivo expansion and adoptive transfer. This allowed us to determine how DNTs can modulate the TME but limits the extent by which we can differentiate indirect and direct mechanisms of DNT-induced anti-tumour immunity. We have highlighted a pro-inflammatory role for DNTs in mediating a more immunogenic response in MC38. Although the high expression of IFNγ and cytotoxic proteins by DNTs, and subsequent increased chemokine concentrations and associated immune cells recruitment within the TME are key contributors to this process, there are likely multi-modal means by which DNTs confer direct and indirect anti-tumour responses. In many ways, DNT1 are acting as a potent adjuvant in the TME, promoting a “hot” tumour and generating an increased immune cell milieu in an untargeted fashion. This points to the potential benefits of combining DNT-adoptive therapy with ICB to maintain a sustained immune response upon immune recruitment to the tumour. However, within the scope of this study, our aims are preliminarily to understand how DNTs affect or transform the TME, rather than optimising an immune-cell therapy. Nevertheless, it provides key information for future translational utility of these cells for that purpose, as well as significant groundwork for future studies in examining their impact on the TME.

Whilst we have provided evidence of unique selection requirements for the DNTs in the thymus, we have not identified their specific restriction molecule(s). Due to the MHC-independent nature of DNT1 and the lack of obvious selection candidates for DNT17, to answer this question would be beyond the scope of the current study. However, we believe further work in this area is critical to allow for positive-marker detection of DNTs and interrogate DNT-specific roles in tissue homeostasis and disease states, as well as for accurately translating DNT phenotypes in human studies. Additionally, the superior innate ability of DNT cells to produce more IFNγ compared to other T cells, as well as anti-viral transcriptional programs observed in sc-seq, positions them as potentially important players in host defence to bacterial and viral infections. Further work in this area could leverage their rapid response kinetics and robust cytokine production to modulate early responses to foreign pathogens.

DNT17 pose distinct challenges compared with DNT1 because their development relies on a β2m-dependent and Tap1-independent presentation pathway. This implies selection via a non-classical β2m-supported molecule whose identity and ligand preferences remains undefined. Without a known restriction element, it is difficult to positively identify DNT17 from other T cell subsets. Therefore, future work should prioritise screening for potential restriction molecules and subsequent probing of selected candidates in knockout mouse models, to assess impact on DNT17 populations in the periphery. In addition, IL-17–skewed lymphocytes can often show context-dependent biology. In homeostasis they can support barrier integrity, and microbial and metabolic balance[48,221], whereas in cancer, IL-17-associated inflammation can skew towards pro-tumour programs via modulation of the myeloid compartment, suppression of anti-tumour immunity and promotion of angiogenesis[202,222]. Although DNT17 populations were largely absent in the Yumm1.7, B16 and EO771 tumour models in this study, future work should investigate the role of DNT17 across alternative tumour models, as well as their contribution to known homeostatic IL-17-driven modalities across tissues.

Whilst human DNTs have been reported across tissues and disease settings, classifying DNTs in humans is complicated by the fact that conventional CD4 or CD8 T cells can significantly downregulate coreceptor expression upon activation[223], creating phenotypic DNT-like cells that may not represent a developmentally distinct lineage. Despite this, there has been translational interest in DNTs as cell therapy platforms for immunotherapy, including CAR-engineered DNTs[174,175], but these studies do not clearly distinguish between unconventional T cell populations and are therefore difficult to interpret. Therefore, accurately translating the murine DNT1/DNT17 frameworks to humans will first require establishing robust markers that distinguish true human “innate-like” DNT lineages from activation-derived DNTs. This motivates the need for deeper definition using restriction elements and antigen responsivity rather than CD4/CD8 negativity alone.

6.5 Conclusion

Overall, this study demonstrates the importance of including the appropriate markers to accurately identify and examine the remaining unexplored unconventional T cell populations, with NK1.1^{+} DNTs being the dominant subtype and largely overlooked in DNT literature. DNT1s are capable infiltrators of multiple syngeneic tumour models and have high cytotoxic action and ability to modulate the TME in MC38. Our data suggests a distinct lineage and selection requirement for both DNT-1 and DNT-17 cells in the thymus, that distinguishes both subtypes from other innate and adaptive T cells. Additionally, we suggest the DNTs are important members of the T cell niche, with widespread distribution across organs, subset specialisation and a clear ability to compensate for the loss of other T cells within multiple tissue compartments. DNT prevalence in both naïve and disease settings, as well as their robust cytokine responses, position them as underappreciated players in innate T cell immunity.

Chapter 7:
Bibliography

1 Hackstein, C. P. & Klenerman, P. MAITs and their mates: "Innate-like" behaviors in conventional and unconventional T cells. *Clin Exp Immunol* **213**, 1-9 (2023). https://doi.org:10.1093/cei/uxad058

2 Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J. & Moody, D. B. The burgeoning family of unconventional T cells. *Nat Immunol* **16**, 1114-1123 (2015). https://doi.org:10.1038/ni.3298

3 Chien, Y. H. & Konigshofer, Y. Antigen recognition by gammadelta T cells. *Immunol Rev* **215**, 46-58 (2007). https://doi.org:10.1111/j.1600-065X.2006.00470.x

4 Herrmann, T. & Karunakaran, M. M. Butyrophilins: gammadelta T Cell Receptor Ligands, Immunomodulators and More. *Front Immunol* **13**, 876493 (2022). https://doi.org:10.3389/fimmu.2022.876493

5 Rigau, M. *et al.* Butyrophilin 2A1 is essential for phosphoantigen reactivity by gammadelta T cells. *Science* **367** (2020). https://doi.org:10.1126/science.aay5516

6 Witherden, D. A. & Havran, W. L. EPCR: a stress trigger for gammadelta T cells. *Nat Immunol* **13**, 812-814 (2012). https://doi.org:10.1038/ni.2398

7 Van Rhijn, I. & Le Nours, J. CD1 and MR1 recognition by human gammadelta T cells. *Mol Immunol* **133**, 95-100 (2021). https://doi.org:10.1016/j.molimm.2020.12.008

8 Lee, M. *et al.* Single-cell RNA sequencing identifies shared differentiation paths of mouse thymic innate T cells. *Nat Commun* **11**, 4367 (2020). https://doi.org:10.1038/s41467-020-18155-8

9 Constantinides, M. G. & Belkaid, Y. Early-life imprinting of unconventional T cells and tissue homeostasis. *Science* **374**, eabf0095 (2021). https://doi.org:10.1126/science.abf0095

10 Li, J., Xiao, C., Li, C. & He, J. Tissue-resident immune cells: from defining characteristics to roles in diseases. *Signal Transduct Target Ther* **10**, 12 (2025). https://doi.org:10.1038/s41392-024-02050-5

11 Holtmeier, W. & Kabelitz, D. gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy* **86**, 151-183 (2005). https://doi.org:10.1159/000086659

12 Martina, M. N., Noel, S., Saxena, A., Rabb, H. & Hamad, A. R. Double negative (DN) alphabeta T cells: misperception and overdue recognition. *Immunol Cell Biol* **93**, 305-310 (2015). https://doi.org:10.1038/icb.2014.99

