THE UNIVERSITY OF CHICAGO

ETS1 PROMOTES DEVELOPMENT AND FUNCTION OF NATURAL KILLER T 1 CELLS VIA TISSUE-SPECIFIC REGULATION OF THE ADHESOME

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Abstract

Invariant natural killer T (iNKT) cells are a subset of innate-like T lymphocytes with a limited TCR repertoire that recognize glycolipid antigen presented on the MHC-I-like molecule CD1d. iNKT cells produce a broad range of cytokines upon activation and play an essential role in directing the cytokine response. In addition to guiding the immune response, iNKT cells can mount strong antitumor responses via interferon γ production, underscoring the importance of understanding how iNKT cells develop and mature into effector populations. Using a novel model for conditional deletion of transcription factor ETS1 in iNKT1 cells, I show that ETS1 regulates the adhesion gene program of iNKT1 cells, promoting a more epithelial transcriptome profile. This is achieved, in part, by suppressing expression of the transcription factor T-bet. T-bet promoted the expression of migration genes *S1pr1* and *S1pr5* and repressed classical iNKT liver retention mediated by LFA-1 and ICAM-1. In the thymus, ETS1 promoted optimal iNKT1 cell numbers. Conditional knockout of TGF-β signaling in iNKT1 cells suggests that expression of adhesion receptors CD49a and CD103 are important in thymic iNKT1 cell maturation. As a similar phenotype was observed in *Ets1*$^{\Delta/\Delta}$ thymic iNKT1 cells, ETS1 may regulate CD49a and CD103 expression by promoting optimal TGF-β signals. Together, I conclude that ETS1 has tissue-specific functions in iNKT1 cells; supporting proper maturation in the thymus and enforcing a more NKT-like adhesome expression profile to support classical retention in the liver.

Chapter One

Introduction

Figure 1.1 adapted from Hosokawa and Rothenberg (2021)
Figure 1.3 adapted from Godfrey et al. (2010)
Figure 1.4 adapted from Kumar et al. (2017)

Innate vs. Adaptive Immunity: The In-Betweeners

The mammalian immune system is comprised of two distinct components, innate and adaptive immunity, that facilitate the body's response to foreign pathogens. Innate immunity acts as the body's first line of defense, after natural barriers like the skin and mucosa, and is associated with a rapid, nonspecific response. Cells of the innate immune system, comprised of macrophages, monocytes, dendritic cells (DCs), basophils, eosinophils, neutrophils, mast cells, innate lymphoid cells (ILCs) and natural killer (NK) cells achieve a fast response using pattern recognition receptors that recognize conserved structures on surface of pathogens, called pathogen associated molecular patterns (PAMPs), and facilitate their direct killing or initiate production of proinflammatory cytokines (Mogensen, 2009). Additionally, certain antigen-presenting cells (APCs) like DCs and macrophages can then process pathogen-derived peptides and present them to the cells of the adaptive immune system. This antigen presentation allows APCs, virally infected cells and tumorigenic cells to communicate their health to adaptive immune cells. The adaptive immune response takes longer to activate compared to innate immunity but is characterized by the establishment of highly specific responses to pathogens, a result of V(D)J recombination of the T- and B-cell receptors,

allowing for recognition of upwards of 10^{11} unique pathogens. Not only are T cells and B cells capable of high pathogen specificity; they are also capable of establishing memory of these pathogens, which allows for more rapid recognition and clearance of pathogens upon subsequent encounters.

While the innate and adaptive immune systems have been well studied and characterized, over the past 30 years we have come to appreciate a subset of cells referred to as innate-like T lymphocytes, which includes γδ T cells, mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells, that arise from traditional T cell development pathways yet recognize antigens broadly in a manner similar to that of innate immune cells. Innate-like T lymphocytes, through production of cytokines, play a key role facilitating the activation of the adaptive immune response in addition to facilitating tissue-specific immune responses and maintaining tissue homeostasis via immune surveillance, making it critical to understand how innate-like lymphocytes develop and the mechanisms that facilitate their selection compared to a conventional T cell fate. While many innate-like T lymphocytes have been identified, the primary focus of this work will be on NKT cells.

Development of Conventional T Cells

The hematopoietic progenitors that generate T cells originate in the fetal liver or bone marrow. These progenitors migrate to the thymus where interactions between Notch1 and Delta-like 4 (*Dll4*) promote the expansion of pro-T cells, T cells that have not expressed TCR genes, and begin the process of T cell commitment. At this point,

development of T cells can be traced based on the expression of CD44, KIT and CD25 where early T cell progenitors (ETPs) are KIT^{++} CD44^{+} CD25^{-} (Fig. 1). Expression of CD25 in addition to upregulation of the IL-7 receptor and early signaling components of the CD3 (*CD3d, CD3g, CD3e*) complex marks progression into the double-negative (CD4^{-} CD8^{-}) 2a (DN2a) stage of T cell development. However, DN2a cells can divert to alternative cell fates if Notch signaling is not sustained (Hosokawa and Rothenberg 2021). Once DN2a cells are no longer responsive to the KIT ligand SCF, they have entered the DN2b stage (KIT^{int} CD44^{+} CD25^{+} CD4^{-} CD8^{-}) and are now committed to the T cell lineage. Then, during the transition from DN2b to DN3a (KIT^{-} CD44^{-} CD25^{+}), expression of CD3 subunits peaks and expression of *Rag1* and *Rag2* are induced, promoting V(D)J recombination at the *Tcrb*, *Tcrd* and *Tcrg* loci to create the TCRβ, TCRδ and TCRγ chains of the T cell receptor (TCR). Rearrangement of these TCR gene loci marks an important decision point in T cell development as DN3a cells will either rearrange and express a functional TCRδ and TCRγ chain and undergo TCRγδ-selection to become TCRγδ+ T cells (to be expanded on later) or rearrange the *Tcrb* locus and undergo TCRβ (β)-selection to eventually become TCRαβ+ T cells (Fig. 1). In DN3 cells TCRβ pairs with a pre-TCR α-chain, forming a pre-TCR that can undergo α-selection. At this stage, T cells are no longer reliant on Notch signaling for expansion and instead express the T-cell costimulatory receptor CD28 (DN3b; KIT^{-}CD44^{-} CD25^{low} CD28^{+}), utilizing signals from the pre-TCR for proliferation. DN3b cells then downregulate expression of CD25 (DN4), cease recombination and begin to express CD8 and enter what is referred to as the immature single positive stage (ISP; CD28^{+} CD4^{-} CD8^{+}). ISPs upregulate CD4 and become double-positive (DP; CD4^{+} CD8^{+}) cells,

where they undergo V(D)J recombination of the Tcra locus in order to form a mature TCRαβ+ T cell. T cells bearing a successfully rearranged TCRα-chain are subjected to selection by cortical thymic epithelial cells (cTECs) expressing self-peptides presented on either Major Histocompatibility Complex (MHC) Class I or II molecules to test for adequate reactivity, called positive selection. Negative selection occurs when DPs react to APC with too strong of an affinity, where these cells undergo clonal deletion and apoptosis mediated by interactions through either CD28 and the TCR or APCs lead to cell death (Punt et al., 1997). Fas-FasL signaling also is thought to contribute to clonal deletion, although this remains highly controversial (Volpe et al., 2016; Chu et al., 2020). Positively selected cells go on to mature, becoming $CD4^+$ helper T cells or cytotoxic $CD8^+$ T cells based on MHC reactivity and circulate to survey peripheral tissues.

Other Innate-Like T Cells

γδ T Cells

As described previously, γδ T cells can arise during rearrangement of the *Tcrb*, *Tcrg* and *Tcrd* loci. γδ T cells can be divided into IFNγ- ($CD27^+$ $CD45RB^+$) or IL-17 ($CD27^-$)-producing subsets and develop at very specific points of ontogeny in mice, highlighting the directed development of these cells. There have been two previously described models for γδ T cell development; the stochastic model, which suggests that T cell fate is already determined early on and subsequent signals continue to facilitate this commitment, and the more widely accepted signal strength model, which proposes

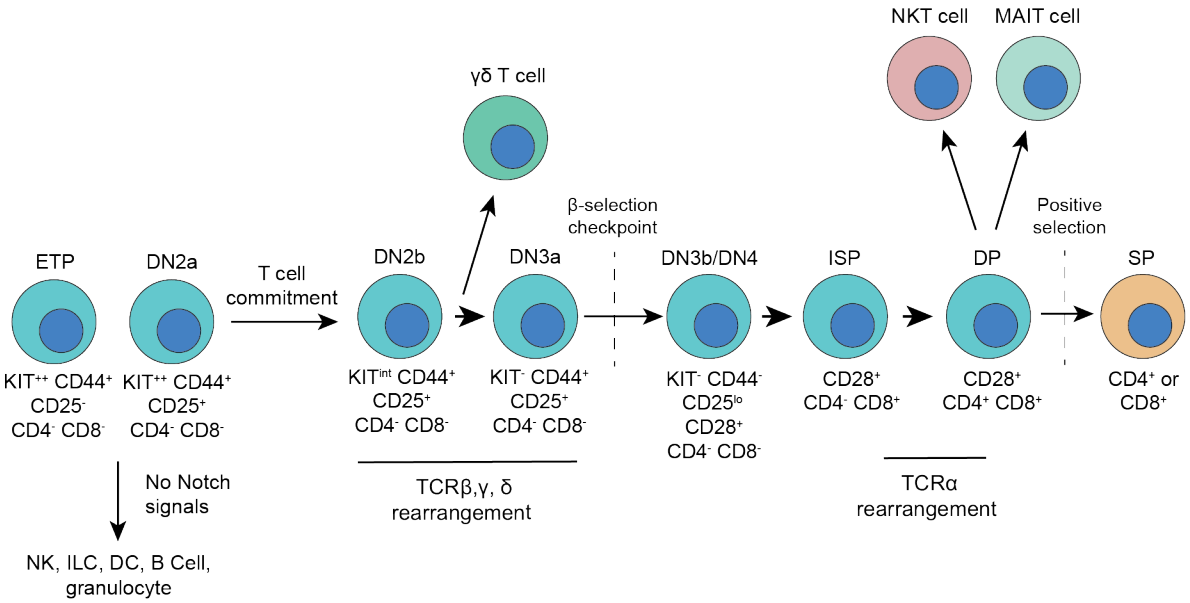

Figure 1.1: Overview of T cell development. ETPs recently migrated from the bone marrow are characterized by KIT, CD44, CD25, CD4 and CD8 expression. ETPs can express CD25 to become DN2a cells,but are reliant on receiving Notch signals to be committed to the T cell lineage. ETPs/DN2as that do not get Notch will develop into NK cells, B cells, ILCs, DCs or granulocytes. Once committed, T cells downregulate KIT, begin to express components of the CD3 complex, and begin to rearrange the β or γδ chains of the TCR. Successful rearrangement of the γδ TCR commits cells to the γδ T cell lineage, while DN3a cells undergo β-selection with a pre-TCR α-chain. Following β-selection, T cells express CD28, CD8 and CD4 as they rearrange a proper TCR α-chain and become CD4+ CD8+ (DP). At the DP stage, conventional cells undergo selection with cortical thymic epithelial cells to test for self-reactivity of the TCR. Non-self-reactive T cells are positively selected and become naive CD4 or CD8 SP cells, while self-reactive T cells are negatively selected. T cells bearing a NKT or MAIT cell TCR rearrangement are selected into their respective lineages based on interactions with other DP thymocytes.

that lineage choice is determined by the strength of TCR signaling (Muñoz-Ruiz et al., 2017). In the signal strength model, T cells with high self-reactivity at the β-selection checkpoint may be directed to rearrange the *Tcrd* and *Tcrg* loci and commit to a γδ fate instead. However, γδ T cells exhibit a high degree of functional and transcriptional differences compared to conventional T cells.

γδ T cells are unique in that they undergo “developmental preprogramming”, meaning they acquire all effector functions prior to exiting the thymus. Furthermore, the γ- and δ-chains rearrange in very specific ways depending on developmental stage. In mice, IFNγ-producing Vγ5 cells emerge as early as embryonic day 12 (E12) and home to the dermis. By E16, γδ rearrangements are biased toward IL-17-producing Vγ6 and Vγ4 cells that home to tissues including the skin, mouth, lungs and liver, followed by a short burst of Vγ7 cells from E18 to birth that home to the intestines and produce IFNγ (Fig. 1.2A). Interestingly, these γδ subsets do not engage the TCR for activation, instead using IL-1β/IL-7/IL-23 and IL-18/IL-12 for IL-17 and IFNγ stimulation, respectively, drawing some parallels to ILCs. One theory behind this innate-like lack of TCR engagement is that these γδ subsets receive such strong TCR signals following initial rearrangement that any subsequent signals are dampened in response (Muñoz-Ruiz et al., 2017; Ribot et al., 2021). On the other hand, γδ subsets that develop following birth tend to be Vγ1 (with some Vγ4) and display a more adaptive phenotype and utilize the TCR to elicit specific responses in the liver and lymphoid tissues (Fig 1.2A).

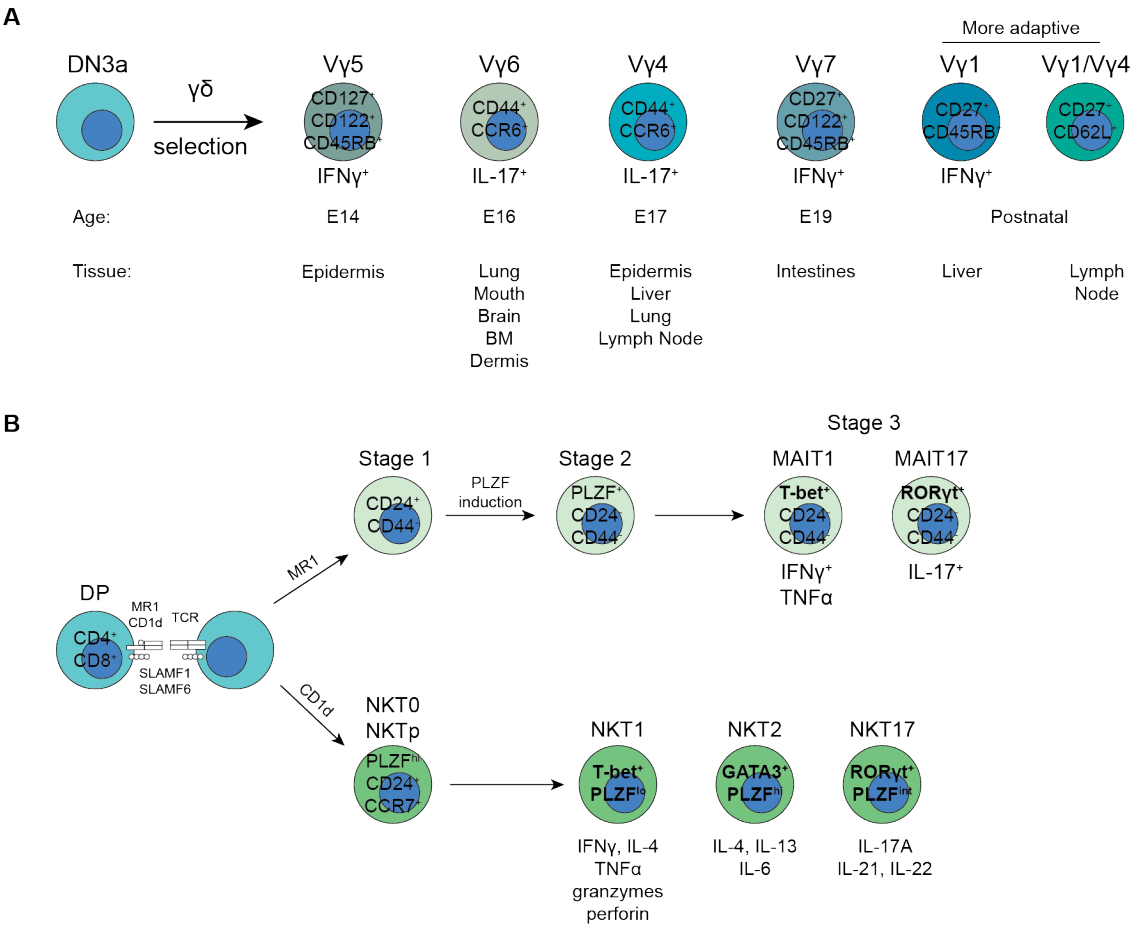

Figure 1.2: Development of unconventional T cell subsets. (A) DN3 cells that rearrange a γδ TCR undergo selection and enter the γδ T cell lineage. Specific types of γδ T cells develop depending on developmental age (E = embryonic day) that are more innate in nature, being activated only through cytokines as their TCR signaling has been suppressed. These cells target to specific tissues and produce either IFNγ or IL-17. Following birth, Vγ1 and Vγ4 are the primary γδ subsets and are able to recognize antigens through the TCR. (B) T cells that reach the DP stage can differentiate into NKT or MAIT cells based on rearrangement of TCRs that recognize lipids presented on non-canonical CD1d or MR1, respectively. MAIT cell development is charcterized by expression of PLZF, CD24 and CD44, using transcription factors T-bet and RORγt to identify MAIT1 and MAIT17 effectors. NKT effector subsets are characterized by varying PLZF expression in addition to T-bet, GATA3 and RORγt to denote NKT1, NKT2 and NKT17 cells, respectively.

Indeed, within the Vγ1 T cells, a subset expressing Vγ1Vδ6.3/6.4 has been named "NKT-like γδ T cells" based on their expression of the innate-like associated transcription factor PLZF, NK1.1 and their ability to produce both IFNγ and IL-17.

Similar to conventional $CD4^+$ T_H subsets, γδ T cells require the transcriptional activity of T-bet and RORγt for the production of IFNγ and IL-17, respectively. E-protein inhibitor Id3, which is induced by Egr2/3 activity and strong TCR signaling, has been shown to be indispensable for IFNγ-producing γδ populations while also inhibiting *Rorc* (encoding RORγt) expression via dimerizing with E47. As such, IL-17-producing subsets must utilize a TCR-independent pathway for expression of *Rorc*. Current evidence suggests that γδ cells can induce expression of *Rorc* based on expression of transcription factors Sox13 and Sox4 prior to TCR signaling (Muñoz-Ruiz et al., 2017). Taken together, while γδ T cells arise from the same DN precursor population, they employ distinct regulatory mechanisms to control effector fate differentiation and cytokine production.