13 Ashby, K. M. & Hogquist, K. A. A guide to thymic selection of T cells. *Nat Rev Immunol* **24**, 103-117 (2024). https://doi.org:10.1038/s41577-023-00911-8

14 Winter, S. J. & Krueger, A. Development of Unconventional T Cells Controlled by MicroRNA. *Front Immunol* **10**, 2520 (2019). https://doi.org:10.3389/fimmu.2019.02520

15 Takahama, Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* **6**, 127-135 (2006). https://doi.org:10.1038/nri1781

16 Wang, H. & Zuniga-Pflucker, J. C. Thymic Microenvironment: Interactions Between Innate Immune Cells and Developing Thymocytes. *Front Immunol* **13**, 885280 (2022). https://doi.org:10.3389/fimmu.2022.885280

17 Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* **14**, 377-391 (2014). https://doi.org:10.1038/nri3667

18 Hewitt, E. W. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* **110**, 163-169 (2003). https://doi.org:10.1046/j.1365-2567.2003.01738.x

19 Maccari, M. E. *et al.* A distinct CD38+CD45RA+ population of CD4+, CD8+, and double-negative T cells is controlled by FAS. *J Exp Med* **218** (2021). https://doi.org:10.1084/jem.20192191

20 Zuniga-Pflucker, J. C., Longo, D. L. & Kruisbeek, A. M. Positive selection of CD4-CD8+ T cells in the thymus of normal mice. *Nature* **338**, 76-78 (1989). https://doi.org:10.1038/338076a0

21 Jayawardena-Wolf, J., Benlagha, K., Chiu, Y. H., Mehr, R. & Bendelac, A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* **15**, 897-908 (2001). https://doi.org:10.1016/s1074-7613(01)00240-0

22 Huang, S. *et al.* MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells. *J Exp Med* **205**, 1201-1211 (2008). https://doi.org:10.1084/jem.20072579

23 Parker, M. E. & Ciofani, M. Regulation of gammadelta T Cell Effector Diversification in the Thymus. *Front Immunol* **11**, 42 (2020). https://doi.org:10.3389/fimmu.2020.00042

24 Boehme, L., Roels, J. & Taghon, T. Development of gammadelta T cells in the thymus - A human perspective. *Semin Immunol* **61-64**, 101662 (2022). https://doi.org:10.1016/j.smim.2022.101662

25 Hu, Y. *et al.* gammadelta T cells: origin and fate, subsets, diseases and immunotherapy. *Signal Transduct Target Ther* **8**, 434 (2023). https://doi.org:10.1038/s41392-023-01653-8

26 Cosway, E. J., James, K. D., Lucas, B., Anderson, G. & White, A. J. The thymus medulla and its control of alphabetaT cell development. *Semin Immunopathol* **43**, 15-27 (2021). https://doi.org:10.1007/s00281-020-00830-z

27 Kurioka, A. & Klenerman, P. Aging unconventionally: gammadelta T cells, iNKT cells, and MAIT cells in aging. *Semin Immunol* **69**, 101816 (2023). https://doi.org:10.1016/j.smim.2023.101816

28 Munoz-Ruiz, M. *et al.* TCR signal strength controls thymic differentiation of discrete proinflammatory gammadelta T cell subsets. *Nat Immunol* **17**, 721-727 (2016). https://doi.org:10.1038/ni.3424

29 Sumaria, N., Grandjean, C. L., Silva-Santos, B. & Pennington, D. J. Strong TCRgammadelta Signaling Prohibits Thymic Development of IL-17A-Secreting gammadelta T Cells. *Cell Rep* **19**, 2469-2476 (2017). https://doi.org:10.1016/j.celrep.2017.05.071

30 Buttrum, L. *et al.* Both Major Histocompatibility Complex Class I (MHC-I) and MHC-II Molecules Are Required, while MHC-I Appears To Play a Critical Role in Host Defense against Primary Coxiella burnetii Infection. *Infect Immun* **86** (2018). https://doi.org:10.1128/IAI.00602-17

31 Nonaka, S. *et al.* Intestinal gamma delta T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles. *J Immunol* **174**, 1906-1912 (2005). https://doi.org:10.4049/jimmunol.174.4.1906

32 Pellicci, D. G., Koay, H. F. & Berzins, S. P. Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge. *Nat Rev Immunol* **20**, 756-770 (2020). https://doi.org:10.1038/s41577-020-0345-y

33 Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C. & Hogquist, K. A. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* **14**, 1146-1154 (2013). https://doi.org:10.1038/ni.2731

34 Lopes, N. *et al.* Distinct metabolic programs established in the thymus control effector functions of gammadelta T cell subsets in tumor microenvironments. *Nat Immunol* **22**, 179-192 (2021). https://doi.org:10.1038/s41590-020-00848-3

35 Koay, H. F. *et al.* A divergent transcriptional landscape underpins the development and functional branching of MAIT cells. *Sci Immunol* **4** (2019). https://doi.org:10.1126/sciimmunol.aay6039

36 Cowan, J. E. *et al.* Differential requirement for CCR4 and CCR7 during the development of innate and adaptive alphabetaT cells in the adult thymus. *J Immunol* **193**, 1204-1212 (2014). https://doi.org:10.4049/jimmunol.1400993

37 Gray, E. E., Suzuki, K. & Cyster, J. G. Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol* **186**, 6091-6095 (2011). https://doi.org:10.4049/jimmunol.1100427

38 Kakugawa, K. *et al.* Essential Roles of SATB1 in Specifying T Lymphocyte Subsets. *Cell Rep* **19**, 1176-1188 (2017). https://doi.org:10.1016/j.celrep.2017.04.038

39 Correia, D. V., Lopes, A. & Silva-Santos, B. Tumor cell recognition by gammadelta T lymphocytes: T-cell receptor vs. NK-cell receptors. *Oncoimmunology* **2**, e22892 (2013). https://doi.org:10.4161/onci.22892

40 Das, R., Sant'Angelo, D. B. & Nichols, K. E. Transcriptional control of invariant NKT cell development. *Immunol Rev* **238**, 195-215 (2010). https://doi.org:10.1111/j.1600-065X.2010.00962.x

41 Raulet, D. H. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* **3**, 781-790 (2003). https://doi.org:10.1038/nri1199

42 Seth, S. *et al.* The murine pan T cell marker CD96 is an adhesion receptor for CD155 and nectin-1. *Biochem Biophys Res Commun* **364**, 959-965 (2007). https://doi.org:10.1016/j.bbrc.2007.10.102

43 Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K. & Kershaw, M. H. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* **89**, 216-224 (2011). https://doi.org:10.1038/icb.2010.78

44 Amini, A., Pang, D., Hackstein, C. P. & Klenerman, P. MAIT Cells in Barrier Tissues: Lessons from Immediate Neighbors. *Front Immunol* **11**, 584521 (2020). https://doi.org:10.3389/fimmu.2020.584521

45 Brennan, P. J., Brigl, M. & Brenner, M. B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* **13**, 101-117 (2013). https://doi.org:10.1038/nri3369