MAIT Cells

Although MAIT cells were discovered nearly 30 years ago, there still remains much to be elucidated regarding their functions in immunity. MAIT cells are semi-invariant in that they possess a restricted TCR α-chain (Vα19 Jα33 in mice, Vα7.2 Jα33 in humans) paired with a limited Vβ repertoire (Vβ6 or Vβ8 in mice, Vβ2 or Vβ13 in humans). MAIT cells differ from conventional T cells in that they use this semi-invariant TCR to recognize vitamin B2 metabolites (such as 5-OP-RU) presented by the non-

canonical MHC Class I-like protein MR1. The importance of MAIT cells in mammalian immunity is underscored by the fact that MR1 and *TRAV1* (encoding $V_{\alpha}19/7.2$) are highly conserved in mammals and appear to have coevolved. Furthermore, MAIT cells have been found to localize in the lungs, liver, blood and mucosal tissues and represent a large subset of T cells in humans.

Positive selection diverges from convention in that cells bearing a TCR with affinity for 5-OP-RU can adopt one of two fates based on the interacting cell. If recognizing MR1 on TECs, these DP thymocytes will undergo selection into a naïve "conventional-like" $CD4^+$ T cell (Salou et al., 2021). If 5-OP-RU:MR1 is presented by another DP thymocyte, TCR engagement is paired with a co-stimulatory signal from the SLAM family of receptors (primarily driven by SLAMF1 and SLAMF6). It is possible that these divergent selection pathways exist as a means to regulate the MAIT cell niche as only a fraction of cells bearing this TCR actually become MAIT cells (Rahimpour et al., 2015; Martin et al., 2009).

Following positive selection, MAIT cells differentiate into either MAIT1 or MAIT17, classified based on expression of T-bet and RORγt, respectively, as well as their functional and transcriptional similarities to conventional $CD4^+$ T_H subsets. Unlike $CD4^+$ helper T cells and NKT cells, MAIT cells do not differentiate into a $GATA3^+$ IL-4-producing effector state. This could likely be due to differences in strength of TCR activation during positive selection as $GATA3^+$ NKTs have higher TCR signal strength (Dashtsoodol et al., 2019). After differentiation MAIT1 and MAIT17 cells migrate to peripheral and secondary lymphoid organs, where MAIT1 cells preferentially seed the

spleen and liver, while MAIT17 cells represent the majority of the MAIT cell population in the lungs, skin and gut lamina propria (Hinks and Zhang, 2020).

MAIT cells are tissue-resident and respond rapidly upon recognition of microbial metabolites to facilitate localized immune responses via secretion of IFNγ, IL-17 and TNFα. MAIT cells have been shown to be important for the clearance of infections mediated by 5-AP-RU-producing bacteria including *Francisella tularensis* (Meierovics et al., 2013) and *Legionella beachae* (Wang et al., 2018). MAIT cells are also capable of activation through cytokine-dependent signaling pathways, allowing them to aid in combating viral infections. Indeed, MAIT cells have been shown to activated by IL-12 and IL-18 produced in an *in vitro* model of influenza infection (Loh et al., 2016).

Natural Killer T Cells: Selection, Development and Function

iNKT cells differ from conventional T cells in that they possess a semi-invariant TCR (Vα14Jα18 paired with Vβ2, Vβ7 or Vβ8, Vα24/Vβ11 in humans) which recognizes glycolipid antigen presented on the non-canonical MHC I-like molecule CD1d. Upon antigen recognition or exposure to stimulatory cytokines, iNKTs produce a broad range of cytokines including IFNγ, IL-4, TNFα, IL-13, IL-17, and IL-10. Due to their rapid activation, iNKTs play an important role in facilitating the immune response by setting up the cytokine milieu that guides the direction of the immune response towards a T_H1, T_H2 or T_H17 profile (Godfrey et al., 2004; Bendelac et al., 2007).

NKT cell development is similar to conventional T cell development up until the DP stage. Rather than being selected by cTECs, thymic iNKTs are positively selected

via interactions with other DP thymocytes presenting glycolipid antigen (most commonly isoglobotriosylceramide, or iGb3) on CD1d which leads them to signal for activation through the TCR and signaling lymphocytic activation molecule (SLAM)-family receptors (SFRs). Induction of SAP signaling by SLAM-TCR activation promotes expression of promyelocytic leukemia zinc finger (PLZF, encoded by *Zbtb16*), a hallmark transcription factor associated with the development of innate-like lymphocytes. Upregulation of PLZF (via Egr2/3) promotes innate-like lymphocyte development through repression of the transcription factor Bach2, which enforces a naïve T cell fate, and activating the effector gene program (Mao et al., 2016; Savage et al., 2008). After positive selection, iNKT cells exist in a $PLZF^{hi}$ $CD24^{+}$ (NKT0 or NKTp) state as they begin to differentiate into one of three primary effector lineages: NKT1 ($PLZF^{lo}$ T-bet^{+}), NKT2 ($PLZF^{hi}$ $GATA3^{+}$) or NKT17 ($PLZF^{int}$ $ROR\gamma t^{+}$), based on similarities in cytokine and transcriptional profile to their conventional helper T cell counterparts.

It has been shown that this divergence in the selection process is important in establishing the NKT population via transgenic expression of MHC II or MHC I in DP thymocytes (Choi et al., 2005; Li et al., 2005; Li et al., 2010; Georgiev et al., 2021). Two groups showed that expression of human class II MHC transactivator (CIITA) under CD4/Lck promoters gave rise to $CD4^{+}$ $PLZF^{+}$ innate-like T cells phenotypically distinct from $CD4^{+}$ NKT cells (Li et al., 2005; Choi et al., 2005). More recently, it was demonstrated that ectopic expression of MHC I in DPs led to the expansion of a subset of peptide-recognizing $PLZF^{+}$ innate-like (PIL) T cells that differentiated similarly to NKT cells and were dependent on SLAM-associated protein, or SAP. NKT cells were present

in reduced frequency in this model, suggesting that PIL T cells were in competition with NKTs for selection (Georgiev et al., 2021). Taken together, it is clear that NKTs emerge as a developmentally distinct population of cells with unique roles within the immune response.

NKT Cells in Disease and Infection

Bacterial and Viral Infection

NKT cells are typically located at the first interface between host and the environment in tissues and have been shown to play a key role in protecting against microbial and helminth infections (Vogt and Mattner, 2021). The liver has become an important tissue to study NKT-mediated defense against microbes as NKT cells represent nearly 12-30% of all lymphocytes in the population, the majority of which are NKT1 (Slauenwhite and Johnston, 2015). Hepatic NKT cells have been shown to patrol the liver by crawling along the surface of the sinusoidal space (Crosby and Kronenberg, 2018; Geissman et al., 2005). Upon recognition of antigen presented on CD1d by either Kupffer cells or liver sinusoidal endothelial cells (LSECs), as in the case of Borrelia burgdorferi infection, patrolling NKTs arrest and produce IFNγ in addition to other cytokines (Lee et al., 2010). Furthermore, NKTs have also been implicated in the attenuation of side effects resulting from Borrelia infection after bacterial clearance (Tupin et al., 2008; Olson et al., 2009). In a murine model of hepatitis B virus (HBV) infection, in vivo injection of α-galactosylceramide (αGalCer) was found to eliminate HBV replication in the liver in an IFNγ-dependent manner. In addition to directly clearing

HBV, liver NKT cells also stimulated NK cells to secrete antiviral cytokines (Kakimi et al., 2000). NKTs in the liver have also been found to clear HCV infection via IFNγ in mice transplanted with human HCV-infected hepatocytes as well (Miyaki et al., 2017; Deignan et al., 2002; Lucas et al., 2003).

NKT-Mediated Inflammation in Hepatic Injury

While NKT cells play essential roles in defense against microbes, they have also been implicated in pathogenesis driven by overactive inflammatory responses. In a model of concanavalin A (ConA)-induced hepatitis, *CD1d*$^{-/-}$ mice lacking NKT cells were found to be resistant to liver injury. Adoptive transfer experiments demonstrated that FasL on NKT cells plays an important role in the onset of hepatitis (Takeda et al., 2000). During liver regeneration, activation of NKTs via IL-12 or αGalCer was also found to exacerbate liver damage and impair regeneration in an IFNγ- and TNFα-dependent manner (Ito et al., 2003). Thus, activation and expansion of NKT cells in acute models of hepatitis leads to direct killing of hepatocytes by IFNγ and Fas-FasL mediated apoptosis in addition to recruitment and activation of other immune cells producing pro-inflammatory cytokines.

Non-alcoholic fatty liver disease (NAFLD) is characterized by an accumulation of excess lipids in the liver. While mild, rarely manifesting symptoms, NAFLD left untreated can progress into non-alcoholic steatohepatitis (NASH) and increase the risk factor for onset of cirrhosis and hepatocellular carcinomas (HCC, Manne et al., 2018; Chen and Tian, 2020). Pathogenesis of NASH is a result of interplay between various

immune cell subsets, but NKT cells have recently been implicated in disease progression. In a diet-induced NASH model, expansion of NKT cells led to increased differentiation of hematopoietic stem cells (HSCs) into myofibroblasts via production of osteopontin and hedgehog ligands, promoting fibrosis of the liver (Syn et al., 2010; Syn et al., 2012). Furthermore, expansion of hepatic NKT cells in human patients was positively correlated with the severity of NAFLD (Adler et al., 2011).

Tumor Immunosurveillance and Immunity

As described, NKT cells have the potential to cause fibrosis in the liver, which can lead to cirrhosis and HCC. However, given the ability of NKT cells to modulate the immune response based on the types of cytokines they produce, they have largely been considered essential mediators of tumor surveillance and anti-tumor immunity (Robertson et al., 2014; Krijgsman et al., 2018). Early studies showed that administration of αGalCer was effective in tumor suppression and limited tumor formation in B16 melanoma models (Kobayashi et al., 1995; Morita et al., 1995; Motoki et al., 1995). Subsequent studies directly linked αGalCer-mediated activation of NKTs to anti-tumor immunity (Nakui et al., 2000; Smyth et al., 2002). Smyth et al. found that αGalCer-mediated antitumor activity was a result of IFNγ production by NKTs and subsequent production of IFNγ by NK cells (Smyth et al., 2002). This indicates that NK and NKT cells may share similar functions in tumor suppression. Treatment with β-mannosylceramide glycolipid, which skewed the NKT response toward a more T_H2 cytokine profile, was not as effective in tumor suppression (O'Konek et al., 2011) while treatment with α-C-GalCer, a C-glycoside analog of αGalCer which elicits a stronger

T_H1 response, was found to be more effective in protecting against metastasis compared to αGalCer (Schmieg et al., 2003), consistent with the requirement of IFNγ in tumor clearance.

Within the context of the tumor microenvironment, the function of NKTs has been found to shift during cancer progression. In the early stages, NKT cells work as potent anti-tumor agents, producing T_H1-associated cytokines, activating NK cells in addition to facilitating the maturation of APCs and generation of tumor-specific $CD8^+$ cytotoxic T cells. NKTs are also able to recognize tumor-derived glycolipids on tumor-associated macrophages (TAM) and facilitate their direct killing (Krijgsman et al., 2018). As tumor progression occurs, chronic stimulation may lead NKT cells to become exhausted and adopt a more immunosuppressive T_H2/T_{reg}-like phenotype. Production of IL-10 by exhausted NKTs can promote T_{reg} activity, which in turn inhibits $CD8^+$ cytotoxic T cells or promote the differentiation of TAMs (Levings et al., 2001).

NKT cells have recently garnered attention in the area of chimeric antigen receptor (CAR)-based immunotherapy. As with NKTs, other cells of the immune system are prone to exhaustion, senescence, and immunosuppression facilitating tumor evasion of the immune system. By introducing tumor-specific NKTs, it may be possible to restore a T_H1 response in the tumor microenvironment and anti-tumor function. As such, Heczey et al were able to generate CAR-NKT cells against GD2 ganglioside, a glycolipid commonly found in neuroblastoma. Injected CAR-NKTs were able to home to the tumor site, mediated T_H1-like polarization and improved the overall survival of the injected mice (Heczey et al., 2014). Additional studies have seen greater anti-tumor

activity with co-administration of IL-15 (Xu et al., 2019) or use of αGalCer in conjunction with CAR-NKTs bearing a tumor-specific TCR (Delfanti et al., 2022) and there has been preliminary success in early-stage clinical trials using CAR-NKT cells (Heczey et al 2020). Thus, determining a strategy to prevent NKT exhaustion or stimulate exhausted NKTs to produce T_H1- associated cytokines in the tumor microenvironment may prove to be a useful immunotherapeutic approach.

Molecular Regulation of NKT Development and Selection

T Cell Signaling

Upon recognition of peptide presented on MHC on cTEC, DP thymocytes signal through the TCR in conjunction with other co-stimulatory receptors to promote survival and differentiation. TCR engagement leads to the recruitment of Fyn, Lck and ZAP-70 (Zhang et al., 1998). Recruitment of these Src family kinases promotes positive signaling of four distinct pathways: the Ras-ERK1/2-AP-1, IP_3-Ca^{2+}-NFAT, PKCθ-NF-κB and the mTOR pathways (Hwang et al., 2020). Activation of these signaling pathways, in turn, leads to transport of key transcription factors into the nucleus that facilitate the expression of genes associated with conventional T cell differentiation and activation. In contrast to conventional T cell differentiation, NKT signaling is reliant on cooperative signals from the TCR and SLAM-family receptors (SFR).

Initially, it was proposed that NKT cells are not reliant on Ras, Mek-1 or Lck for selection; that SFR engagement promotes activation of Fyn and subsequently RasGAP, which acts as an inhibitor of the Ras-MAPK pathway (Alberola-Ila et al., 1996; Borowski

and Bendelac, 2005). However, it was later revealed that loss of Ras activity via a germline dominant-negative mutation resulted in near complete loss of NKT cells in the thymus, highlighting an essential role for Ras-MAPK and TCR signaling in NKT cell development. Interestingly, overexpression of apoptosis regulator Bcl-2 in *Egr2*$^{-/-}$ mice resulted in recovery of conventional T, but not NKT, cell numbers in the thymus suggesting that conventional and NKT cell survival is regulated by distinct factors (Hu et al., 2011). The IP_3-Ca^{2+}-NFAT pathway has also been shown to be essential for development of NKT cells. Calcium signaling promotes dephoshorylation of nuclear factor of activated T cells (NFAT) via calcineurin, facilitating its entry into the nucleus and activation of downstream targets, including Egr2 (Fig. 1.3, Rengarajan et al., 2000). As such, Cre-mediated deletion of calcineurin B1 in thymocytes resulted in near loss of NKT cells (Lazarevic et al., 2009). Inhibition of NF-κB activity appears to be important for selection into the NKT lineage, but the role of NF-κB following selection remains unclear (Schmidt-Supprian et al., 2004, Hu et al., 2011b).

SLAM-family Receptors

There are nine known members of SFRs. *Slamf1* (SLAM/CD150), *Slamf2* (CRACC/CD48), *Slamf3* (Ly9/CD229), *Slamf4* (CD244/2B4), *Slamf5* (CD84) and *Slamf6* (Ly108/NTB-A) are believed to signal in a SAP-dependent manner while *Slamf7*, *Slamf8* and *Slamf9* are SAP-independent (De Calisto et al., 2014; Gerth and Mattner, 2019). Homotypic interactions mediated by SFRs (CD244 prefers to recognize CD48) promote recruitment and binding of SAP and subsequently Fyn (Chan et al., 2003; Latour et al., 2003). SAP appears to have a greater effect compared to SFRs on NKT cells, in

addition to other populations, as germline deletion of *Sh2d1a*, encoding SAP, results in loss of NKT cells, NK cells and T_{fH} cells and X-linked lymphoproliferative disease (XLP), which is characterized by increased susceptibility to Epstein-Barr virus (EBV), B cell lymphomas and impaired humoral immunity (Nichols et al., 2005; Pasquier et al., 2005; Gerth and Mattner, 2019). Single-receptor mutations in SFRs have yielded mild or no effect on NKT cells suggesting that they may play partially redundant roles in signaling (Wang et al., 2004; Howie et al., 2005; Graham et al., 2006; Griewank et al., 2007). Indeed, deletion of multiple SFRs, most notably *Slamf1* and *Slamf6*, had a greater impact on NKT cell numbers (De Calisto et al., 2014; Huang et al., 2016; Hu et al., 2016; Chen et al., 2017; Lu et al., 2019). Interestingly, Lu et al. also showed that SFRs are important for attenuating the strength of NKT TCR signaling and promote survival via suppression of inhibitory immune checkpoint receptors such as PD-1 (Lu et al., 2019). Taken together, TCR signaling and SFR signaling both play important roles in NKT cell selection and development. Understanding how these pathways work in concert to establish the NKT lineage will be essential to leveraging NKT cells in therapeutic approaches.