46 Schipper, H. S. *et al.* Natural killer T cells in adipose tissue prevent insulin resistance. *J Clin Invest* **122**, 3343-3354 (2012). https://doi.org:10.1172/JCI62739

47 Clancy-Thompson, E. *et al.* Monoclonal Invariant NKT (iNKT) Cell Mice Reveal a Role for Both Tissue of Origin and the TCR in Development of iNKT Functional Subsets. *J Immunol* **199**, 159-171 (2017). https://doi.org:10.4049/jimmunol.1700214

48 Douglas, A. *et al.* Rhythmic IL-17 production by gammadelta T cells maintains adipose de novo lipogenesis. *Nature* **636**, 206-214 (2024). https://doi.org:10.1038/s41586-024-08131-3

49 Jeffery, H. C. *et al.* Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J Hepatol* **64**, 1118-1127 (2016). https://doi.org:10.1016/j.jhep.2015.12.017

50 Pinco, P. & Facciotti, F. Unconventional T Cells' Role in Cancer: Unlocking Their Hidden Potential to Guide Tumor Immunity and Therapy. *Cells* **14** (2025). https://doi.org:10.3390/cells14100720

51 Liu, Z. *et al.* Protective immunosurveillance and therapeutic antitumor activity of gammadelta T cells demonstrated in a mouse model of prostate cancer. *J Immunol* **180**, 6044-6053 (2008). https://doi.org:10.4049/jimmunol.180.9.6044

52 Morikawa, R. *et al.* Intraepithelial Lymphocytes Suppress Intestinal Tumor Growth by Cell-to-Cell Contact via CD103/E-Cadherin Signal. *Cell Mol Gastroenterol Hepatol* **11**, 1483-1503 (2021). https://doi.org:10.1016/j.jcmgh.2021.01.014

53 Girardi, M. *et al.* Regulation of cutaneous malignancy by gammadelta T cells. *Science* **294**, 605-609 (2001). https://doi.org:10.1126/science.1063916

54 Gao, Y. *et al.* Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med* **198**, 433-442 (2003). https://doi.org:10.1084/jem.20030584

55 Schonefeldt, S. *et al.* The Diverse Roles of gammadelta T Cells in Cancer: From Rapid Immunity to Aggressive Lymphoma. *Cancers (Basel)* **13** (2021). https://doi.org:10.3390/cancers13246212

56 Medina, B. D. *et al.* Oncogenic kinase inhibition limits Batf3-dependent dendritic cell development and antitumor immunity. *J Exp Med* **216**, 1359-1376 (2019). https://doi.org:10.1084/jem.20180660

57 Petley, E. V. *et al.* MAIT cells regulate NK cell-mediated tumor immunity. *Nat Commun* **12**, 4746 (2021). https://doi.org:10.1038/s41467-021-25009-4

58 Ruf, B. *et al.* Activating Mucosal-Associated Invariant T Cells Induces a Broad Antitumor Response. *Cancer Immunol Res* **9**, 1024-1034 (2021). https://doi.org:10.1158/2326-6066.CIR-20-0925

59 Fujii, S. I. & Shimizu, K. Immune Networks and Therapeutic Targeting of iNKT Cells in Cancer. *Trends Immunol* **40**, 984-997 (2019). https://doi.org:10.1016/j.it.2019.09.008

60 Bae, E. A. *et al.* Activation of NKT Cells in an Anti-PD-1-Resistant Tumor Model Enhances Antitumor Immunity by Reinvigorating Exhausted CD8 T Cells. *Cancer Res* **78**, 5315-5326 (2018). https://doi.org:10.1158/0008-5472.CAN-18-0734

61 Janakiram, N. B. *et al.* Loss of natural killer T cells promotes pancreatic cancer in LSL-Kras(G12D/+) mice. *Immunology* **152**, 36-51 (2017). https://doi.org:10.1111/imm.12746

62 Zhang, Z. X., Yang, L., Young, K. J., DuTemple, B. & Zhang, L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med* **6**, 782-789 (2000). https://doi.org:10.1038/77513

63 Fischer, K. *et al.* Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8- double-negative regulatory T cells. *Blood* **105**, 2828-2835 (2005). https://doi.org:10.1182/blood-2004-07-2583

64 Ascon, D. B. *et al.* Normal mouse kidneys contain activated and CD3+CD4-CD8- double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol* **84**, 1400-1409 (2008). https://doi.org:10.1189/jlb.0907651

65 Martina, M. N. *et al.* Double-Negative alphabeta T Cells Are Early Responders to AKI and Are Found in Human Kidney. *J Am Soc Nephrol* **27**, 1113-1123 (2016). https://doi.org:10.1681/ASN.2014121214

66 Johansson, M. & Lycke, N. A unique population of extrathymically derived alpha beta TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. *J Immunol* **170**, 1659-1666 (2003). https://doi.org:10.4049/jimmunol.170.4.1659

67 Hamad, A. R. Analysis of gene profile, steady state proliferation and apoptosis of double-negative T cells in the periphery and gut epithelium provides new insights into the biological functions of the Fas pathway. *Immunol Res* **47**, 134-142 (2010). https://doi.org:10.1007/s12026-009-8144-3

68 Brandt, D. & Hedrich, C. M. TCRalphabeta(+)CD3(+)CD4(-)CD8(-) (double negative) T cells in autoimmunity. *Autoimmun Rev* **17**, 422-430 (2018). https://doi.org:10.1016/j.autrev.2018.02.001

69 Li, H. & Tsokos, G. C. Double-negative T cells in autoimmune diseases. *Curr Opin Rheumatol* **33**, 163-172 (2021). https://doi.org:10.1097/BOR.0000000000000778

70 Bafor, E. E., Valencia, J. C. & Young, H. A. Double Negative T Regulatory Cells: An Emerging Paradigm Shift in Reproductive Immune Tolerance? *Front Immunol* **13**, 886645 (2022). https://doi.org:10.3389/fimmu.2022.886645

71 Wu, Z. *et al.* CD3(+)CD4(-)CD8(-) (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer. *Front Immunol* **13**, 816005 (2022). https://doi.org:10.3389/fimmu.2022.816005

72 Morse, H. C., 3rd *et al.* Abnormalities induced by the mutant gene lpr: expansion of a unique lymphocyte subset. *J Immunol* **129**, 2612-2615 (1982).

73 Ihle, J. N. *et al.* Phenotypic characteristics of cell lines requiring interleukin 3 for growth. *J Immunol* **129**, 1377-1383 (1982).