E-proteins and Id-Proteins in Selection

A major distinction between innate-like lymphocytes (ILLs) and their adaptive counterparts is the constitutive versus inducible expression of the E protein transcription factor inhibitors ID2 and ID3 (Kee, 2009). During conventional TCR selection, ID proteins are induced and promote differentiation while enforcing the cessation

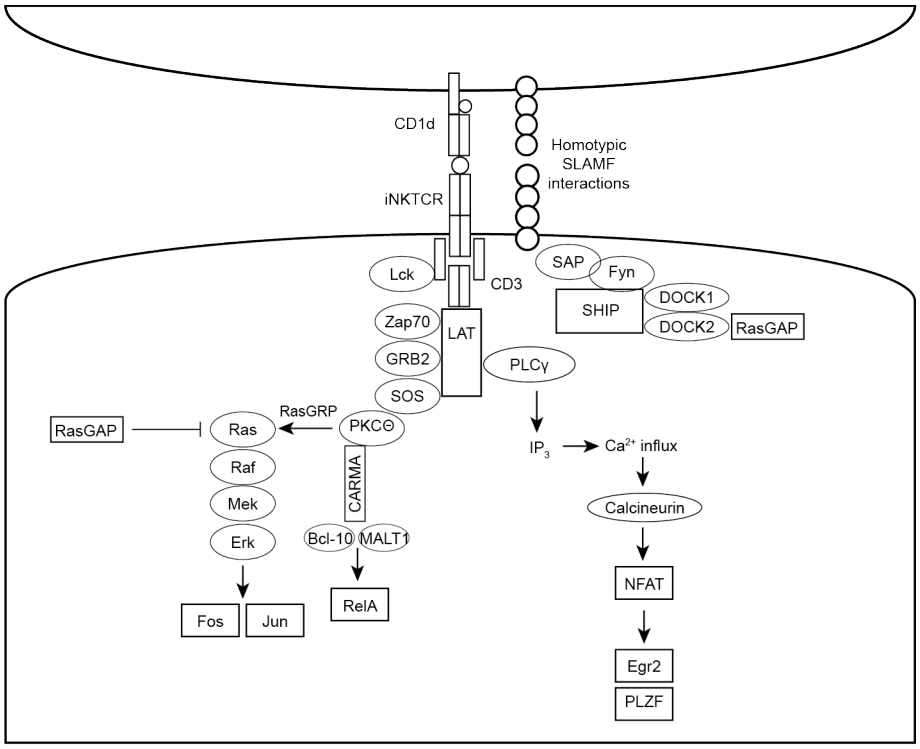

Figure 1.3: Molecular signaling in iNKT development. iNKT development is driven by the interplay between traditional TCR signaling pathways; comprised of the Ras-MAPK pathway, the PKCΘ pathway and the IP3-Ca2+ pathway, in addition to signaling driven by SLAM family receptors. Signaling through these pathways controls TCR signaling while inducing expression of Egr2 and PLZF, the signature transcription factor of the innate-like lineage of lymphocytes.

of rearrangement at *Tcra* through repression of E proteins (Jones and Zhuang, 2007). When *Id2* and *Id3* are deleted in DP thymocytes, conventional TCR-mediated selection cannot occur and thymocytes continue to rearrange *Tcra* until they create a TCR that can undergo unconventional selection, for example the iNKT cell receptor or the MAIT cell receptor (Verykokakis et al., 2013). iNKT cell selection does not require ID2 or ID3; rather, E proteins participate in the induction of *Zbtb16* (D'Cruz et al., 2014). However, ID2 and ID3 are required for proper iNKT cell differentiation. Failure to express both ID2 and ID3 results in an accumulation of Vα14Jα18 TCR^+ cells with low PLZF and a phenotype consistent with ST0 or ST1 cells, although they produce IL-4 (D'Cruz et al., 2014; Verykokakis el al., 2013; Verykokakis et al., 2010; Li et al., 2013).

Id2 is highly expressed in T-bet^+ iNKT1 cells whereas *Id3* is expressed at much lower levels in these cells (D'Cruz et al., 2014). Despite this, deletion of *Id2* did not affect development of thymic iNKT1 cells (Monticelli et al., 2009). In contrast, deletion of *Id3* had a major impact on thymic iNKT1 cell numbers (Verykokakis et al., 2010). Although not formally demonstrated, this observation of high *Id2* without a requirement for *Id2* could be explained by induction of *Id3* by E proteins and functional redundancy between ID2 and ID3. Alternatively, ID3 may be critical for the emergence of iNKT1 cells in ST1. Indeed, ID3 is highly expressed in ST1 cells and required for the emergence of T-bet-expressing cells in this population (D'Cruz et al., 2014; Verykokakis et al., 2013). *Id2* promotes cytotoxic effector differentiation in NK and $CD8^+$ T cells by binding to an intronic enhancer of *Tcf7*, encoding TCF1, and restricting E protein activity (Zook et al., 2018; Cannarile et al., 2006; Masson et al., 2013; Delconte et al., 2016; Li

et al., 2021). Whether ID2 and ID3 limit *Tcf7* transcription early in iNKT cell development remains to be determined, but they do play a similar role in γδ T cells (Fahl et al., 2021). Of note, TCF1 and LEF1 are both required for early iNKT cell expansion and iNKT2 differentiation, and thus overexpression of TCF1 or LEF1, which is also regulated by E proteins, might derail the iNKT1 cell program (Carr et al., 2015; Berga-Bolaños et al., 2015). In mice lacking ID3, iNKT2 cells are expanded in number and produce sufficient IL-4 to induce innate-like CD8 T cells, a phenotype that could be consistent with increased TCF1 or LEF1 (Verykokakis et al., 2010; Li et al., 2013). Thus, the differential regulation of the ID protein-E protein axis is a key feature of all ILLs and is associated with their acquisition of a primed effector state.

Transcriptional Regulation of NKT Cell Development

Regulators of PLZF

Given the central role of PLZF in iNKT cells and MAIT cells, much focus has been placed on how PLZF is regulated, but little is known about the genomic context that drives PLZF expression. In an elegant study by Mao et al. (Mao et al., 2017), a combination of ATAC-seq and CRISPR/Cas9-mediated deletion was used to interrogate genomic requirements at the *Zbtb16* gene, encoding PLZF, in ILCPs and iNKT cells. This analysis revealed a region downstream of the transcriptional start site that was important for PLZF expression and identified RUNX1 binding sites within the enhancer. Conditional deletion of *Runx1* in DP thymocytes resulted a loss of PLZF expression in

ST0 and ST1 iNKT cells and arrested iNKT development; a phenotype partially rescued via transgenic expression of PLZF confirming that *Zbtb16* is a RUNX1 target.

The BTB-POZ transcription factor BCL6 plays a role in setting up the genomic context for iNKT cell development and for the initial expression of PLZF (Gioulbasani et al., 2020). DP thymocytes express BCL6, but it is rapidly extinguished as a consequence of conventional positive selection. In contrast, in iNKT cells undergoing positive selection, BCL6 increases and is not extinguished until PLZF is expressed. Despite its restricted expression, deletion of *Bcl6* in $CD4^+$ $CD8^+$ thymocytes dramatically affects iNKT cell numbers. Mechanistically, in the absence of BCL6 the boundary between ST0 and ST1 iNKT cells is blurred and ST1 cells continue to express genes that should only be expressed in ST0 and fail to express some genes associated with ST1. Analysis of chromatin accessibility in ST0 and ST1 iNKT cells was consistent with the mixed ST0/ST1 phenotype. Notably, regions of accessibility near some genes that are expressed in ST1 or later in iNKT cell development failed to become accessible in the absence of BCL6, including *Zbtb16*. Indeed, PLZF target genes are a subset of the genes that fail to be repressed in BCL6-deficient ST1 cells. Interestingly, PLZF expression was reduced and delayed in $CD4^{Cre}$ $Bcl6^{F/F}$ iNKT cells, but some mature iNKT cells emerge that express PLZF (Mao et al., 2017). Thus, BCL6 is required to initiate accessibility at key iNKT cell genes, including *Zbtb16*, and for the repression of PLZF target genes in ST1 iNKT cells.

In DP thymocytes, *Zbtb16* has both H3K27me3 and H3K4me3 at its promoter, indicating that it is in a repressed, but poised, chromatin state (Dobenecker et al., 2015).

Deletion of EZH2, the histone methyltransferase component of the polycomb repressive complex 2 (PRC2), in DP thymocytes leads to reduced H3K27me3 in iNKT cells and a significant increase in PLZF$^+$ thymocytes (Dobenecker et al., 2015; Savage et al., 2011; Kovalovsky et al., 2010). A similar phenotype is observed in mice lacking JARID2, a component of PRC2 and other histone methyltransferase complexes (Pereira et al., 2014). Many of the PLZF$^+$ cells arising in the absence of EZH2 fail to be detected by CD1d tetramers, indicating that they have an altered TCR repertoire and suggesting that PLZF induction occurs in cells undergoing conventional positive selection. Notably, deletion of the histone lysine demethylases UTX and JMJD3 in DP thymocytes had a negative impact on iNKT cell development and PLZF expression, suggesting that demethylation of H3K27me3 is required (Beyaz et al., 2017, Northrup et al., 2017). However, deletion of EED or SUZ12, two essential components of the PRC2 complex, resulted in a loss of iNKT cells and a more severe loss of H3K27me3 in iNKT cells than observed with EZH2 deletion (Vasanthakumar et al., 2017). Thus, EZH2 may have a function distinct from PRC2 in iNKT cells. EZH2 is capable of methylating non-histone proteins and PLZF contains a functional degron that can be methylated by EZH2 (Vasanthakumar et al., 2017; Su et al., 2005). A role for EZH2 in PLZF degradation is consistent with the increased DNA binding by PLZF observed after deletion of EZH2 (Koubi et al., 2018). Although degradation of PLZF may be an important role of EZH2 in iNKT cells, it would not explain the use of alternative TCRs in PLZF$^+$ T cells after EZH2 deletion unless there are cells that initiate and then extinguish PLZF via this mechanism. Taken together, the early and/or heightened expression of PLZF must be regulated to ensure appropriate iNKT cell numbers.

Downstream Targets of PLZF

PLZF is sufficient to induce the innate properties of iNKT cells, as revealed in mice with ectopic expression of PLZF starting in $CD4^+$ $CD8^+$ T cells (Savage et al., 2011; Kovalovsky et al., 2010). In these mice, PLZF promotes a memory-like phenotype on conventional CD4 T cells and the ability to coproduce IFNγ and IL-4 rapidly after activation. PLZF concentration could affect target site choice or its ability to interact with cooperating factors. PLZF binding sites have been examined in PLZF transgenic mice and revealed that PLZF binds DNA enriched for ETS, E protein, and RUNX family binding motifs, implicating these transcription factors not only in induction of PLZF but also in the recruitment of PLZF to DNA (Mao et al., 2016). RNA-seq analysis of wild-type and PLZF-deficient iNKT cells, as well as wild-type and PLZF transgenic CD4 T cells, revealed a small number of PLZF-dependent genes with approximately half being direct targets based on chromatin immunoprecipitation sequencing (Mao et al., 2016, Gleimer et al., 2012). These targets include genes that are downregulated early in iNKT cell development and encode proteins associated with migration such as *Sell* and *Klf2*, as well as *Bach2*, which codes for a repressor of Th effector fates. Multiple chemokine and cytokine receptors involved in iNKT cell activation are targets of PLZF, including *Il12rb*, *Il18r1*, *Il4ra*, *Il21r*, and *Ifngr1*, as are transcription factors involved in iNKT cell effector fate with the exception of *Tbx21*. PLZF also directly regulates *c-Maf*, which encodes a transcription factor that is essential for IL-17 production in iNKT17 cells and directly regulates expression of *Il4* (Gleimer et al., 2012, Yu et al., 2017). Therefore,

although PLZF has a limited number of targets in iNKT cells, these targets function in the most fundamental aspects of the primed state and effector fate of iNKT cells.

PLZF activity is also regulated by acetylation (Sadler et al., 2015; Guidez et al., 2005). Both p300 and HAT1 acetylate lysine residues in the C-terminal zinc finger domain of PLZF to promote DNA binding and gene repression. In myeloid cells, PLZF is acetylated in response to Toll-like receptor or Tumor-necrosis factor receptor signaling, resulting in recruitment of HDAC3 and NF-kB p50 to DNA-bound PLZF to repress the NF-κB response (Sadler et al., 2015). Mutations that inhibit acetylation of these lysines abrogate DNA binding by PLZF. The role of PLZF acetylation has recently been investigated in iNKT cells using a mutant form of PLZF that mimics constitutive acetylation (called $PLZF^{ON}$) (Klibi et al., 2021). In these mice, iNKT1 and iNKT17 cell numbers are decreased and a novel $TBET^{-}$ $ROR\gamma t^{-}$ immature cell emerges. These immature cells show reduced proliferation, increased apoptosis, and a heightened capacity to produce IL-4 and IL-13. At the transcriptome level, these immature cells show reduced expression of transcription factors and cytokine receptors associated with iNKT1 and iNKT17 cells and increased expression of known PLZF target genes, including *Sell*, *Bach2*, and *Bcl6*. This gene signature suggests that acetylation of PLZF in iNKT cells interferes with its repressive ability. However, $PLZF^{ON}$ iNKT cells showed greatly increased overall CpG methylation compared with controls, which was postulated to be a mechanism to prevent premature differentiation (Klibi et al., 2021). Acetylation is an important mechanism controlling transcription factor function and gene regulation in iNKT cell development beyond PLZF. The histone lysine acetyltransferase

GCN5 positively controls the function of EGR2 to promote expression of *Zbtb16*, *Runx1*, and *Tbx21* while limiting iNKT17 cell differentiation (Wang et al., 2017). Further studies are needed to clarify the role of acetylation on PLZF or other transcription factors in iNKT cells and other ILLs.

Migration, Residency and Function

Mechanisms of Thymic Emigration

Once positive selection has occurred, T cells begin to express the surface receptors CCR7 and CD69. CD69 acts to enforce retention in lymphoid tissues while CCR7 facilitates the migration of newly selected cells from the cortex to the medulla (Kwan and Kileen, 2004; Ueno et al., 2004). This migration is essential to T cell development as it acts as a secondary checkpoint in central tolerance in the thymus. Mice lacking CCR7 were found to have reduced accumulation of T cells in the medulla and increased autoimmunity due to altered negative selection (Kurobe et al., 2006; Nitta et al., 2009). Following additional tolerization via interactions with mTECs and DCs presenting self-peptide, functionally mature T cells (HSA^{lo} $CD69^{-}$ $Qa2^{+}$ $S1PR1^{+}$ $CD62L^{+}$) can migrate out of the thymus and seed peripheral tissues (McCaughtry et al., 2007; Weinreich and Hogquist, 2008; James et al., 2018). However, CCR7-deficient thymocytes are capable of migrating directly from the cortex (Kurobe et al., 2006).

Central to the process of thymocyte emigration are CD69 and sphingosine-1-phosphate receptors (S1PR). The role of S1PRs in lymphocyte egress was first identified using the immunosuppressant FTY720. Use of FTY720 in animal models was

found to prevent graft rejection and deplete lymphocytes in the blood, due to retention of T cells in the lymph nodes (Chiba et al., 1998). Similar blocks to egress were later identified in the thymus (Yagi et al., 2000; Rosen et al., 2003; Alfonso et al., 2006). FTY720 was found to be an agonist for several S1PRs. This, paired with the knowledge that mature T cells in S1PR1-deficient mice failed to downregulate CD69, revealed that CD69 and S1PR1 act as mutual antagonists to modulate migratory signals (Matloubian et al., 2004). As T cells mature, they upregulate S1PR1, which leads to decreased CD69 and the initiation of migratory signaling by S1P ligand. When S1PR signaling is at its peak and thymocytes have entered the bloodstream, a negative feedback loop is induced that promotes the internalization of S1PR as cells home to peripheral tissues (Fig.1.4, Kumar et al., 2017). Similar retention of lymphocytes across multiple tissues may suggest that this mechanism of egress is similar across peripheral tissues. The transcription factors Forkhead box protein O1 (Foxo1) and Krüpple-like factor 2 (Klf2) have also been found to be upregulated during thymocyte maturation and identified as direct activators of *Sell*, encoding the secondary lymphoid organ homing receptor CD62L/L-selectin, and *S1pr1*, highlighting a central role for these transcription factors as regulators of lymphocyte migration (Carlson et al., 2006; Bai et al., 2007; Fabre et al 2008).

While it is evident that thymic NKT cells also utilize S1PR1 for thymic egress (Allende et al., 2008), the presence of CD1d Tet^{+} NK1.1^{-} cells, that encompass NKT2s and NKT17s in addition to developing NKT1s, in peripheral tissues (Pellicci et al., 2002; Benlagha et al., 2002) suggest that NKT cells are able to emigrate from the thymus

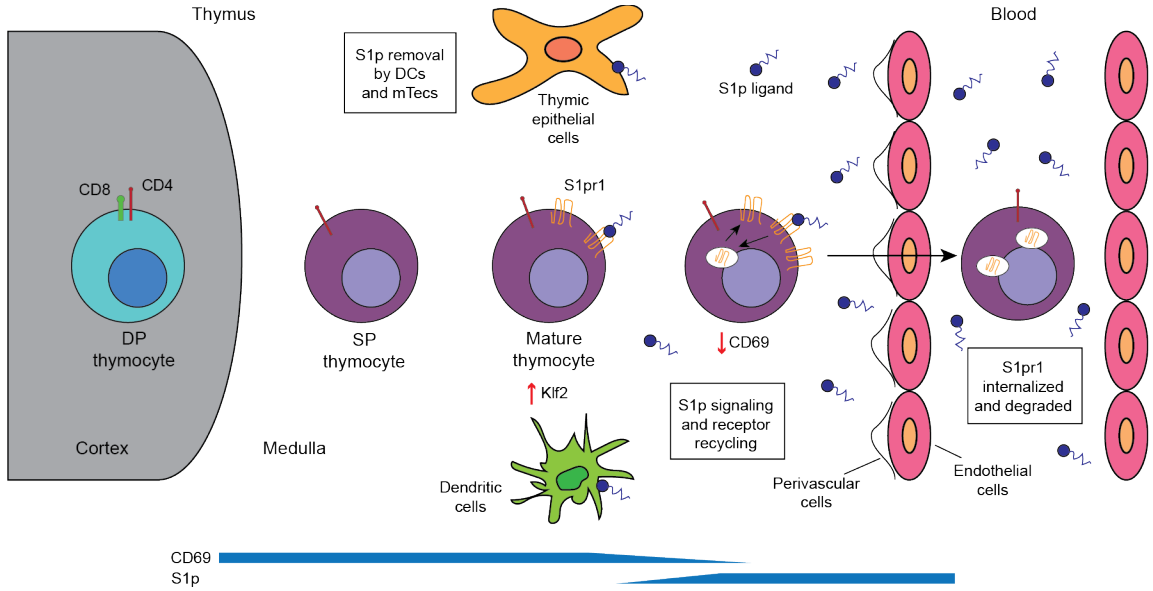

Figure 1.4: Thymic egress of T cells. Migration in the thymus is tightly controlled by the concentration of S1p ligand in the medulla. Expression of CD69 remains high to enforce thymic residency until T cells have completed initial maturation while S1p (produced by perivascular and endothelial cells) is either removed or irreversibly degraded by dendritic cells and medullary thymic epithelial cells. As thymocytes mature, they upregulate Klf2, which promotes the expression of S1pr1, and begin to migrate toward higher concentrations of S1p ligand. This induces S1pr1 signaling via a positive feedback loop and antagonizes CD69, leading to its repression. Once a thymocyte has entered the blood, high concentration of S1p ligand promotes internalization and degradation of S1pr1, completing egress and allowing for circulation through the blood.

before maturation into their full effector states, similarly to how mature T cells can migrate before expansion and differentiation. In fact, a study by Wang and Hogquist identified a subset of NKT cell precursors based on a *Rag2*^{GFP} reporter and expression of CCR7 (Wang and Hogquist, 2018). These NKTps expressed high levels of PLZF, CD24 and CD69 in addition to LEF1, which is important for NKT cell development (Carr et al., 2015) and were able to differentiate into NKT1, NKT2 and NKT17 populations. Using an intra-thymic labeling scheme, it was demonstrated that CCR7 defined a significant portion of recent thymic emigrants (RTE) in the spleen. The authors also observed that a small population of RTE T-bet^{+} NKT1 cells existed in the spleen. However, these RTE NKT1 cells were PLZF^{hi} Qa2^{-}, implying that they were recently differentiated and likely derived from RTE NKTps. Taken together, CCR7^{+} NKTps represent the primary NKT population emigrating from the thymus, a process dependent on Klf2 (Wang and Hogquist, 2018).