74 Fisher, G. H. *et al.* Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* **81**, 935-946 (1995). https://doi.org:10.1016/0092-8674(95)90013-6

75 Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. & Nagata, S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**, 314-317 (1992). https://doi.org:10.1038/356314a0

76 Mohamood, A. S. *et al.* Fas-mediated apoptosis regulates the composition of peripheral alphabeta T cell repertoire by constitutively purging out double

negative T cells. *PLoS One* **3**, e3465 (2008). https://doi.org:10.1371/journal.pone.0003465

77 Li, H. *et al.* Systemic lupus erythematosus favors the generation of IL-17 producing double negative T cells. *Nat Commun* **11**, 2859 (2020). https://doi.org:10.1038/s41467-020-16636-4

78 Dean, G. S., Anand, A., Blofeld, A., Isenberg, D. A. & Lydyard, P. M. Characterization of CD3+ CD4- CD8- (double negative) T cells in patients with systemic lupus erythematosus: production of IL-4. *Lupus* **11**, 501-507 (2002). https://doi.org:10.1191/0961203302lu234oa

79 Alunno, A. *et al.* IL-17-producing double-negative T cells are expanded in the peripheral blood, infiltrate the salivary gland and are partially resistant to corticosteroid therapy in patients with Sjogren's syndrome. *Reumatismo* **65**, 192-198 (2013). https://doi.org:10.4081/reumatismo.2013.192

80 Crispin, J. C. *et al.* Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* **181**, 8761-8766 (2008). https://doi.org:10.4049/jimmunol.181.12.8761

81 Ford, M. S., Zhang, Z. X., Chen, W. & Zhang, L. Double-negative T regulatory cells can develop outside the thymus and do not mature from CD8+ T cell precursors. *J Immunol* **177**, 2803-2809 (2006). https://doi.org:10.4049/jimmunol.177.5.2803

82 Achita, P. *et al.* Infusion of ex-vivo expanded human TCR-alphabeta(+) double-negative regulatory T cells delays onset of xenogeneic graft-versus-host disease. *Clin Exp Immunol* **193**, 386-399 (2018). https://doi.org:10.1111/cei.13145

83 D'Alessio, F. R., Kurzhagen, J. T. & Rabb, H. Reparative T lymphocytes in organ injury. *J Clin Invest* **129**, 2608-2618 (2019). https://doi.org:10.1172/JCI124614

84 Tian, D. *et al.* Double negative T cells mediate Lag3-dependent antigen-specific protection in allergic asthma. *Nat Commun* **10**, 4246 (2019). https://doi.org:10.1038/s41467-019-12243-0

85 Anderson, G. & Jenkinson, E. J. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* **1**, 31-40 (2001). https://doi.org:10.1038/35095500

86 Chapman, J. C., Chapman, F. M. & Michael, S. D. The production of alpha/beta and gamma/delta double negative (DN) T-cells and their role in the maintenance of pregnancy. *Reprod Biol Endocrinol* **13**, 73 (2015). https://doi.org:10.1186/s12958-015-0073-5

87 Hayes, S. M., Li, L. & Love, P. E. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* **22**, 583-593 (2005). https://doi.org:10.1016/j.immuni.2005.03.014

88 Wang, R., Wang-Zhu, Y. & Grey, H. Interactions between double positive thymocytes and high affinity ligands presented by cortical epithelial cells generate double negative thymocytes with T cell regulatory activity. *Proc Natl Acad Sci U S A* **99**, 2181-2186 (2002). https://doi.org:10.1073/pnas.042692799

89 Rodriguez-Rodriguez, N. *et al.* TCR-alpha/beta CD4(-) CD8(-) double negative T cells arise from CD8(+) T cells. *J Leukoc Biol* **108**, 851-857 (2020). https://doi.org:10.1002/JLB.1AB0120-548R

90 Zhang, D. *et al.* New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. *Blood* **109**, 4071-4079 (2007). https://doi.org:10.1182/blood-2006-10-050625

91 Hillhouse, E. E., Beauchamp, C., Chabot-Roy, G., Dugas, V. & Lesage, S. Interleukin-10 limits the expansion of immunoregulatory CD4-CD8- T cells in autoimmune-prone non-obese diabetic mice. *Immunol Cell Biol* **88**, 771-780 (2010). https://doi.org:10.1038/icb.2010.84

92 Voelkl, S., Gary, R. & Mackensen, A. Characterization of the immunoregulatory function of human TCR-alphabeta+ CD4- CD8- double-negative T cells. *Eur J Immunol* **41**, 739-748 (2011). https://doi.org:10.1002/eji.201040982

93 Li, W. *et al.* Ex vivo converted double negative T cells suppress activated B cells. *Int Immunopharmacol* **20**, 164-169 (2014). https://doi.org:10.1016/j.intimp.2014.02.034

94 Gao, J. F. *et al.* Regulation of antigen-expressing dendritic cells by double negative regulatory T cells. *Eur J Immunol* **41**, 2699-2708 (2011). https://doi.org:10.1002/eji.201141428

95 Su, Y. *et al.* Double negative Treg cells promote nonmyeloablative bone marrow chimerism by inducing T-cell clonal deletion and suppressing NK cell

function. *Eur J Immunol* **42**, 1216-1225 (2012). https://doi.org:10.1002/eji.201141808

96 Hill, M. *et al.* Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. *Am J Transplant* **11**, 2036-2045 (2011). https://doi.org:10.1111/j.1600-6143.2011.03651.x

97 Ford McIntyre, M. S., Young, K. J., Gao, J., Joe, B. & Zhang, L. Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression. *J Immunol* **181**, 2271-2275 (2008). https://doi.org:10.4049/jimmunol.181.4.2271

98 He, K. M. *et al.* Donor double-negative Treg promote allogeneic mixed chimerism and tolerance. *Eur J Immunol* **37**, 3455-3466 (2007). https://doi.org:10.1002/eji.200737408

99 Dugas, V., Beauchamp, C., Chabot-Roy, G., Hillhouse, E. E. & Lesage, S. Implication of the CD47 pathway in autoimmune diabetes. *J Autoimmun* **35**, 23-32 (2010). https://doi.org:10.1016/j.jaut.2010.01.002

100 Nemoto, Y. *et al.* Intestinal CD4(-)CD8alphabeta(-)TCRalphabeta(+) T cells function as tolerogenic antigen presenting cells in mice. *Nat Commun* **16**, 7072 (2025). https://doi.org:10.1038/s41467-025-62089-y

101 Hillhouse, E. E. *et al.* Double-Negative T Cell Levels Correlate with Chronic Graft-versus-Host Disease Severity. *Biol Blood Marrow Transplant* **25**, 19-25 (2019). https://doi.org:10.1016/j.bbmt.2018.09.008

102 Young, K. J., DuTemple, B., Phillips, M. J. & Zhang, L. Inhibition of graft-versus-host disease by double-negative regulatory T cells. *J Immunol* **171**, 134-141 (2003). https://doi.org:10.4049/jimmunol.171.1.134

103 Sun, G. *et al.* CD4 derived double negative T cells prevent the development and progression of nonalcoholic steatohepatitis. *Nat Commun* **12**, 650 (2021). https://doi.org:10.1038/s41467-021-20941-x

104 Zhao, X. *et al.* A novel differentiation pathway from CD4(+) T cells to CD4(-) T cells for maintaining immune system homeostasis. *Cell Death Dis* **7**, e2193 (2016). https://doi.org:10.1038/cddis.2016.83

105 Lei, H. Y. & Chang, C. P. Lectin of Concanavalin A as an anti-hepatoma therapeutic agent. *J Biomed Sci* **16**, 10 (2009). https://doi.org:10.1186/1423-0127-16-10