Homing Signals from Chemokine and Chemokine Receptors.

Following selection, NKTps will either remain in the thymus and mature or migrate out of the thymus and seed peripheral tissues where they will finish their maturation. It is unclear what determines this decision, but induction of T-bet in NKT cells promotes the expression of CCR5 and CXCR3 which could lead to the thymic retention (Matsuda et al., 2006). Alteration of CXCR3 via germline deletion or intravenous injection of a CXCR3 antagonist resulted in a decrease in the number of NKTs in the thymus and a corresponding accumulation of RTE NKT cells in the blood, with no observable effect on hepatic NKT cells, highlighting a role for CXCR3 in

the retention of NKTs in the thymus (Drennan et al., 2009).

Emigrating NKTps home to tissues through a combination of chemokine and adhesion receptors expressed on their cell surface and are directed toward cognate ligands expressed by cells of the target tissues (Fig. 1.5). It is unknown what chemokine signals direct NKTs to the spleen, but $CXCR5^{+}$ splenic NKT cells have been identified (Johnston et al., 2003). Unlike the localization of hepatic NKTs in the vasculature, splenic NKTs are distributed more broadly, including the red and white pulp, periarteriolar lymphoid sheath and the marginal zone (Slauenwhite and Johnston, 2015). However, stimulation of splenic NKT cells results in their localization to marginal zone macrophages and DCs (Barral et al., 2012; King et al., 2013). As such, it may be possible that $CXCR5^{+}$ NKTs are mobilized to the marginal zone by chemokine ligand CXCL13, which mediates homing to B cell zones (Slauenwhite and Johnston, 2015). Current evidence suggests that NKT17s, which are common to the lymph nodes and skin, utilize CCR7-CCL21 for chemotaxis (Johnston et al., 2003). In the skin, NKT17s are recruited in a CCR6-dependent manner and retained in the epithelium by CD103 and E-cadherin (Doisne et al., 2009). NKT cells expressing the chemokine receptor CXCR6 tend to home to the liver where NK cells expressing leukocyte function-associated antigen-1 (LFA-1) are believed to facilitate entry of NKT cells into the liver (Doherty, 2016, Emoto et al., 2000). At this point, they will reside in the liver sinusoids via sustained interactions with LFA-1 and intracellular adhesion molecule 1 (ICAM-1) (Thomas et al., 2011). CXCR6 may also play a role in liver retention as $CXCR6^{-/-}$

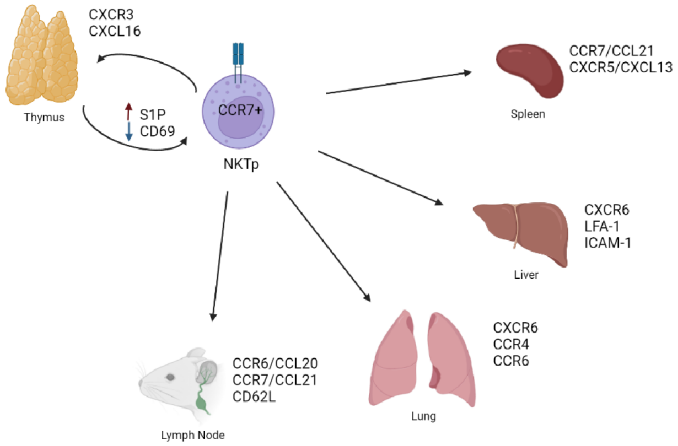

Figure 1.5: Migration of NKT precursors to the periphery. NKTps that continue to mature in the thymus will upregulate CXCR3 and CD69. Once NKTps have migrated out of the thymus, they will seed peripheral tissues based on expression of the chemokine and adhesion receptors shown above. It has been suggested that NKTps are imprinted with these expression profiles prior to egress rather than emigrating to tissues at random. Once they have seeded a secondary organ, NKTps will develop into mature effector states based on the microenvironment of the target tissue.

hepatic NKTs were found to redistribute to the bone marrow (Germanov et al., 2008). In addition to its roles in migration, CXCR6 plays an important role in the development and survival of NKT1s. CXCR6-deficiency had no noticeable effect on thymic NKT cells or NK1.1^{-} RTEs, but NK1.1^{+} NKTs failed to accumulate in the liver. CXCR6$^{-/-}$ splenic and hepatic NKTs also had reduced cytokine production following stimulation with αGalCer (Germanov et al., 2008).

A Universal Program for Tissue Residency

Once NKTps have migrated to their target tissues (or have chosen to remain in the thymus), they will finish maturation into their effector states and typically remain tissue resident. Composition of iNKT effector subsets is largely dependent on the microenvironment of the target tissue; for example, NKTs in the liver are almost entirely NKT1 whereas thymic NKTs give rise to NKT1s, NKT2s and NKT17s and lymph nodes are primarily NKT17 (Crosby and Kronenberg, 2018). This composition can also vary across different strains of mice as well as between humans. A study from the Godfrey lab (Berzins et al., 2006) first observed the tissue resident nature of mature NKTs in the thymus using thymus transplantation with congenically marked host and donor mice. They showed that donor-derived mature NK1.1^{+} NKTs were able to be retained in thymus over a period of weeks while donor-derived NK1.1^{-} NKTs were more likely to emigrate and seed peripheral tissues. Subsequent studies by Thomas et al (Thomas et al., 2011) assessed the tissue residency of NKT cells across various lymphoid tissues using a parabiosis model. When compared to B cells, T cells and NK cells, which tend to circulate and have roughly 50/50 chimerism across tissues, NKT cells showed very

little chimerism across all tissues observed, excluding blood, demonstrating that tissue residency is a general characteristic of mature NKT cells.

However, tissue residency is not a characteristic unique to NKT cells. Numerous studies have explored $CD8^+$ $CD69^+$ $CD103^+$ tissue-resident memory T cells (referred to as CD8 T_{RM}), including their circulation to sites of infection and differentiation into CD8 T_{RM} cells in peripheral tissues and extra-lymphoid tissues (Mackay et al., 2013, reviewed in Gebhardt et al., 2018). Tissue residency has also been observed in ILC1s and a subset of liver cells with an NK cell signature also expressed CD49a and CD69, both of which have been associated with residency (Sojka et al., 2014). Given the functional similarities between these lymphoid populations, it was suggested that they may also share a common transcriptional program for tissue residency. Indeed, Mackay et al identified a tissue residency gene profile in CD8 T_{RM} cells (Mackay et al., 2013) and found that the transcription factors Hobit and/or Blimp1 were essential for proper development of CD8 T_{RM} cells in the skin and act in a synergistic manner (Mackay et al., 2016). Deletion of *Hobit* had no impact on conventional NK cells (cNK) in the spleen or liver, but tissue-resident NK cells (trNK) were markedly reduced in number compared to wild-type. Interestingly, deletion of *Hobit* or *Blimp1* did not affect splenic NKT cells, but liver NKTs were significantly reduced in the double knockout. This may suggest that splenic NKT cells utilize alternative genes to establish residency in the spleen. RNA sequencing of CD8 T_{RM} cells, CD8 T effector memory (CD8 T_{EM}) cells and circulating memory T (T_{CM}) cells post-infection in addition to liver trNK and NKT cells revealed a common gene program associated with circulating vs tissue-resident lymphoid cells

driven by Hobit and Blimp1 (Mackay et al., 2016). In addition to their roles in tissue residency, Blimp-1 and Hobit are important for the terminal differentiation and effector function of lymphoid cells (Bikoff et al., 2009; Nutt et al., 2007) and NKT cells, respectively (van Gisbergen et al., 2012).

These studies point to similar developmental and functional mechanisms between lymphoid cells, particularly NKT cells and CD8 T_{RM} cells, that may be able to inform one another. For example, after migration to the skin, gut and lungs $CD8^+$ T cells downregulate expression of integrin α4β7 and induce expression of CD103 (integrin αE), which promotes formation of integrin αEβ7 (Masopust et al., 2010). Interactions between αEβ7 and E-cadherin on epithelial cells have been shown to contribute to CD8 T_{RM} cell retention as defects in TGF-β signaling, which promotes expression of CD103, result in a failure of CD8 T_{RM} cells to be retained in tissues (Casey et al., 2012; Zhang and Bevan, 2013; Mackay et al., 2013). At the transcriptional level, Nath and colleagues (Nath et al., 2019) identified a common residency signature among CD8 T_{RM} cells in different tissues and compared this signature to differentially expressed genes (DEGs) between TGF-β-stimulated and unstimulated CD8 T cells. The authors found a significant overlap between the DEGs and the T_{RM}-associated residency signature, demonstrating that TGF-β signaling plays an important role in the development and maintenance of CD8 T_{RM} cells (Nath et al., 2019). Given that thymic iNKT cells also express CD103 and share a residency signature with CD8 T_{RM} cells, it is possible that TGF-β signaling is important for the maintenance of iNKT cells in the thymus.

Additionally, it has recently been shown that CD69, which is strongly associated with tissue residency, is not consistently required for CD8 T_{RM} cell residency across different tissues. In fact, CD69 was only required for the generation of CD8 T_{RM} cells in the kidney and had no effect on T_{RM} cells in other tissues (Walsh et al., 2019). In the context of mature NKT cells, which also express CD69, it is possible that their residency is variably dependent on CD69 as well. While S1PR1 has an established role in facilitating thymic emigration, a new role has been demonstrated for S1PR5 in directing trafficking of CD8 T_{RM} cells out of peripheral tissues (Evrard et al., 2021). Repression of S1PR5 was essential to T_{RM} development as germline deletion of *S1pr5* promoted T_{RM} formation and was also found to influence localization of NK cells and ILC1s (Evrard et al., 2021). As such, repression of S1PR5 may play a role in facilitating residency of NKT cells.

Ets1 in Immunity

Ets1 is the founding member of the Ets (E26 transformation-specific) family of transcription factors. The Ets family is comprised of 27 genes in mice (28 in humans) and share the characteristic winged-helix-turn-helix DNA binding domain that binds the GGAA/T consensus motif (Fig. 1.6A, Hollenhorst et al., 2011). However, while all Ets family members are capable of binding this motif (Fig. 1.6C), suggestive of redundant function between factors, sequence variation and restriction in timing of gene expression demonstrates that Ets transcription factors have distinct roles (Garrett-Sinha, 2013). Ets1 bears an acidic transactivation domain and a Pointed domain

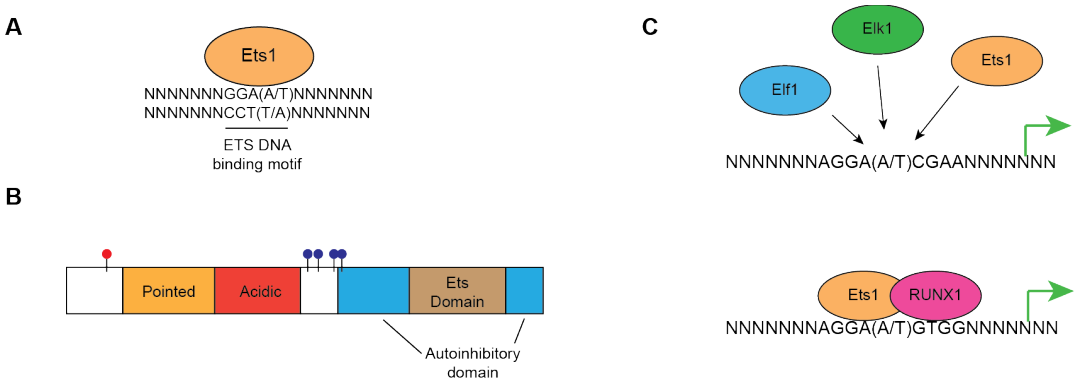

Figure 1.6: Regulation and function of Ets1. Ets1 is the founding member of the Ets family of transcription factors. (A) All Ets family members bind to DNA at the GGA(A/T) motif. (B) Ets1 is comprised of four primary domains: the Ets domain used for DNA binding, the Acidic transactivation domain, the Pointed domain which facilitates protein-protein interactions and two autoinhibitory domains. Ets1 can be activated via Ras-MAPK mediated phosphorylation of a threonine residue near the Pointed domain (red) or inhibited by calcium-mediated phosphorylation of serine residues at the autoinhibitory domain (blue). Inhibitory phosphorylation induces conformational changes in Ets1 that prevents it from binding to DNA. (C) Ets1 can regulate transcriptional activity by binding to DNA on its own (top) or via cooperative binding with other transcription factors such as RUNX1. As Ets family members can bind the same DNA motif, their expression is strictly regulated by cell type and developmental stages.

which allows for protein-protein interactions. The Pointed domain allows docking of ERK2 (induced through the Ras pathway described earlier), which phosphorylates a highly conserved serine residue (T38) and enhances Ets1-mediatedtranscriptional activation of Ets1 (Yang et al., 1996; Wasylyk et al., 1997). Phosphorylation of amino acid residues at the auto-inhibitory domain, mediated by calcium-dependent kinases, induce conformational changes in Ets1 that prevent its binding to DNA (Fig. 1.6B).

Ets1 is expressed in many tissues during embryonic development in mice (Kola et al., 1993; Maroulakou et al., 1994). In adult mice, its expression is limited to lymphoid tissues, where Ets1 continues to be expressed at high levels. In particular, Ets1 is highly expressed in NK cells, B cells, T cells and NKT cells (Chen, 1985; Faust et al., 1993; Barton et al., 1998; Anderson et al., 1999), suggesting that Ets1 may play an important role in the development or differentiation of various immune cell subsets.

Ets1 in T Cells, B Cells and NK Cells

Mice lacking Ets1 have many defects in the T cell compartment including altered development in the thymus, skewing towards a more effector-memory-like phenotype and a reduced capacity to produce T_H1- and T_H2-associated cytokines (Garrett-Sinha, 2013). Differentiation and function of regulatory T cells is also impaired in these mice (Mouly et al., 2010). Interestingly, despite defects in development T_H1 and T_H2 cells, *Ets1*$^{-/-}$ mice were observed to have an increased number of T_H17 cells, suggesting that Ets1 represses T_H17 development (Moisan et al., 2007). As Ets1 has been shown to cooperatively bind with T-bet to induce expression of IFNγ (Grenningloh et al., 2005), it

could be possible that developing T_H1 cells aren't receiving strong enough signals. Ets1 may also be required for the repression of T_H2-associated genes via recruitment of HDAC (Lee et al., 2012). Defects have also been observed in CD8 T cells as *Ets1*$^{-/-}$ CD8 T cells have reduced expression of CD8 (Muthusamy et al., 1995). The presence of mature DPs in the periphery may suggest that reduced CD8 expression is a result of failure to properly induce Runx3 to silence CD4 expression, which is important for CD8 SP development (Clements et al., 2006, Zamisch et al., 2009).

Given the importance of T cells to B cell development, it is not immediately clear whether defects in B cell development/function are due to intrinsic or extrinsic factors. In early B cell development, Ets1-deficient B cells in the bone marrow had a reduced capacity to transition from the pro-B to pre-B cell stage (Eyquem et al., 2004). A study by Wang et al (Wang et al., 2005) showed that *Ets1*$^{-/-}$ cultured splenic cells are hyper-responsive to TLR9 signaling and preferentially differentiate into IgM plasma cells in a B-cell-intrinsic manner. This enhanced plasma cell differentiation was also observed *in vivo* in other studies (Barton et al., 1998; Bories et al., 1995). Ets1 may also act as a regulator of T-bet expression in B cells as loss of Ets1 in B cells led to a defect in IgG2a isotype switching due to a failed induction of T-bet expression in response to IFNγ (Nguyen et al., 2012). Recently, conditional deletion of *Ets1* in B cells was generated to parse some of these B cell phenotypes (Sunshine et al., 2019). The authors found that *Ets1* cKO B cells were more activated and that there was an increased number of antibody-secreting cells present. Ultimately, Ets1 was found to be intrinsically required

in B cells and that combined defects across multiple immune subsets results in a greater autoimmune phenotype (Sunshine et al., 2019).

Ets1 has also been implicated in NK cell development. *Ets1*$^{-/-}$ mice have reduced numbers of both NK and NK progenitor cells (Barton et al., 1998; Ramirez et al., 2012). In early NK cell development, Ets1 was shown to be important for the induction of T-bet, ID2 and NK cell receptors NKp46, Ly49H and Ly49D. Reduced expression of these receptors led to a failure to degranulate upon stimulation, a result which likely explains the impaired cytolytic capacity of *Ets1*$^{-/-}$ NK cells observed by the Leiden lab (Barton et al., 1998; Ramirez et al 2012). Furthermore, our lab showed that despite the impaired activation phenotype, the remaining NK cells present in Ets1-deficient mice were hyper-responsive to IL-15 and displayed a phenotype characteristic of chronically activated cells (Ramirez et al., 2012).

Ets1 in NKT Cell Development

An early study by Walunas et al (Walunas et al., 2000) demonstrated that Ets1 is required for the development of CD4^{+} NK1.1^{+} NKT cells in the thymus, spleen and liver. *Ets1*$^{-/-}$ liver lymphocytes also had reduced Vα14 cDNA compared to wild-type mice, as measured by semi-quantitative PCR. Furthermore, this loss of NKT cells was not due to reduced expression of CD1d on DP thymocytes (Walunas et al., 2000). Similarly to NK cells, yet unlike T cells, NKT cells are unable to develop in the absence of Ets1. Given the plethora of defects observed in T cell development of germline-deficient mice, it could be possible that dysfunction prior to the DP stage could be affecting the

development and/or selection of NKT cells. The selection of *Ets1*$^{-/-}$ conventional T cells paired with normal levels of Vα8 cDNA might suggest that DP thymocytes lack the proper survival signals required to rearrange a more distal TCR α-chain. Indeed, introduction of a Vα14Jα18 transgenic TCR in *Ets1*$^{-/-}$ mice restored NKT numbers, although many of these NKTs were blocked at ST0, implicating a role for Ets1 after positive selection (Tai et al., 2020). Conditional deletion of the Pointed domain of Ets1 in T cells revealed that protein-protein interactions mediated by this domain are important for proper cytokine production of NKT cells in the and may act to repress a more Th17-like effector fate (Tai et al., 2020). Subsequent studies from this group showed that Ets1-deficient NKTs have impaired TCR signaling and differentiation capacity using gene-set enrichment analysis. Notably, *Ets1*$^{-/-}$ NKTs had reduced expression of PLZF and introduction of a GFP-PLZF retrovirus partially restored the differentiation of Vα14 TG/KO NKTs into CD44- and NK1.1-expressing subsets (Chuang et al., 2021). While Ets1 is critical for NKT development, these experiments demonstrate that Ets1 continues to play a role in their differentiation and homeostasis following positive selection.

Here, I continue to explore the functions of Ets1 using a conditional deletion model in iNKT1 cells. I demonstrate distinct roles for Ets1 in iNKT1 development independent of selection-associated defects in the thymus and liver. Overall, Ets1 regulates the adhesion gene profile of iNKT1 cells and acts to suppress the expression of lymphocyte migration genes by modulating the activity of the transcription factor T-bet. Similar to observations in CD8 T_{RM} cells, I show that TGFβ signaling is important for

the expression of CD103 and maturation of thymic iNKT1 cells, which may contribute to the phenotype of *Ets1*-deficient mice. The work presented in this dissertation contributes to our knowledge of tissue-resident lymphocytes, further corroborating downregulation of common lymphocyte migration genes while also highlighting unique functions of residency-associated genes across different tissue-resident immune cells.

Chapter Two

Materials and Methods

Mice

Ets1$^{f/f}$ mice were generated as previously described (Zook et al., 2016). The mice were crossed to Tbet-Cre (B6;CBA-Tg(Tbx21-cre)1Dlc/J, JAX#024507) and *Rosa26*-eYFP mice generated by Madisen et al. (Haddad et al., 2013; Madisen et al., 2010; Srinivas et al., 2001). B6;129-*Tgfbr2*^{tm1Karl}/J (*Tgfbr2*$^{F/F}$) mice were purchased from Jackson Labs (Leveen et al., 2002). For rescue experiments, a single *Tbx21*^{f} allele (B6.129-Tbx21^{tm2Srnr}/J, JAX#022741) was crossed into *ROSA26-YFP*+/- *Ets1*$^{f/f}$ *Tbx21*Cre+ mice. These and other control mice were housed at the University of Chicago under the guidelines of the University of Chicago Institutional Animal Care and Use Committee. All mice used were on the C57BL/6 background.

Antibodies and Flow Cytometry

Mice were sacrificed using CO_2 and thymus, spleen and liver were dissociated into single-cell suspensions using frosted glass microscope slides and passed through a mesh filter to remove debris. Liver lymphocytes were enriched using a Lympholyte-M (Cedar Labs, CL#5035) density gradient. Thymic NKT cells were isolated using magnetic enrichment, staining for PE- or APC-conjugated CD1d tetramer followed by incubation with anti-PE or anti-APC MicroBeads (Miltenyi Biotech). Labeled cells were enriched using the AutoMACS Pro Separator. Suspensions were incubated with fluorochrome-conjugated (PE, APC, PECy7, APCCy7, BV421, BV605, BV650, BV711)

antibodies against CD4, CD8α, TCRβ, CD45.1, CD45.2, CD24, CD44, NK1.1, CD69, LFA-1a, ICAM-1, CD49a, CD103, T-bet, IL-4, Gmzb, and IFNγ. Antibodies were purchased from Biolegend, eBioscience or Invitrogen. CD1d tetramer (PE/APC), unloaded or with PBS-57, was obtained from the NIH Tetramer Core Facility. For intracellular antibodes, cells were fixed following surface staining using either the BD Cytofix/Cytoperm Fixation Kit (Thermo Fisher, Cat#: BDB555028) for cytokines or the FoxP3 Permeablization Kit for transcription factors. Data was analyzed using FlowJo (TreeStar).

Mixed Bone Marrow Chimeras

CD45.2$^+$ *Ets1*$^{+/+}$ or *Ets1*$^{\Delta/\Delta}$ bone marrow (BM) was mixed with CD45.1$^+$ WT bone marrow at a 1:1 ratio. 2 x 10^6 cells from the *Ets1*$^{+/+}$ or *Ets1*$^{\Delta/\Delta}$ mixed BM was injected retro-orbitally into lethally irradiated CD45.1$^+$ host mice. At 8 weeks post-reconstitution, mice were analyzed for the reconstitution efficiency of total and YFP-expressing *Ets1*$^{\Delta/\Delta}$ NKT cells.

Cytokine Production Assays

For the *in vitro* cytokine assay, 2 x 10^6 cells from thymus and liver were transferred to 12-well cell culture plates and incubated with PMA (20ng/mL), ionomycin (1μg/mL) and GolgiPlug (1:1000) for 5 hours. Following incubation, cells were washed with FACS Buffer and stained as described for IL-4 and IFNγ. For *in vivo* assays, mice were injected with either 2.5μg αGalCer intravenously (KRN7000, Avanti Polar Lipids) or

MCMV Smith strain intraperitoneally at 5 x 10^4 PFU (colony-forming units) for 2 hours and 36 hours, respectively, before analysis.

RNA-seq Preparation and Analysis

Ets1$^{+/+}$ and *Ets1*$^{\Delta/\Delta}$ NKT1 cells were sorted from the thymus or liver and RNA was isolated using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. Libraries were constructed using Nugen's Ovation Ultralow Library Systems and were subsequently subjected to 76 cycles of NextSeq500 sequencing. Raw sequence reads were trimmed using Trimmomatic v 0.33 (TRAILING:30 MINLEN:20) (Bolger et al., 2014) and then aligned to mouse genome assembly mm10 with Tophat v 2.1.0 (Trapnell et al., 2009). Reads were assigned to genes using the high throughput sequencing count tool from HTSeq v 0.6.1 (Anders et al., 2015) and gene annotations from Ensembl release 78 (Kinsella et al., 2011). The R package EdgeR (Robinson et al., 2010) was used to normalize the gene counts and to calculate differential expression statistics for each gene for each pairwise comparison of sample groups. Genes were considered differentially expressed if the |fold change| ≥ 2 and the false-discovery rate (FDR), < 0.01. Gene set enrichment analysis was performed using gene sets from the Hallmark and ImmuneAll Pathways of MSigDB (Subramanian et al., 2005). RNA-sequencing data can be accessed in the Gene Expression Omnibus (for TGF-β, GSEXXXX).

Parabiosis

6-week-old CD45.1^{+} WT and CD45.2^{+} *Ets1*$^{\Delta/\Delta}$ female mice were anesthetized and had their circulatory systems surgically combined via a linear incision made from the scapulae to the lower abdomen on opposite sides of each pair. Mice were analyzed 4 weeks post-operation.

In Vivo Antibody Treatment

8-9 week *Ets1*$^{+/+}$, *Ets1*$^{\Delta/\Delta}$, and *Ets1*$^{\Delta/\Delta}$ T-bet$^{+/-}$ were co-housed for 3-4 days and injected retro-orbitally with 150µg of anti-mouse LFA-1α/CD11a (BioXCell, Cat#BE0006, Clone M17/4) or anti-mouse ICAM-1/CD54 (Cat#B0020-1, Clone YN1/1.7.4). Control mice were injected with either 150µg mouse IgG2a (Cat#BE0085, Clone C1.18.4) or 50µg of recombinant mouse IgG2a Fc (Cat#BE0097). Mice were analyzed one hour after injection.

S1PR Migration Inhibition Assay

Mice ranging from 6-8 weeks were co-housed and either Glucose (10g/L) or a combination of Glucose + 4-deoxypyridoxine (DOP, 30mg/L, Sigma-Aldrich, Cat#D0501) was administered via drinking water for 10 days. On day 11, mice were sacrificed and analyzed as described.

ATAC-seq Preparation and Analysis

Samples for ATAC-seq were prepared as described in Corces et al (Corces et al., 2017) with minor changes. 50,000 *Ets1*$^{+/+}$ and *Ets1*$^{\Delta/\Delta}$ NKT1 cells were sorted from the liver and washed in cold ATAC-RSB. After lysis, the cells were centrifuged at 500xg, the

supernantant was aspirated and the remaining nuclei were resuspended in 50µl of transposition mix (25µl 2x Tagment Buffer, 2.5µl Tagment DNA Enzyme, 22.5µl Nuclease-free H_2O, Illumina Cat#FC-121-1030) and incubated at 37˚C for 30 minutes. All subsequent steps were performed as described. Samples were submitted for single-end sequencing on the Illumina NovaSeq 6000 system. Indexing primers were dual (10/10) from the Illumina DNA/RNA UD Indexes Set A (Ref#20026121).

Read alignment positions were adjusted according to their strand: +4 bp for + strand alignments, and −5 bp for − strand alignments. We called open chromatin regions using Macs2 with the "—nomodel" option set (Zhang et al., 2008). Motif enrichment analysis was performed using HOMER (Hypergeometric Optimization of Motif EnRichment) (Heinz et al., 2010). Both positively and negatively enriched peaks identified as significant (FDR adjusted p-value<0.05) were converted into BED files and analyzed using the findMotifsGenome program in HOMER (size restricted to 200bp). Peaks were visualized using the Integrative Genomics Viewer software (Robinson et al., 2011).

Quantitative real-time PCR

RNA was isolated from sorted cells using the RNeasy Micro Kit (Qiagen) and reverse transcribed with Superscript III (Invitrogen). Quantitative RT-PCR was performed in an iCycler (Bio-Rad Laboratories) with SYBR Green Supermix (Bio-Rad Laboratories). Expression values were normalized to *Hprt* and were calculated by the ΔΔCt method. Primer sequences used are shown in Table 2.1.

Statistical Analysis

Unless otherwise noted a Student's *t* test was used to establish significant differences for in vitro and in vivo phenotypes. Statistical tests were calculated with GraphPad Prism software, where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

	Forward	Reverse
Klf2	ACCAACTGCGGCAAGACCTA	CATCCTTCCCAGTTGCAATGA
S1pr1	GTGTAGACCCAGAGTCCTGCG	AGCTTTTCCTTGGCTGGAGAG
S1pr5	GCCTGGTGCCTACTGCTACAG	CCTCCGTCGCTGGCTATTTCC
Zeb2	CATGAACCCATTTAGTGCCA	AGCAAGTCTCCCTGAAATCC

Table 2.1: List of primers used for qPCR analysis. All primers listed are in the 5' to 3' direction.

Chapter Three

Ets1 represses a T-bet dependent migration program and stabilizes the iNKT1 adhesome

Figure 3.2 data generated with Rosie Morman
Figure 3.3 data generated with Micke Sigvardsson and Elizabeth Bartom
Figure 3.5 data generated with Mun Chowdhury
Figure 3.6 data generated with Mark Maienschein-Cline

Introduction

Invariant natural killer T (iNKT) cells are a subgroup of tissue-resident, innate-like lymphocytes bearing a semi-invariant TCR (Vα14Jα18 paired with Vβ2, Vβ7 or Vβ8) that recognize glycolipid antigen presented on non-canonical MHCI-like protein CD1d. NKT cells in the liver are predominantly NKT1 and have the capacity to mount a strong local Th1 response. This response can be beneficial as hepatic NKT cells have been shown to play important roles in response to *Borrelia burgdorferi* and hepatitis C virus, combating infection through IFNγ production (Crosby and Kronenberg, 2018). However, in the context of chronic hepatitis B infection, persistently activated NKTs are also known to hinder regeneration and promote hepatic injury (Dong et al., 2007; Ito et al., 2003). Additionally, NKT cells play dual roles in non-alcoholic fatty liver disease (NAFLD) progression, combating excess lipid accumulation in early disease stages while contributing to the onset of non-alcoholic steatohepatitis (NASH) and NASH hepatocellular carcinoma (NASH-HCC) in advanced cases (Chen and Tien, 2020). Given the promising applications for iNKT cells in new therapeutic models (Heczey et

al., 2014; Xu et al., 2019), it is important to understand iNKT function in the liver and how they can be harnessed to combat disease.

Upon positive selection, iNKT cells exist in a PLZF^{hi} CD24^{+} (NKT0 or NKTp) state as they begin to differentiate into one of three primary effector lineages: NKT1 (PLZF^{lo} T-bet^{+}), NKT2 (PLZF^{hi} GATA3^{+}) or NKT17 (PLZF^{int} RORγt. At this stage, NKTps will either remain in the thymus and mature or emigrate and seed peripheral tissues where they will finish their maturation. For example, NKT cells expressing the chemokine receptor CXCR6 home to the liver where NK cells expressing leukocyte function-associated antigen-1 (LFA-1) facilitate entry of NKT cells into the liver (Doherty et al., 2016; Emoto et al., 1999; Emoto et al., 2000), at which point they will reside in the liver sinusoids via sustained interactions with LFA-1 and intracellular adhesion molecule 1 (ICAM-1).

The transcription factor Ets1 has been implicated to influence the development of lymphocytes based on restriction of its expression to immune cells in adult mice (Garrett-Sinha, 2013). Alongside its roles in development and survival, Ets1 is known to be involved with cell migration as it has been shown to be overexpressed in metastasizing cancers and plays a role in facilitating epithelial-mesenchymal transition (EMT) in these cells (Yalim-Camci et al., 2019). Ets1 has also been shown to be required early in iNKT (CD4^{+} NK1.1^{+}) cell development (Walunas et al., 2000) and the Pointed domain of Ets1 was recently shown to repress a Th17-like program (Tai et al., 2020). Ets1 is expressed at high levels in T and NKT cells throughout their lifetime

(Garrett-Sinha, 2013), however the functions of Ets1 following positive selection in NKT cells are not well characterized.

To investigate the role of Ets1 in iNKT1 cells, we examined the requirements for Ets1 in thymic and hepatic iNKT1 cells by creating a conditional knockout of *Ets1* specifically in the NKT1 effector lineage. Using this novel mouse model, found that Ets1 regulates expression of a broad range of genes involved EMT, migration and adhesion including the liver retention receptor ICAM-1. Mechanistically, we demonstrate that at least a subset of this program is maintained by Ets1-dependent restraint of the transcription factor T-bet. Indeed, dysregulation of T-bet supported LFA-1/ICAM1-independent residency of liver iNKT1 cells. Here, we provide additional insight into the roles of Ets1 in postselection NKT cells and set the stage for further analysis of how Ets1 regulates the adhesome of NKT1 cells.

Results

Ets1 is required for proper maintenance of thymic NKT1 numbers and IFNγ production in the liver

Ets1 is required for the development of $CD4^+$ $NK1.1^+$ NKT cells (Walunas et al., 2000) and protein interactions via the Pointed domain of Ets1 contribute to optimal cytokine production (Tai et al., 2020). However, the role of Ets1 in NKT cells independent of selection-associated defects has not been explored. To determine whether Ets1 is required for NKT cell maturation and homeostasis following positive selection, we generated a novel mouse model using a conditional deletion of *Ets1*

driven by *Tbx21*-Cre, allowing for targeted deletion in NKT1 cells. In the *Rosa26-YFP*+/- *Ets1*$^{f/f}$ *Tbx21*Cre+ (referred to as *Ets1* cKO) mouse thymus, we observed a significant reduction in the number of NKT1 (CD1d-Tet^{+} CD24^{-}/TCRβ^{+} YFP^{+}) cells compared to *Rosa26-YFP*+/- *Ets1 WT Tbx21*Cre+ (*Ets1* WT) mice. However, there was no change in the number of NKT1 cells present in the liver of *Ets1* cKO mice (Fig. 3.1A, 3.1B). To investigate whether *Ets1* cKO NKT cells were competent to generate mature NKT1 cells, we created mixed bone marrow chimeras in which *Ets1* cKO NKTs compete with wild-type NKT1 cells. We measured the reconstitution efficiency of NKT cells in the thymus and liver as a ratio of the frequency of CD45.2^{+} cells in the NKT cell compartment to that of the CD4^{+} CD8^{+} (DP) thymocytes, which do not yet express *Tbx21*-Cre. In the thymus, we observed a ~30% reduction in the reconstitution efficiency of NKT1 cells derived from the *Ets1* cKO bone marrow (Fig. 3.1C). In the liver, we found no difference in the ability of *Ets1* cKO NKT1s to reconstitute compared to CD45.1^{+}-derived WT NKT cells (Fig. 3.1D).