106 Cowley, S. C., Meierovics, A. I., Frelinger, J. A., Iwakura, Y. & Elkins, K. L. Lung CD4-CD8- double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with Francisella tularensis live vaccine strain. *J Immunol* **184**, 5791-5801 (2010). https://doi.org:10.4049/jimmunol.1000362

107 Mou, Z. *et al.* MHC class II restricted innate-like double negative T cells contribute to optimal primary and secondary immunity to Leishmania major. *PLoS Pathog* **10**, e1004396 (2014). https://doi.org:10.1371/journal.ppat.1004396

108 Sundaravaradan, V. *et al.* Multifunctional double-negative T cells in sooty mangabeys mediate T-helper functions irrespective of SIV infection. *PLoS Pathog* **9**, e1003441 (2013). https://doi.org:10.1371/journal.ppat.1003441

109 Hall, M. *et al.* Expansion of tumor-infiltrating lymphocytes (TIL) from human pancreatic tumors. *J Immunother Cancer* **4**, 61 (2016). https://doi.org:10.1186/s40425-016-0164-7

110 Di Blasi, D. *et al.* Unique T-Cell Populations Define Immune-Inflamed Hepatocellular Carcinoma. *Cell Mol Gastroenterol Hepatol* **9**, 195-218 (2020). https://doi.org:10.1016/j.jcmgh.2019.08.004

111 Liu, Z. *et al.* Tumor-infiltrating lymphocytes (TILs) from patients with glioma. *Oncoimmunology* **6**, e1252894 (2017). https://doi.org:10.1080/2162402X.2016.1252894

112 Prins, R. M. *et al.* Characterization of defective CD4-CD8- T cells in murine tumors generated independent of antigen specificity. *J Immunol* **172**, 1602-1611 (2004). https://doi.org:10.4049/jimmunol.172.3.1602

113 Vallacchi, V. *et al.* Transcriptional profiling of melanoma sentinel nodes identify patients with poor outcome and reveal an association of CD30(+) T lymphocytes with progression. *Cancer Res* **74**, 130-140 (2014). https://doi.org:10.1158/0008-5472.CAN-13-1672

114 Ponzetta, A. *et al.* Neutrophils Driving Unconventional T Cells Mediate Resistance against Murine Sarcomas and Selected Human Tumors. *Cell* **178**, 346-360 e324 (2019). https://doi.org:10.1016/j.cell.2019.05.047

115 Miller, B. C. *et al.* Author Correction: Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol* **20**, 1556 (2019). https://doi.org:10.1038/s41590-019-0528-5

116 Godfrey, D. I., MacDonald, H. R., Kronenberg, M., Smyth, M. J. & Van Kaer, L. NKT cells: what's in a name? *Nat Rev Immunol* **4**, 231-237 (2004). https://doi.org:10.1038/nri1309

117 Rossjohn, J., Pellicci, D. G., Patel, O., Gapin, L. & Godfrey, D. I. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* **12**, 845-857 (2012). https://doi.org:10.1038/nri3328

118 Ussher, J. E., Klenerman, P. & Willberg, C. B. Mucosal-associated invariant T-cells: new players in anti-bacterial immunity. *Front Immunol* **5**, 450 (2014). https://doi.org:10.3389/fimmu.2014.00450

119 Akitsu, A. & Iwakura, Y. Interleukin-17-producing gammadelta T (gammadelta17) cells in inflammatory diseases. *Immunology* **155**, 418-426 (2018). https://doi.org:10.1111/imm.12993

120 O'Brien, R. L. & Born, W. K. Dermal gammadelta T cells--What have we learned? *Cell Immunol* **296**, 62-69 (2015). https://doi.org:10.1016/j.cellimm.2015.01.011

121 Mensurado, S. & Silva-Santos, B. Battle of the gammadelta T cell subsets in the gut. *Trends Cancer* **8**, 881-883 (2022). https://doi.org:10.1016/j.trecan.2022.08.006

122 Kabelitz, D., Kalyan, S., Oberg, H. H. & Wesch, D. Human Vdelta2 versus non-Vdelta2 gammadelta T cells in antitumor immunity. *Oncoimmunology* **2**, e23304 (2013). https://doi.org:10.4161/onci.23304

123 Bialy, S. & Bogunia-Kubik, K. Uncovering the mysteries of human gamma delta T cells: from origins to novel therapeutics. *Front Immunol* **16**, 1543454 (2025). https://doi.org:10.3389/fimmu.2025.1543454

124 Adams, E. J., Gu, S. & Luoma, A. M. Human gamma delta T cells: Evolution and ligand recognition. *Cell Immunol* **296**, 31-40 (2015). https://doi.org:10.1016/j.cellimm.2015.04.008

125 Takagaki, Y., DeCloux, A., Bonneville, M. & Tonegawa, S. Diversity of gamma delta T-cell receptors on murine intestinal intra-epithelial lymphocytes. *Nature* **339**, 712-714 (1989). https://doi.org:10.1038/339712a0

126 Gapin, L. Check MAIT. *J Immunol* **192**, 4475-4480 (2014). https://doi.org:10.4049/jimmunol.1400119

127 Fang, D. *et al.* Differential regulation of transcription factor T-bet induction during NK cell development and T helper-1 cell differentiation. *Immunity* **55**, 639-655 e637 (2022). https://doi.org:10.1016/j.immuni.2022.03.005

128 Lazarevic, V., Glimcher, L. H. & Lord, G. M. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol* **13**, 777-789 (2013). https://doi.org:10.1038/nri3536

129 Wang, L. *et al.* The multiple roles of interferon regulatory factor family in health and disease. *Signal Transduct Target Ther* **9**, 282 (2024). https://doi.org:10.1038/s41392-024-01980-4

130 Llao-Cid, L. *et al.* EOMES is essential for antitumor activity of CD8(+) T cells in chronic lymphocytic leukemia. *Leukemia* **35**, 3152-3162 (2021). https://doi.org:10.1038/s41375-021-01198-1

131 Wagner, J. A. *et al.* Stage-Specific Requirement for Eomes in Mature NK Cell Homeostasis and Cytotoxicity. *Cell Rep* **31**, 107720 (2020). https://doi.org:10.1016/j.celrep.2020.107720

132 Chen, Z. *et al.* Development of PVTX-405 as a potent and highly selective molecular glue degrader of IKZF2 for cancer immunotherapy. *Nat Commun* **16**, 4095 (2025). https://doi.org:10.1038/s41467-025-58431-z

133 Butcher, M. J., Wu, C. I., Waseem, T. & Galkina, E. V. CXCR6 regulates the recruitment of pro-inflammatory IL-17A-producing T cells into atherosclerotic aortas. *Int Immunol* **28**, 255-261 (2016). https://doi.org:10.1093/intimm/dxv068

134 Hou, L. & Yuki, K. CCR6 and CXCR6 Identify the Th17 Cells With Cytotoxicity in Experimental Autoimmune Encephalomyelitis. *Front Immunol* **13**, 819224 (2022). https://doi.org:10.3389/fimmu.2022.819224