We questioned whether Ets1 was required for iNKT1 function, in particular, cytokine production. We examined IL-4 and interferon-gamma (IFNγ) production in NKT cells utilizing different pathways of activation: *in vitro* with PMA and ionomycin and *in vivo* via intravenous injection of α-galactosylceramide (αGalCer) or viral infection with MCMV. Following stimulation with PMA and ionomycin, we observed that a similar frequency of *Ets1* cKO and *Ets1* WT thymic iNKT cells produced IL-4 and IFNγ

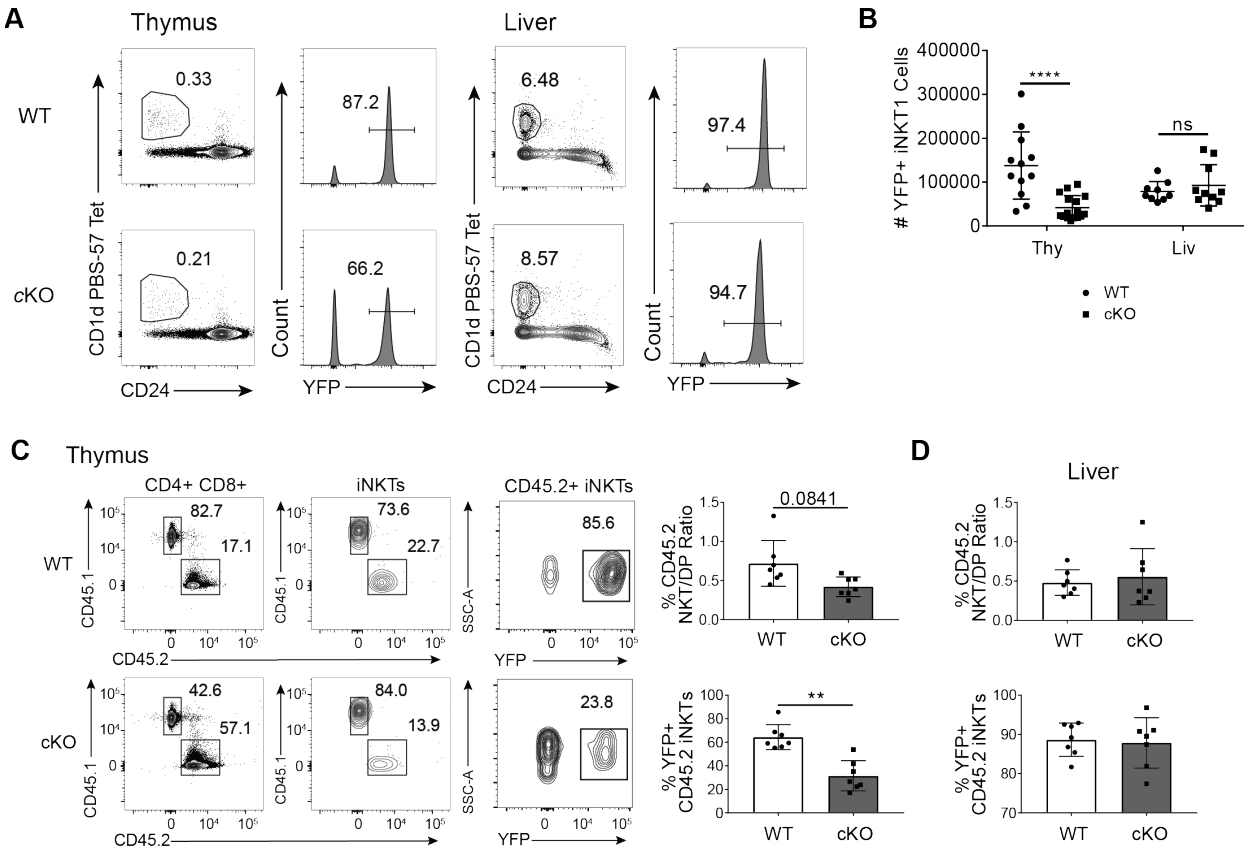

Figure 3.1: Ets1 plays a role in iNKT1 cells following positive selection. (A) Flow cytometry of 6-week old NKT1 (CD1d-Tet+ CD24- YFP+) cells in the liver. Expression of CD44 and NK1.1 were also examined in these mice. (B) Quantification of NKT1 cells in the liver at 6 weeks of age. (C-D) Mixed bone marrow chimeras in 8-10 week Ets1 WT and cKO mice and reconstitution efficiency of NKT cells compared to DP thymocytes in (C) thymus and (D) liver.

and that the MFI for these cytokines was unchanged (Figure 3.2A). In contrast, there was an increase in the frequency of IFNγ-producing NKTs in liver of *Ets1 cKO* mice after injection of αGalCer (Figure 3.2A). These activated liver NKTs also produced more IFNγ compared to *Ets1* WT NKTs (Fig. 3.2A).

A key characteristic of NKT cells is that they can be activated through TCR-mediated antigen presentation, cytokines produced by other immune cells or a combination of both. The differences in manner of activation allow for regulation of both response type and amount of cytokines produced in NKT cells (Brennan et al., 2013). Administration of the glycolipid agonist αGalCer resulted in a lower frequency of *Ets1 cKO* NKT1s producing IFNγ *in vivo* and of those IFNγ-producing cells, there was a reduced MFI for IFNγ compared to *Ets1 WT* NKTs (Fig. 3.2B). In a model of MCMV infection, NKT cell activation is dependent on production of IL-12 by Flt3L-dependent dendritic cells (Tyznik et al., 2014). Secreted IL-12 can stimulate IFNγ production in NKTs through a variety of pathways such as the JAK-STAT signaling pathway (Nan et al., 2017). *Ets1 WT* and *Ets1 cKO* mice were analyzed 36 hours post-MCMV infection and we found that a higher frequency of NKT cells produced IFNγ in *Ets1 cKO* mice (Fig. 3.2C). These results demonstrate that Ets1 continues to play a role in NKT1 cells following positive selection in a cell-intrinsic manner, potentially influencing proper maturation in the thymus and cytokine production in the liver.

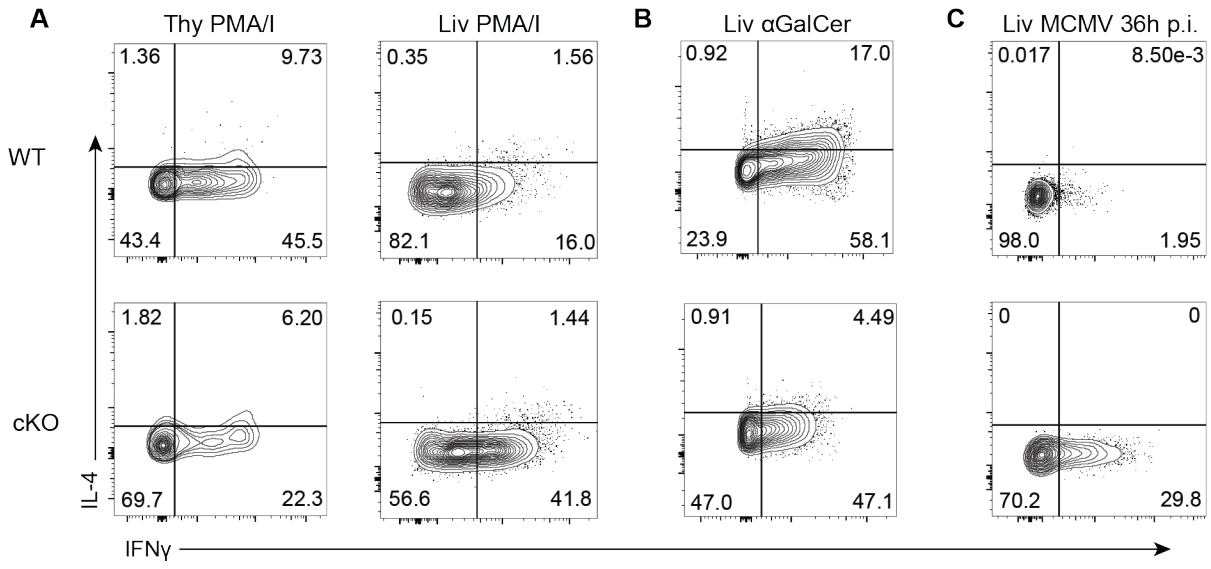

Figure 3.2: Ets1 regulates cytokine production of stimulated iNKT cells. Representative flow cytometry data from thymic and hepatic NKT cells activated via TCR-dependent and -independent approaches. (A) Total lymphocytes were isolated from thymus or liver and cultured in PMA and ionomycin for 5h prior to staining and analysis. (B) Mice were injected i.v. with 2.5ug of αGalCer and analyzed 2h post-injection. (C) Mice were injected with MCMV (Smith strain) i.p. and analyzed 36h post-infection

Ets1 Regulates the Adhesion Profile of NKT1 Cells

To rigorously determine the requirements for Ets1 in iNKT1 cells, we compared the transcriptome of *Ets1 cKO* NKT1s to control iNKT1 cells by RNA-sequencing. In the thymus, we identified 34 differentially expressed genes (DEG) (FDR, $p<0.01$), where 5 genes were upregulated and 29 were downregulated. For the liver, we found 309 differentially expressed genes, of which 170 genes were upregulated and 139 genes were downregulated (Fig. 3.3A). Given the greater number of DEGs in the liver compared to the thymus, we chose to focus analysis on the liver. Gene ontology analysis in the liver revealed that many of the DEGs were associated with cell migration, adhesion and NK cell activation (Fig. 3.3B). Gene set enrichment analysis also showed a high correlation between upregulated genes in *Ets1*-deficient liver NKTs and gene sets associated with $CD8^+$ T cell effector signatures, fatty acid metabolism and EMT (Fig. 3.3C). Of these genes, we observed that expression of *S1pr1*, *S1pr5* and *Sell*, key genes associated with lymphocyte migration, were increased in *Ets1*-deficient NKT1s. *Klf2*, a known activator of *S1pr1* expression, had increased expression in *Ets1 cKO* liver, but decreased expression in the thymus (Carlson et al., 2006, Fig. 3.3D). While T lymphocytes are developing in the thymus, expression of S1PR1 is carefully regulated by the presence of CD69. In regions of high CD69 concentration, CD69 will readily bind to S1PR1 on the cell surface, facilitating the internalization of S1PR1. In addition to repressing egress by S1PR1 internalization, an abundance of S1P lyase is present in the medulla to irreversibly degrade S1P ligand, inhibiting S1P binding to S1PR1. As T lymphocytes prepare to exit the thymus they express Klf2, promoting *S1pr1* expression

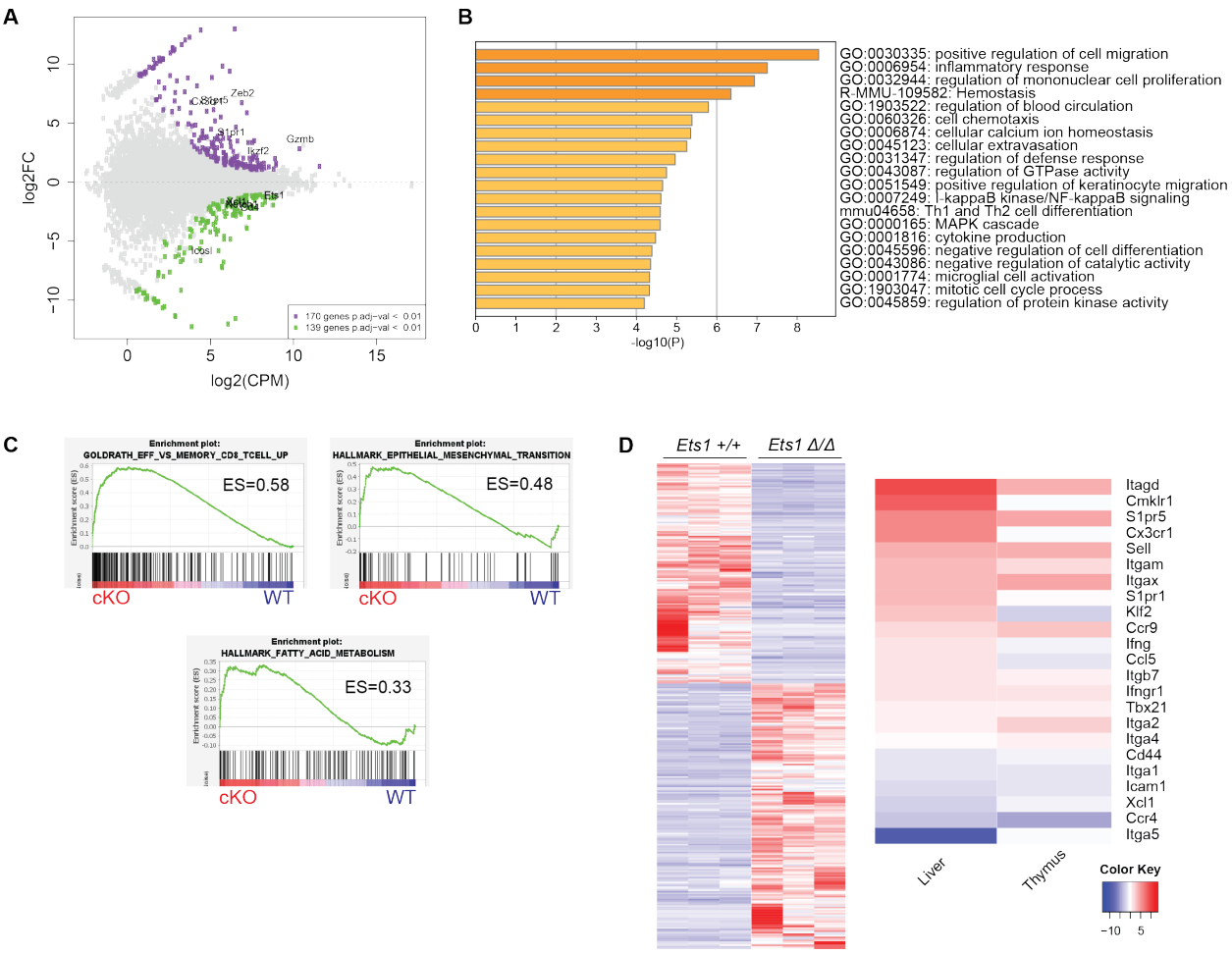

Figure 3.3: Ets1 regulates the adhesion profile of iNKT1 cells. RNA-seq analysis of 6-week *Ets1*$^{+/+}$ and *Ets1*$^{\Delta/\Delta}$ NKT1 cells. (A) Volcano plot of differentially expressed genes. 170 genes were upregulated and 139 were downregulated (FDR<0.01). (B) Gene ontology analysis of DEG in the liver. (C) Gene set enrichment analysis (GSEA) of DEGs in WT and KO liver using Hallmark and ImmuneAll pathways from MSigDB. (D) Gene expression changes in *Ets1*$^{+/+}$ and *Ets1*$^{\Delta/\Delta}$ iNKT1 cells across three replicates (left) and logFC of a list of select genes associated with migration/adhesion in NKTs (right).

and initiating their movement towards high concentrations of S1P ligand where S1PR signaling activates and emigration proceeds (Kumar et al., 2017). We found that a number of adhesion receptors were differentially expressed in *Ets1 cKO* NKT1s. Among the downregulated genes was *Icam1*, which is known to be important for retention of NKT cells in the liver (Thomas et al., 2011, Figure 3.3D).

To confirm whether these changes in gene expression resulted in changes at the protein level, we did flow cytometry analysis on thymus and liver NKT1 cells for multiple integrins and adhesion receptors. While CD69 was not differentially expressed by RNA-seq, we hypothesized that increased S1PR expression might negatively impact CD69 expression. Indeed, flow analysis showed reduced expression of CD69 as well as LFA-1α, ICAM-1 and CD44 compared to *Ets1 WT* NKT1s (Figure 3.4A). Surprisingly, we also identified a population of NKT1s in the liver that had increased expression of CD69 (Figure 3.4A). CD11b, CD11c and CD49b, integrins more commonly associated with NK cells, were found to have increased in expression on *Ets1* cKO NKT1s (Fig. 3.4B), although some changes in expression were only observed in a subset of liver NKT1 cells. Based on the results from the RNA-seq, Ets1 acts as a regulator of multiple adhesion receptors on NKT1 cells and suppresses expression of migratory genes.

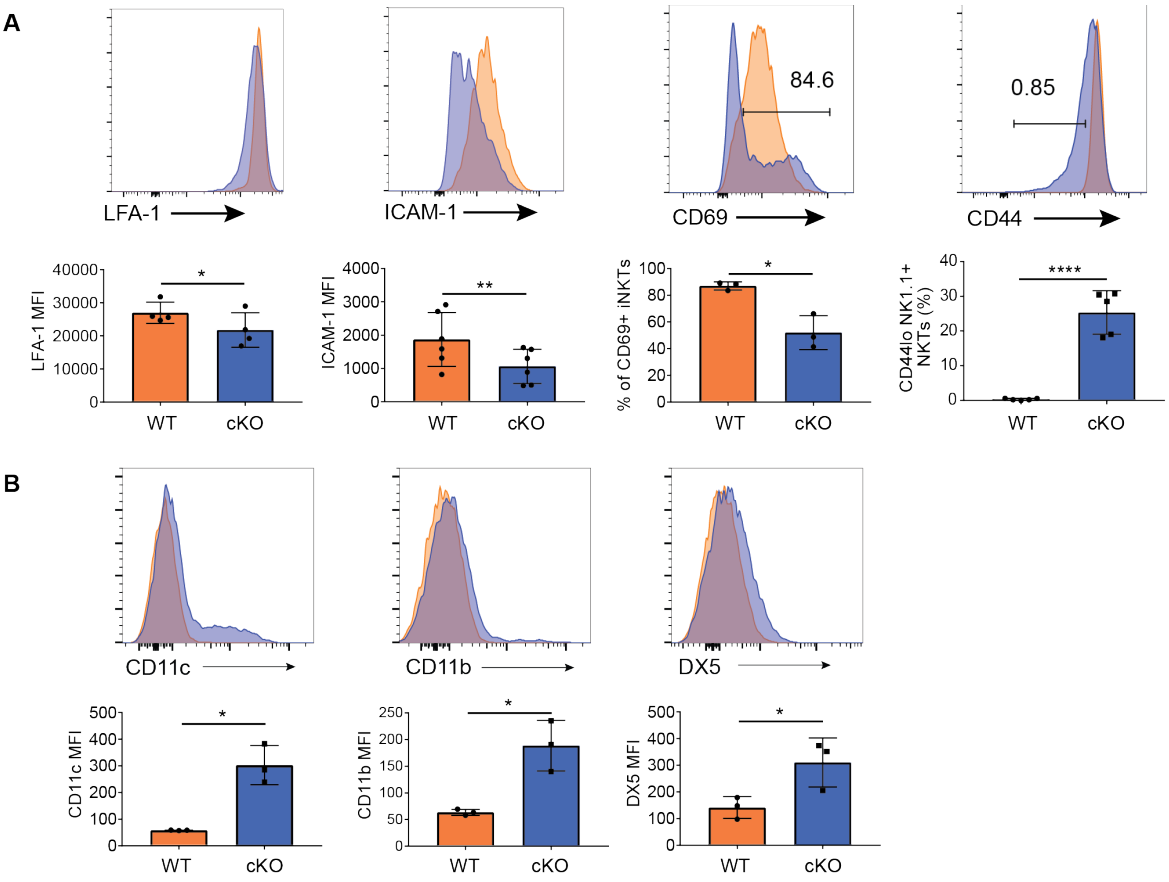

Figure 3.4: Ets1 regulates the adhesion profile of iNKT1 cells. Flow cytometry analysis of (A) adhesion receptors associated with NKT cell adhesion and retention in the liver (LFA-1, ICAM-1, CD69 and CD44) and (B) integrins associated with NK cell adhesion (CD11c, CD11b, DX5) in *Ets1* WT and cKO liver NKTs with summary data included below.

Ets1 enforces reliance on LFA-1 and ICAM-1 for liver retention

Our data indicate that Ets1 regulates the adhesome of iNKT1 cells and limits migration associated genes raising the question of whether these cells have altered retention or migration properties. However, *Ets1* cKO NKT1s in the liver are present at numbers similar to Ets1 WT mice. These observations raised the question of whether *Ets1* cKO iNKT1 cells continue to be tissue resident or whether they are migratory with a balanced input and output rate in the liver, for example, akin to T cells or NK cells (Thomas et al., 2011). To address this question, we performed a parabiosis experiment in which the circulatory system of *Ets1* cKO mice was connected to that of a WT CD45.1$^+$ mouse to allow for equilibration of cells that can migrate through the blood. Consistent with efficient parabiosis, CD45.1$^+$ and CD45.2$^+$ T cells were equally represented in the liver of both the CD45.2$^+$ *Ets1* cKO mouse and the CD45.1$^+$ WT mouse (Fig. 3.5A/B). In contrast, in the liver of both the CD45.1$^+$ WT and CD45.2$^+$ *Ets1* cKO mice, the majority of iNKT1 cells were host-derived. In *Ets1* cKO mice, on average, 88.8% of liver iNKT1 cells were CD45.2$^+$ and in the CD45.1$^+$ WT mouse 88.3% of liver iNKT1 cells were CD45.1+ (Fig. 3.5B). These results suggest that despite the dramatic changes in adhesion receptor expression, *Ets1* cKO liver NKT1 cells remain tissue resident.

A previous study by Thomas et al. demonstrated that that LFA-1α is important for the retention of NKT cells in the liver (Thomas et al., 2011). Given the decrease in LFA-1 and ICAM-1, an LFA-1 interacting adhesion molecule, we tested the requirement for these receptors for liver residency of *Ets1* cKO iNKT1 cells. To do this, we injected

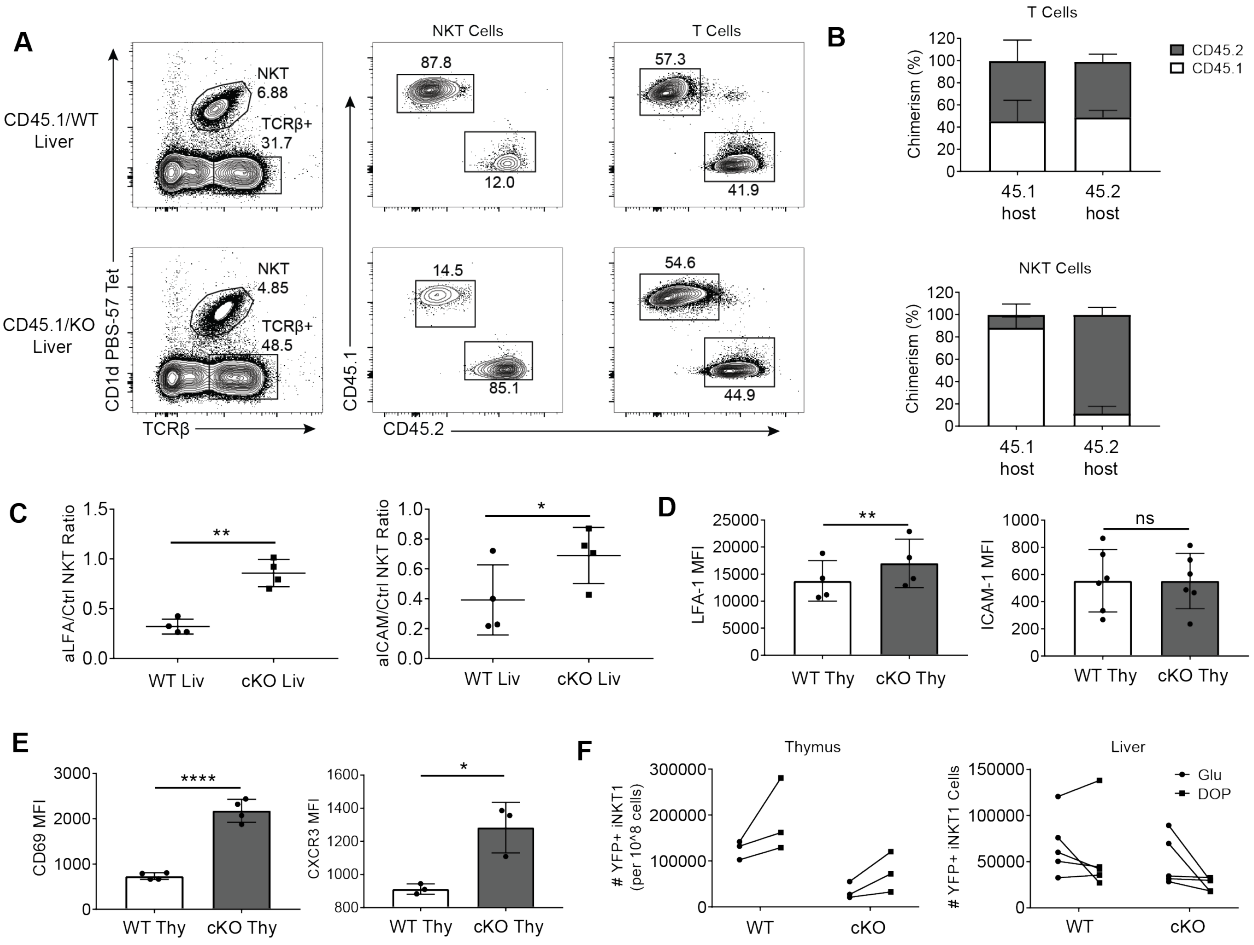

Figure 3.5: Ets1 enforces LFA-1/ICAM-1 mediated retention of liver iNKT1 cells.
(A) Parabiosis of CD45.1 WT and CD45.2 *Ets1* cKO mice. Representative frequencies of CD45.1+ and CD45.2+ populations for iNKT (center) and T (right) cells are shown. (B) Bar graph representation of CD45.1 and CD45.2 T cells (top) and iNKT cells (bottom) across three parabiotic replicates. (C) Mice were injected i.v. with 150ug of either anti-LFA-1 (left) or anti-ICAM-1 (right) and analyzed 1h later. Data represents the frequency of liver NKTs in treated mice relative to PBS/IgG2a isotype control mice. (D) Mean expression of LFA-1 and ICAM-1 in Ets1 WT and KO thymic iNKT1 cells. (E) Mean expression of thymic residency receptors CD69 and CXCR3 in Ets1 WT and KO thymic NKT1 cells. (F) Number of iNKT1 cells present in the thymus and liver of Ets1 WT and KO mice administered either glucose (Glu) or glucose with 5-deoxypyridoxine (DOP) via drinking water for 11 days.

Ets1 WT and *Ets1* cKO mice intravenously with anti-LFA-1α, anti-ICAM-1 or IgG2a isotype control antibody to determine whether *Ets1* cKO NKT1s still relied on this interaction for retention. We found that both LFA-1α and ICAM-1 were required for retention of NKT1 cells in Ets1 WT mice (Figure 3.5C). In contrast, neutralizing LFA-1 or ICAM-1 did not impact the number of iNKT1 cells in the liver of *Ets1* cKO mice (Fig. 3.5C), indicating that *Ets1* cKO NKT1 cells do not require LFA-1 or ICAM-1 interactions for retention. While NKT1 cells express both LFA-1 and ICAM-1, it may be likely that the interacting receptor expressed on each cell type is important. Data from Emoto et al., suggest that LFA-1α on NKTs is more important for retention than LFA-1α on endothelial cells (Emoto et al., 2000), which may corroborate our observations that anti-LFA-1α has a greater impact on NKT cell numbers than anti-ICAM-1.

Ets1-deficient thymic NKT1 cells do not show altered migration

The loss of NKT1 cells in thymus paired with the increased RNA of migratory genes led us to hypothesize that Ets1-deficient NKT1 cells emigrate from the thymus at an increased frequency. However, CD69 was highly expressed in thymic *Ets1* cKO NKT1s and we also observed that these cells had increased expression of CXCR3, a chemokine receptor associated with thymic retention of NKTs (Fig. 3.5E) (Drennan et al., 2009). To determine if NKT1s lacking Ets1 were more migratory, we utilized 4-deoxypyridoxine (DOP), an inhibitor of S1P lyase and S1PR signaling. DOP treatment resulted in an excess of S1P ligand, which promotes a negative feedback loop in S1PRs and leads to their subsequent degradation (Kumar et al., 2017). Following administration of DOP, we observed no difference in the number of NKT1 cells retained

in the thymus or liver compared to glucose-treated controls (Fig. 3.5F). Thus, like liver iNKT1 cells, *Ets1* cKO thymic NKT1s appear to remain tissue resident. As NKT1 numbers were significantly reduced in the thymi of *Ets1* cKO mice, Ets1 may have distinct roles in NKT1 function across different tissues.

Ets1 Restricts the Chromatin Access of T-bet Targets

The gene encoding the transcription factor T-bet, *Tbx21*, was upregulated in *Ets1* cKO liver and thymus NKT1 cells. T-bet expression was also increased in these cells when assessed by flow cytometry (Fig. 3.6A). We also found that the mRNA levels of adhesion and migration receptors that are known to be targets of T-bet were increased--including *Cxcr3* (Dhume et al., 2019), and *S1pr5* (Evrard et al., Fig. 3.6B). These data suggest that Ets1 may regulate expression of T-bet or targets of T-bet. To this end, we isolated nuclei from roughly 50,000 *Ets1* WT and *Ets1* cKO liver NKT1s and performed ATAC-seq to understand how Ets1 influences chromatin accessibility in NKT1 cells. Out of 63,599 identified peaks, 1,326 peaks were more accessible in *Ets1* WT iNKT cells and 2,334 peaks were more accessible in *Ets1* cKO NKT1s (Figure 3.6C). *S1pr5* was found to be more accessible across large regions of the locus in *Ets1* cKO liver (Fig. 3.6E). We also saw increased accessibility at the *Tox2* locus. Tox2 has been implicated as a direct activator of *Tbx21* in human NK cells (Vong et al., 2014). Motif analysis of these peaks using HOMER revealed that regions of increased accessibility in *Ets1* WT NKT1s were associated with Ets1 and Ets-family (Etv2) DNA binding motifs (Fig. 3.6D).

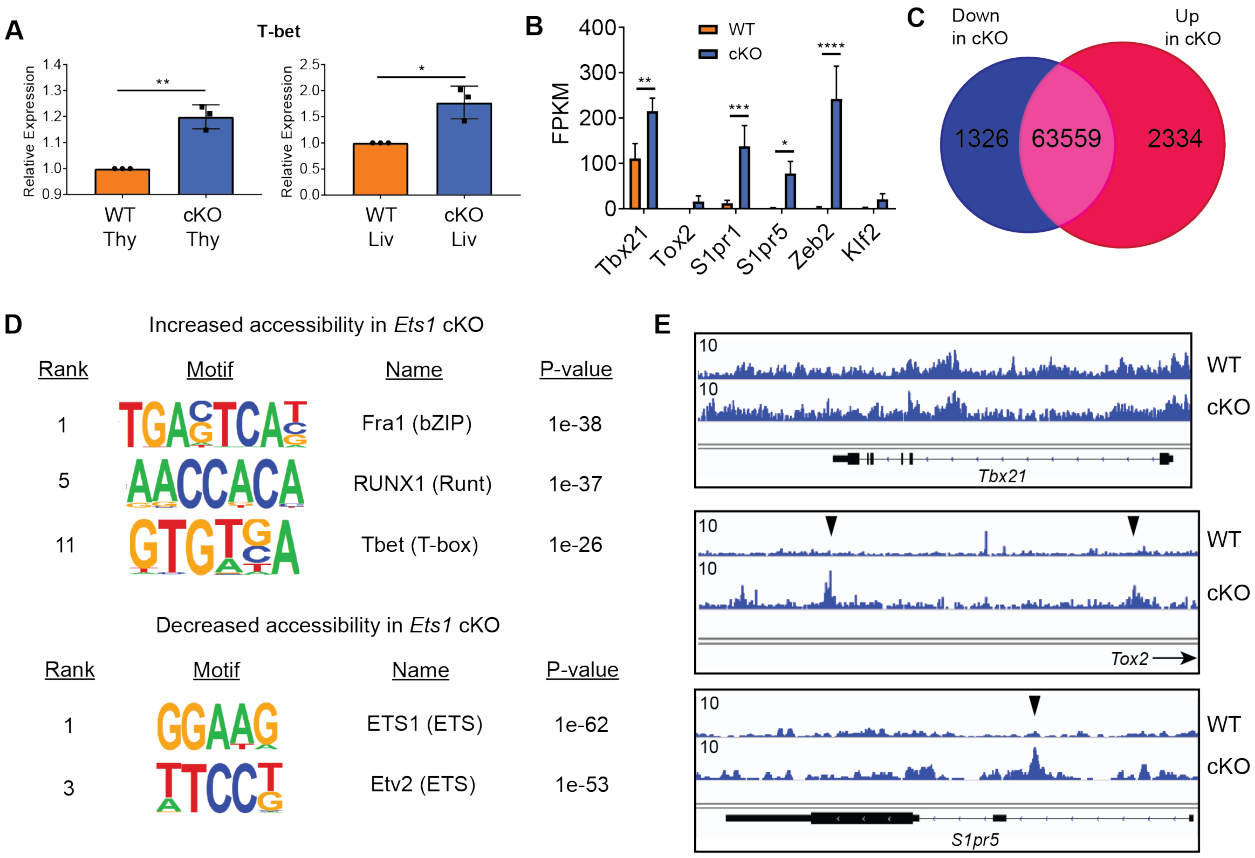

Figure 3.6: Ets1 restricts the chromatin access of T-bet targets. (A) Summary data showing mean expression of T-bet in thymus and liver relative to WT iNKTs. (B) RNA-seq analysis of T-bet and genes known to regulate T-bet or are direct targets of T-bet. (C) Differential chromatin accessibility in Ets1 cKO liver iNKT1 cells by ATAC-seq. The red circle represents the number of gene regions that saw increased accessibility in the KO, while the blue circle represents regions with reduced accessibility in KO NKTs (N=2). (D) Motif enrichment analysis of differentially accessible peaks using HOMER. Each rank shown represents the first unique transcription factor motif identified in the analysis. (E) Visualization of changes in chromatin accessibility at selected loci using the Integrated Genomics Viewer.

In regions that had increased accessibility in the *Ets1* cKO NKT1 cells, there was an enrichment for Fra1 (bZIP), RUNX1 (Runt) and T-bet (T-box) motifs (Figure 3.6D). As T-bet is the signature transcription factor of the NKT1 cell lineage, our data suggests that regulation of T-bet is an important component of Ets1 function in NKT cells.

T-bet represses LFA-1 and ICAM-1 dependence of tissue-resident liver iNKT1 cells

To determine whether the increased expression of T-bet contributed to gene dysregulation in *Ets1 cKO* NKT1s, we generated *Ets1 cKO* mice with one floxed allele of *Tbx21* that could be deleted by *Tbx21Cre* (*ROSA26-YFP*+/- *Ets1*$^{f/f}$ *Tbx21*$^{f/+}$ *Tbx21*$^{Cre+}$, referred to as *Ets1* cKO/Het). We found that *Ets1* cKO/Het iNKT1 cells had restored expression of ICAM-1 to wild-type levels. CD44 expression also increased on *Ets1* cKO iNKT1 cells. Interestingly, *Ets1* cKO/Het iNKT1 cells had increased CD69 expression beyond that of *Ets1* WT NKT1s (Fig. 3.7A). These data demonstrate that the increased expression of T-bet caused by deletion of Ets1 was contributing to the altered expression of ICAM-1, CD44 and CD69 on these cells.

Restored expression of CD69 and ICAM-1 led us to hypothesize that reduced T-bet in liver *Ets1* cKO/Het NKT1 cells may impact the requirement for LFA-1/CAM-1 for retention. To address this, we injected Ets1 cKO/Het mice with anti-ICAM-1 neutralizing antibodies. Treatment of *Ets1* cKO/Het mice with anti-ICAM-1 antibody results in a decreased frequency of NKT cells in the liver compared to treated *Ets1 cKO* liver (Fig.