135 Hou, S. *et al.* S100A4 protects mice from high-fat diet-induced obesity and inflammation. *Lab Invest* **98**, 1025-1038 (2018). https://doi.org:10.1038/s41374-018-0067-y

136 Krautbauer, S. *et al.* Annexin A6 regulates adipocyte lipid storage and adiponectin release. *Mol Cell Endocrinol* **439**, 419-430 (2017). https://doi.org:10.1016/j.mce.2016.09.033

137 Guo, L., Li, X. & Tang, Q. Q. Transcriptional regulation of adipocyte differentiation: a central role for CCAAT/enhancer-binding protein (C/EBP) beta. *J Biol Chem* **290**, 755-761 (2015). https://doi.org:10.1074/jbc.R114.619957

138 Fang, L. *et al.* Annexin A1 binds PDZ and LIM domain 7 to inhibit adipogenesis and prevent obesity. *Signal Transduct Target Ther* **9**, 218 (2024). https://doi.org:10.1038/s41392-024-01930-0

139 Qin, D. D. *et al.* NR4A1 retards adipocyte differentiation or maturation via enhancing GATA2 and p53 expression. *J Cell Mol Med* **22**, 4709-4720 (2018). https://doi.org:10.1111/jcmm.13715

140 Habibian, J. S. *et al.* DUSP5 functions as a feedback regulator of TNFalpha-induced ERK1/2 dephosphorylation and inflammatory gene expression in adipocytes. *Sci Rep* **7**, 12879 (2017). https://doi.org:10.1038/s41598-017-12861-y

141 Yang, L. *et al.* Transcriptome landscape of double negative T cells by single-cell RNA sequencing. *J Autoimmun* **121**, 102653 (2021). https://doi.org:10.1016/j.jaut.2021.102653

142 Sanz, M. *et al.* Deep characterization of human gammadelta T cell subsets defines shared and lineage-specific traits. *Front Immunol* **14**, 1148988 (2023). https://doi.org:10.3389/fimmu.2023.1148988

143 Bozinovski, S. *et al.* Innate cellular sources of interleukin-17A regulate macrophage accumulation in cigarette- smoke-induced lung inflammation in mice. *Clin Sci (Lond)* **129**, 785-796 (2015). https://doi.org:10.1042/CS20140703

144 Kim, H. J. *et al.* Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science* **350**, 334-339 (2015). https://doi.org:10.1126/science.aad0616

145 Chandra, S. & Kronenberg, M. Activation and Function of iNKT and MAIT Cells. *Adv Immunol* **127**, 145-201 (2015). https://doi.org:10.1016/bs.ai.2015.03.003

146 Fang, Y. *et al.* Biological functions and therapeutic applications of human mucosal-associated invariant T cells. *J Biomed Sci* **32**, 32 (2025). https://doi.org:10.1186/s12929-025-01125-x

147 Pasman, L. & Kasper, D. L. Building conventions for unconventional lymphocytes. *Immunol Rev* **279**, 52-62 (2017). https://doi.org:10.1111/imr.12576

148 Gutierrez-Arcelus, M. *et al.* Lymphocyte innateness defined by transcriptional states reflects a balance between proliferation and effector functions. *Nat Commun* **10**, 687 (2019). https://doi.org:10.1038/s41467-019-08604-4

149 Ascui, G. *et al.* Innateness transcriptome gradients characterize mouse T lymphocyte populations. *J Immunol* **214**, 223-237 (2025). https://doi.org:10.1093/jimmun/vkae015

150 Krovi, S. H., Loh, L., Spengler, A., Brunetti, T. & Gapin, L. Current insights in mouse iNKT and MAIT cell development using single cell transcriptomics data. *Semin Immunol* **60**, 101658 (2022). https://doi.org:10.1016/j.smim.2022.101658

151 Lu, Y., Cao, X., Zhang, X. & Kovalovsky, D. PLZF Controls the Development of Fetal-Derived IL-17+Vgamma6+ gammadelta T Cells. *J Immunol* **195**, 4273-4281 (2015). https://doi.org:10.4049/jimmunol.1500939

152 Barao, I. *et al.* Mouse Ly49G2+ NK cells dominate early responses during both immune reconstitution and activation independently of MHC. *Blood* **117**, 7032-7041 (2011). https://doi.org:10.1182/blood-2010-11-316653

153 Lusty, E. *et al.* IL-18/IL-15/IL-12 synergy induces elevated and prolonged IFN-gamma production by ex vivo expanded NK cells which is not due to enhanced STAT4 activation. *Mol Immunol* **88**, 138-147 (2017). https://doi.org:10.1016/j.molimm.2017.06.025

154 McVay, L. D. & Carding, S. R. Extrathymic origin of human gamma delta T cells during fetal development. *J Immunol* **157**, 2873-2882 (1996).

155 Laky, K., Lefrancois, L., von Freeden-Jeffry, U., Murray, R. & Puddington, L. The role of IL-7 in thymic and extrathymic development of TCR gamma delta cells. *J Immunol* **161**, 707-713 (1998).

156 Matsuzaki, G. *et al.* Extrathymic development of self-reactive gamma(delta) T cells in athymic BALB/c nu/nu mice. *Cell Immunol* **173**, 49-54 (1996). https://doi.org:10.1006/cimm.1996.0250

157 Li, L., Dong, M. & Wang, X. G. The Implication and Significance of Beta 2 Microglobulin: A Conservative Multifunctional Regulator. *Chin Med J (Engl)* **129**, 448-455 (2016). https://doi.org:10.4103/0366-6999.176084

158 Rodgers, J. R. & Cook, R. G. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5**, 459-471 (2005). https://doi.org:10.1038/nri1635

159 Xu, C. *et al.* Expansion of MAIT cells in the combined absence of NKT and gammadelta-T cells. *Mucosal Immunol* **16**, 446-461 (2023). https://doi.org:10.1016/j.mucimm.2023.05.003

160 Pamer, E. & Cresswell, P. Mechanisms of MHC class I--restricted antigen processing. *Annu Rev Immunol* **16**, 323-358 (1998). https://doi.org:10.1146/annurev.immunol.16.1.323

161 Ritz, U. & Seliger, B. The transporter associated with antigen processing (TAP): structural integrity, expression, function, and its clinical relevance. *Mol Med* **7**, 149-158 (2001).

162 Treiner, E. & Lantz, O. CD1d- and MR1-restricted invariant T cells: of mice and men. *Curr Opin Immunol* **18**, 519-526 (2006). https://doi.org:10.1016/j.coi.2006.07.001

163 Behar, S. M., Dascher, C. C., Grusby, M. J., Wang, C. R. & Brenner, M. B. Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *J Exp Med* **189**, 1973-1980 (1999). https://doi.org:10.1084/jem.189.12.1973

164 Nakagawa, H. *et al.* Instability of Helios-deficient Tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity. *Proc Natl Acad Sci U S A* **113**, 6248-6253 (2016). https://doi.org:10.1073/pnas.1604765113

165 Thornton, A. M. *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* **184**, 3433-3441 (2010). https://doi.org:10.4049/jimmunol.0904028

166 Lam, A. J., Uday, P., Gillies, J. K. & Levings, M. K. Helios is a marker, not a driver, of human Treg stability. *Eur J Immunol* **52**, 75-84 (2022). https://doi.org:10.1002/eji.202149318

167 Hetemaki, I. *et al.* Loss-of-function mutation in IKZF2 leads to immunodeficiency with dysregulated germinal center reactions and reduction of MAIT cells. *Sci Immunol* **6**, eabe3454 (2021). https://doi.org:10.1126/sciimmunol.abe3454

168 Peters, C., Oberg, H. H., Kabelitz, D. & Wesch, D. Phenotype and regulation of immunosuppressive Vdelta2-expressing gammadelta T cells. *Cell Mol Life Sci* **71**, 1943-1960 (2014). https://doi.org:10.1007/s00018-013-1467-1

169 Ross, S. H. & Cantrell, D. A. Signaling and Function of Interleukin-2 in T Lymphocytes. *Annu Rev Immunol* **36**, 411-433 (2018). https://doi.org:10.1146/annurev-immunol-042617-053352

170 Nakanishi, K. Unique Action of Interleukin-18 on T Cells and Other Immune Cells. *Front Immunol* **9**, 763 (2018). https://doi.org:10.3389/fimmu.2018.00763

171 Korman, A. J., Garrett-Thomson, S. C. & Lonberg, N. The foundations of immune checkpoint blockade and the ipilimumab approval decennial. *Nat Rev Drug Discov* **21**, 509-528 (2022). https://doi.org:10.1038/s41573-021-00345-8

172 Li, Y. R., Zhou, K., Zhu, Y., Halladay, T. & Yang, L. Breaking the mold: Unconventional T cells in cancer therapy. *Cancer Cell* **43**, 317-322 (2025). https://doi.org:10.1016/j.ccell.2024.11.010

173 Molgora, M. & Colonna, M. Innate-like T cells: A promising asset in anti-cancer immunity. *Cancer Cell* **40**, 714-716 (2022). https://doi.org:10.1016/j.ccell.2022.05.017

174 Vasic, D. *et al.* Allogeneic double-negative CAR-T cells inhibit tumor growth without off-tumor toxicities. *Sci Immunol* **7**, eabl3642 (2022). https://doi.org:10.1126/sciimmunol.abl3642

175 Tin, E. *et al.* Single-cell RNA sequencing of human double-negative T cells reveals a favorable cellular signature for cancer therapy. *J Immunother Cancer* **13** (2025). https://doi.org:10.1136/jitc-2024-010684

176 Zhou, X. *et al.* CAR-redirected natural killer T cells demonstrate superior antitumor activity to CAR-T cells through multimodal CD1d-dependent mechanisms. *Nat Cancer* **5**, 1607-1621 (2024). https://doi.org:10.1038/s43018-024-00830-0

177 Look, A., Burns, D., Tews, I., Roghanian, A. & Mansour, S. Towards a better understanding of human iNKT cell subpopulations for improved clinical outcomes. *Front Immunol* **14**, 1176724 (2023). https://doi.org:10.3389/fimmu.2023.1176724

178 Schiller, J. H., Bittner, G., Storer, B. & Willson, J. K. Synergistic antitumor effects of tumor necrosis factor and gamma-interferon on human colon carcinoma cell lines. *Cancer Res* **47**, 2809-2813 (1987).

179 Ohmori, Y., Schreiber, R. D. & Hamilton, T. A. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of

transcription 1 and nuclear factor kappaB. *J Biol Chem* **272**, 14899-14907 (1997). https://doi.org:10.1074/jbc.272.23.14899

180 Komita, H. *et al.* Interferon-gamma produced by interleukin-12-activated tumor infiltrating CD8+T cells directly induces apoptosis of mouse hepatocellular carcinoma. *J Hepatol* **45**, 662-672 (2006). https://doi.org:10.1016/j.jhep.2006.05.018

181 Berg, R. E., Cordes, C. J. & Forman, J. Contribution of CD8+ T cells to innate immunity: IFN-gamma secretion induced by IL-12 and IL-18. *Eur J Immunol* **32**, 2807-2816 (2002). https://doi.org:10.1002/1521-4141(2002010)32:10<2807::AID-IMMU2807>3.0.CO;2-0

182 Timperi, E. *et al.* IL-18 receptor marks functional CD8(+) T cells in non-small cell lung cancer. *Oncoimmunology* **6**, e1328337 (2017). https://doi.org:10.1080/2162402X.2017.1328337

183 Lin, L. *et al.* Ex vivo conditioning with IL-12 protects tumor-infiltrating CD8(+) T cells from negative regulation by local IFN-gamma. *Cancer Immunol Immunother* **68**, 395-405 (2019). https://doi.org:10.1007/s00262-018-2280-3

184 Gilfillan, S. *et al.* DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med* **205**, 2965-2973 (2008). https://doi.org:10.1084/jem.20081752

185 Del Prete, A. *et al.* Dendritic cell subsets in cancer immunity and tumor antigen sensing. *Cell Mol Immunol* **20**, 432-447 (2023). https://doi.org:10.1038/s41423-023-00990-6

186 Lee, A. H., Hong, J. H. & Seo, Y. S. Tumour necrosis factor-alpha and interferon-gamma synergistically activate the RANTES promoter through nuclear factor kappaB and interferon regulatory factor 1 (IRF-1) transcription factors. *Biochem J* **350 Pt 1**, 131-138 (2000).

187 Appay, V. & Rowland-Jones, S. L. RANTES: a versatile and controversial chemokine. *Trends Immunol* **22**, 83-87 (2001). https://doi.org:10.1016/s1471-4906(00)01812-3

188 Tokunaga, R. *et al.* CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy. *Cancer Treat Rev* **63**, 40-47 (2018). https://doi.org:10.1016/j.ctrv.2017.11.007

189 Wang, X. *et al.* The role of CXCR3 and its ligands in cancer. *Front Oncol* **12**, 1022688 (2022). https://doi.org:10.3389/fonc.2022.1022688

190 Dyck, L. *et al.* Suppressive effects of the obese tumor microenvironment on CD8 T cell infiltration and effector function. *J Exp Med* **219** (2022). https://doi.org:10.1084/jem.20210042

191 Ikeda, H., Old, L. J. & Schreiber, R. D. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* **13**, 95-109 (2002). https://doi.org:10.1016/s1359-6101(01)00038-7

192 Shen, J. *et al.* Anti-cancer therapy with TNFalpha and IFNgamma: A comprehensive review. *Cell Prolif* **51**, e12441 (2018). https://doi.org:10.1111/cpr.12441

193 Bottcher, J. P. & Reis e Sousa, C. The Role of Type 1 Conventional Dendritic Cells in Cancer Immunity. *Trends Cancer* **4**, 784-792 (2018). https://doi.org:10.1016/j.trecan.2018.09.001

194 Tosolini, M. *et al.* Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* **71**, 1263-1271 (2011). https://doi.org:10.1158/0008-5472.CAN-10-2907

195 Qu, Y. *et al.* Baseline Frequency of Inflammatory Cxcl9-Expressing Tumor-Associated Macrophages Predicts Response to Avelumab Treatment. *Cell Rep* **32**, 107873 (2020). https://doi.org:10.1016/j.celrep.2020.107873

196 House, I. G. *et al.* Macrophage-Derived CXCL9 and CXCL10 Are Required for Antitumor Immune Responses Following Immune Checkpoint Blockade. *Clin Cancer Res* **26**, 487-504 (2020). https://doi.org:10.1158/1078-0432.CCR-19-1868

197 Bill, R. *et al.* CXCL9:SPP1 macrophage polarity identifies a network of cellular programs that control human cancers. *Science* **381**, 515-524 (2023). https://doi.org:10.1126/science.ade2292

198 Lerner, E. C. *et al.* CD8(+) T cells maintain killing of MHC-I-negative tumor cells through the NKG2D-NKG2DL axis. *Nat Cancer* **4**, 1258-1272 (2023). https://doi.org:10.1038/s43018-023-00600-4

199 Taylor, B. C. & Balko, J. M. Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response. *Front Immunol* **13**, 844866 (2022). https://doi.org:10.3389/fimmu.2022.844866

200 Kraja, F. P. *et al.* Tumor-infiltrating lymphocytes in cancer immunotherapy: from chemotactic recruitment to translational modeling. *Front Immunol* **16**, 1601773 (2025). https://doi.org:10.3389/fimmu.2025.1601773

201 Joller, N., Anderson, A. C. & Kuchroo, V. K. LAG-3, TIM-3, and TIGIT: Distinct functions in immune regulation. *Immunity* **57**, 206-222 (2024). https://doi.org:10.1016/j.immuni.2024.01.010

202 Fabre, J. *et al.* Targeting the Tumor Microenvironment: The Protumor Effects of IL-17 Related to Cancer Type. *Int J Mol Sci* **17** (2016). https://doi.org:10.3390/ijms17091433

203 Lee, J. *et al.* Allogeneic Human Double Negative T Cells as a Novel Immunotherapy for Acute Myeloid Leukemia and Its Underlying Mechanisms. *Clin Cancer Res* **24**, 370-382 (2018). https://doi.org:10.1158/1078-0432.CCR-17-2228

204 Chen, X., Wang, D. & Zhu, X. Application of double-negative T cells in haematological malignancies: recent progress and future directions. *Biomark Res* **10**, 11 (2022). https://doi.org:10.1186/s40364-022-00360-w

205 Lu, J. *et al.* Structure of MHC-Independent TCRs and Their Recognition of Native Antigen CD155. *J Immunol* **204**, 3351-3359 (2020). https://doi.org:10.4049/jimmunol.1901084

206 Tikhonova, A. N. *et al.* alphabeta T cell receptors that do not undergo major histocompatibility complex-specific thymic selection possess antibody-like recognition specificities. *Immunity* **36**, 79-91 (2012). https://doi.org:10.1016/j.immuni.2011.11.013

207 Almeida, C. F. *et al.* Direct recognition of an intact foreign protein by an alphabeta T cell receptor. *Nat Commun* **15**, 8816 (2024). https://doi.org:10.1038/s41467-024-51897-3

208 Quatrini, L. *et al.* Human NK cells, their receptors and function. *Eur J Immunol* **51**, 1566-1579 (2021). https://doi.org:10.1002/eji.202049028

209 Robinson, P. J., Travers, P. J., Stackpoole, A., Flaherty, L. & Djaballah, H. Maturation of Qa-1b class I molecules requires beta 2-microglobulin but is TAP independent. *J Immunol* **160**, 3217-3224 (1998).

210 Sullivan, B. A., Reed-Loisel, L. M., Kersh, G. J. & Jensen, P. E. Homeostatic proliferation of a Qa-1b-restricted T cell: a distinction between the ligands required for positive selection and for proliferation in lymphopenic hosts. *J*

Immunol **173**, 6065-6071 (2004). https://doi.org:10.4049/jimmunol.173.10.6065

211 Manoharan Valerio, M. *et al.* The promiscuous development of an unconventional Qa1b-restricted T cell population. *Front Immunol* **14**, 1250316 (2023). https://doi.org:10.3389/fimmu.2023.1250316

212 de Almeida, S. F. *et al.* HFE cross-talks with the MHC class I antigen presentation pathway. *Blood* **106**, 971-977 (2005). https://doi.org:10.1182/blood-2004-12-4640

213 Rohrlich, P. S. *et al.* Direct recognition by alphabeta cytolytic T cells of Hfe, a MHC class Ib molecule without antigen-presenting function. *Proc Natl Acad Sci U S A* **102**, 12855-12860 (2005). https://doi.org:10.1073/pnas.0502309102

214 Sanchez, L. M., Chirino, A. J. & Bjorkman, P. Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. *Science* **283**, 1914-1919 (1999). https://doi.org:10.1126/science.283.5409.1914

215 Mercogliano, M. F., Bruni, S., Mauro, F., Elizalde, P. V. & Schillaci, R. Harnessing Tumor Necrosis Factor Alpha to Achieve Effective Cancer Immunotherapy. *Cancers (Basel)* **13** (2021). https://doi.org:10.3390/cancers13030564

216 Laha, D., Grant, R., Mishra, P. & Nilubol, N. The Role of Tumor Necrosis Factor in Manipulating the Immunological Response of Tumor Microenvironment. *Front Immunol* **12**, 656908 (2021). https://doi.org:10.3389/fimmu.2021.656908

217 Jorgovanovic, D., Song, M., Wang, L. & Zhang, Y. Roles of IFN-gamma in tumor progression and regression: a review. *Biomark Res* **8**, 49 (2020). https://doi.org:10.1186/s40364-020-00228-x

218 Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* **22**, 333-340 (2010). https://doi.org:10.1016/j.coi.2010.02.013

219 Mehta, A. K., Gracias, D. T. & Croft, M. TNF activity and T cells. *Cytokine* **101**, 14-18 (2018). https://doi.org:10.1016/j.cyto.2016.08.003

220 Avice, M. N. *et al.* IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-gamma

secretion by CD4+ T cells in the absence of TCR ligation. *J Immunol* **161**, 3408-3415 (1998).

221 Dubin, P. J. & Kolls, J. K. Th17 cytokines and mucosal immunity. *Immunol Rev* **226**, 160-171 (2008). https://doi.org:10.1111/j.1600-065X.2008.00703.x

222 Li, X., Bechara, R., Zhao, J., McGeachy, M. J. & Gaffen, S. L. IL-17 receptor-based signaling and implications for disease. *Nat Immunol* **20**, 1594-1602 (2019). https://doi.org:10.1038/s41590-019-0514-y

223 Robbins, P. A. & McMichael, A. J. Immune recognition of HLA molecules downmodulates CD8 expression on cytotoxic T lymphocytes. *J Exp Med* **173**, 221-230 (1991). https://doi.org:10.1084/jem.173.1.221