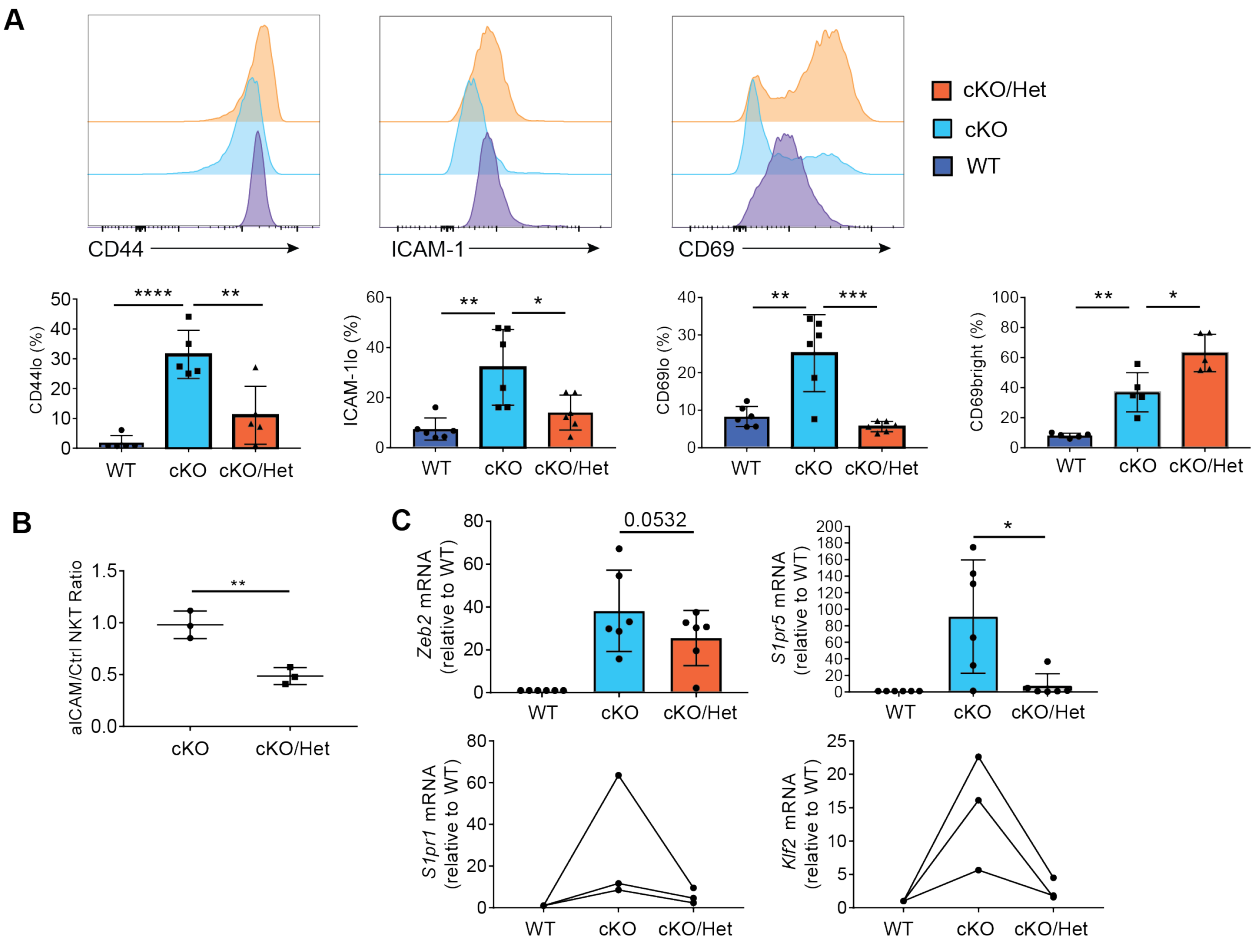

Figure 3.7: Reduced expression of T-bet restores the adhesive profile of iNKT1 cells. (A) Representative histograms of CD44, ICAM-1 and CD69 expression in iNKT1 cells from Ets1 WT, Ets1 cKO and Ets1 cKO/Het mice (top) with summary data (bottom). (B) Ets1 WT, Ets1 cKO and Ets1 cKO/Het mice were injected with 150ug of anti-ICAM-1 antibody and analyzed 1h post-injection. Data represented as the frequency of liver NKTs in anti-ICAM-1-treated mice to PBS/anti-IgG2a-treated control mice. (C) qPCR analysis of Zeb2, *S1pr5*, *S1pr1* and *Klf2* expression in sorted YFP-expressing WT, cKO and cKO/Het liver iNKTs.

3.7B). This observation suggests that reduced expression of T-bet restored the reliance of NKT1 cells on LFA-1/ICAM-1 interactions for retention in the liver.

To further explore the role of T-bet in the altered gene program of *Ets1 cKO* iNKT cells, we performed qPCR analysis of *Ets1 cKO* and *Ets1 cKO Tbx21*$^{\Delta/+}$ NKT1s. We first examined *Zeb2* mRNA, as Zeb2 is a direct target of T-bet and is involved in EMT (Yalim-Camci et al., 2019). In the liver, *Ets1* cKO NKT1 cells had increased *Zeb2* mRNA levels compared to wild-type, consistent with RNA-seq results (Fig. 3.7C). There was a partial reduction of *Zeb2* mRNA observed in *Ets1* cKO/Het NKT1 cells, suggesting that other factors may contribute to its transcription. We also analyzed *S1pr5* as it is a known target of T-bet in CD8^{+} T_{RM} cells (Evrard et al., 2021) and the increased expression of CD69 in *Ets1* cKO/Het NKT1 cells could likely be a result of reduced S1PR expression. As predicted, *Ets1 cKO Tbx21*$^{\Delta/+}$ NKT1s had a significant reduction in *S1pr5* mRNA, resembling wild-type mRNA levels (Fig. 3.7C).

Conclusions

Using a novel mouse model for Cre-mediated deletion of *Ets1* in NKT1 cells, we show that Ets1 regulates the adhesome of postselection NKT1 cells in the thymus and liver. Furthermore, we find that this regulation is due, in part, to modulation of T-bet, the signature transcription factor of the NKT1 lineage. This suggests that while T-bet is important for the development of NKT1 cells, its expression must be controlled to ensure proper development and maturation. Indeed, increased T-bet expression in *Ets1* cKO mice resulted in increased expression of migratory genes including *S1pr1, S1pr5, Sell* and *Klf2*. Loss of Ets1 resulted in a reduction in the number of NKT1 cells in the

thymus. However, this reduction in NKT1 cells was not due to increased thymic emigration as DOP experiments blocking S1PR-mediated migration had no change in the number of NKT1 cells retained in the *Ets1* cKO thymus. These results may be indicative of a developmental defect. As we see significant changes in the adhesion genes expressed in *Ets1* cKO NKT1 cells, it is possible that NKT1 cells are localizing to different regions within the thymus, effectively altering their access to key cytokines required for development. To follow up on these initial results, it would be beneficial to do immunofluorescence staining of the thymus for NKT1 cells to assess potential changes in localization within the tissue. While we see an overall increase in the amount of T-bet expression in *Ets1* cKO NKT1 cells in the thymus, early data suggests that T-bet expression in highest in the most mature subset of NKT1 cells. This could indicate that thymic NKT1 cells are impaired at this developmental stage.

In the liver, we observed expression changes in multiple adhesion receptors. LFA-1 and ICAM-1, whose interactions are important for liver retention, had reduced expression in *Ets1* cKO NKT1 cells. We also found that NKT1 cell no longer had homogenous expression of CD69. Rather, two distinct populations of $CD69^{lo}$ and $CD69^{hi}$ cells emerged in the liver. Moreover, when analyzing expression of NK cell-associated integrins, we found that DX5, CD11b and CD11c had slightly higher expression in *Ets1* cKO NKT1s. Interestingly, there was a small population of NKT1 cells that had much higher expression of CD11c. While CD11c has not been studied extensively in T cells or NKT cells, it has been associated with more activated, cytotoxic T cells in a murine RSV model (Beyer et al., 2005) and increased migratory potential in

humans (Qualai et al., 2016). This data suggests that there is increased heterogeneity of NKT1 cells in *Ets1* cKO liver. In order to better characterize this heterogeneity, we could create a flow cytometry staining panel encompassing as many of our receptors of interest to identify a molecular signature associated with this heterogeneous population. This, in turn, could allow for a more direct correlation between expression of certain surface receptors and any altered localization within the tissue. Parabiosis experiments demonstrated that while *Ets1* cKO NKT1 cells have a more migration-associated transcriptional profile, they do not appear to be more migratory. However, *Ets1* cKO NKT1 cells no longer required LFA-1 or ICAM-1 to be retained in the liver following injection of anti-LFA-1/ICAM-1 antibodies. How these cells retain themselves in the liver in the absence of these signals remains to be determined.

ATAC-seq analysis of *Ets1* cKO NKT1 cells in the liver revealed an enrichment of bZIP, Runx and T-bet DNA binding motifs in accessible regions of chromatin. In conjunction with RNA-seq data highlighting a number of genes that are direct or indirect targets of T-bet, we hypothesized that Ets1 may act to regulate migration and adhesion genes through T-bet. Indeed, flow cytometry analysis of *Ets1* cKO/*Tbx21* Het mice showed restored expression of ICAM-1, CD69 and CD44. Additionally, reduced expression of T-bet restored the reliance of hepatic NKT1 cells on LFA-1/ICAM-1 for retention. To further explore this, it may be possible to determine whether this restored interaction is a result of increased LFA-1 and ICAM-1 expression or another T-bet dependent mechanism. One of the most outstanding questions regarding this work pertains to this data: if *Ets1* cKO NKT1 cells no longer need LFA-1/ICAM-1 interactions

to be retained in the liver, what do they use? While modulation of T-bet represents a subset of the overall Ets1 phenotype, we propose leveraging cKO RNA-seq analysis with RNA-seq of cKO/Het to identify a list of putative targets of T-bet. Genes of interest could be further studied to elucidate how T-bet regulates its downstream targets in NKT1 cells. qPCR analysis showed that cKO/Het NKT1 cells had *S1pr5* mRNA level resembling that of WT. While not achieving statistical significance, early data suggests that *S1pr1* and *Klf2* mRNA in cKO/Het NKT1 cells is also back down to WT levels. RNA-seq of cKO/Het NKT1 cells would also confirm these results.

Chapter Four

Transforming growth factor beta receptor controls the postselection thymic differentiation and peripheral function of interferon-γ-producing invariant natural killer T cells

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Introduction

Invariant natural killer T (iNKT) cells are a subset of innate like T lymphocytes that express an invariant T cell receptor that recognizes glycolipid antigens in the context of the non-classical major histocompatibility complex protein CD1d (Shissler and Webb, 2019). These cells comprise a large portion of T lymphocytes in peripheral tissues. As much as 40% and 5% of liver T cells are iNKT cells in mice and humans (Crosby and Kronenberg, 2018; Syn et al., 2010), respectively, the majority of which are tissue resident, interferon (IFN)-γ and interleukin (IL)-4 – producing, iNKT1 cells (Crosby and Kronenberg, 2018; Lee et al., 2015). These cells patrol the sinusoidal space to protect against invading pathogens such as the spirochete *Borrelia burgdorferi*, the causative agent of Lyme's Disease, and viral infection such as Hepatitis C Virus. (Crosby and Kronenberg, 2018; Thomas et al., 2011; Umeshappa et al., 2022) However, chronic activation of iNKT cells is associated with disease severity in

nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Crosby and Kronenberg, 2018; Syn et al., 2012; Syn et al., 2010). iNKT cells have also been implicated in protection from pathogens in the lung and other tissues but can lead to inflammatory disease such as asthma and atherosclerosis.

iNKT cells develop in the thymus from $CD4^+CD8^+$ thymocytes after rearrangement of the iNKT cell receptor (Bendelac et al., 2007; Benlagha et al., 2002). During their selection, iNKT cells are recruited to the thymic medulla by CCL21 and a subset of these immature postselection iNKT cells emigrate from the thymus to seed peripheral tissues (Baranek et al., 2020; Harsha Krovi et al., 2020; Wang and Hogquist, 2018). However, some of these cells remain in the thymus medulla where they differentiate into thymic resident IFN-γ producing iNKT1 cells, IL-4 producing iNKT2 or IL-17 producing iNKT17 cells, although some iNKT cells can make both IFN-γ and IL-4 (Engel et al., 2016; Lee et al., 2016). Cytokines produced by thymic iNKT cells, in particular IL-4, have the potential to impact multiple cell types including CD8 T cell and dendritic cells (Breed et al., 2022; Verykokakis et al., 2010; Weinreich et al., 2010). While much research has gone in to understanding the mechanisms of iNKT cell selection we know much less about the functions of postselection iNKT cells in the thymus, the mechanisms that promote their thymic residency or how tissue specific factors impact iNKT cell differentiation and function.

Here, using a novel model for gene deletion in postselection iNKT1 cells, we demonstrate that thymic iNKT1 cells differ from liver and spleen iNKT1 cells by expression of a transforming growth factor (TGF)-β gene signature and reduced

expression of cytokine signaling-associated gene signatures. While TGF-β is known to regulate iNKT cell selection (Doisne et al., 2009), we demonstrated that TGF-β signaling in postselection thymic iNKT1 cells enforced a classic TGF-β-associated gene signature, including repression of migratory genes and induction of multiple residency-associated adhesion receptors, and repressed a cytokine signaling-associated gene signature. Moreover, postselection iNKT1 cells that cannot respond to TGF-β due to deletion of *Tgfbr2* remained thymic resident but failed to differentiate into cells that expressed the residency-associated adhesion receptors CD49a and CD103. In contrast to studies in which *Tgfbr2* was deleted in other IFN-γ producing cell types, including preselection iNKT cells (Doisne et al., 2009), NK cells (Viel et al., 2016), or CD8 cells differentiated into tissue resident memory cells (Trm) (Crowl et al., 2022; Mackay et al., 2013; Mackay et al., 2015), we found no role for TGF-β signaling in the control of the transcription factor T-BET or T-BET target genes. Despite the lack of a TGF-β gene signature in liver iNKT1 cells, TGF-β signaling was required for their optimal expression of CD49a and impacted their production of IFN-γ and IL-4 in response to antigen stimulation. Our data reveal a selective requirement for TGF-β signaling in the postselection generation of thymic $CD49a^{+}CD103^{+}$ iNKT1 cells and optimal liver iNKT1 function.

Results

Thymic iNKT1 cells express genes associated with TGF-β signaling.

To create a system in which genes could be deleted postselection in iNKT1 cells we crossed a *Tbx21*^{Cre} bacterial artificial chromosome transgenic mouse to Rosa26-Stop-floxed-YFP transgenic mice (Haddad et al., 2013; Srinivas et al., 2001). This mouse model allowed us to isolate iNKT cells with a history of *Tbx21* expression (iNKT1 cells) in the thymus, liver, and spleen and compare their gene programs by RNA-sequencing (Fig. 4.S1). A prior study demonstrated that iNKT1 cells isolated from different tissues, using a different selection strategy, had essentially identical gene programs (Murray et al., 2021). Consistent with this observation, we found that the transcriptome of liver and spleen iNKT1 cells were nearly identical with < 20 differentially expressed genes (DEGs) (Fig. 4.1A). However, the transcriptome of thymus and liver iNKT1 cells differed by 124 genes (adj.p < 0.01) whereas thymus and spleen differed by 107 genes (Fig. 4.1A). While these are relatively small differences, there were some notable genes that were differentially expressed. Using Gene Set Enrichment Analysis (GSEA), we observed a significant enrichment (FDR < 25%) of multiple Hallmark pathways in the liver as compared to the thymus including in the IL6-JAK-STAT3 signaling, mTORC1 signaling and fatty acid metabolism (Fig. 4.1B, 4.S1). As expected, the enriched pathways were similar but not identical in the liver and the spleen (Fig. 4.S1).

The GSEA algorithm tests for the enrichment of genes from an *a priori* specified

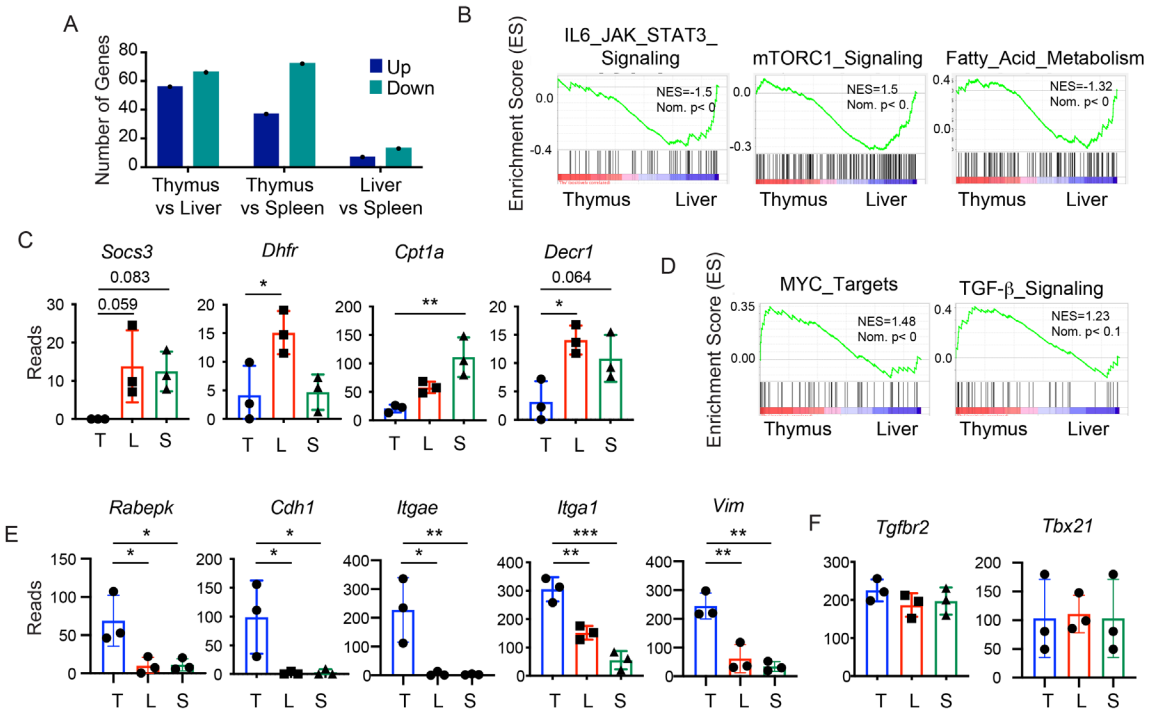

Figure 4.1: Thymic iNKT1 cells are enriched for genes associated with TGF-β signaling compared to liver and spleen iNKT1 cells. RNA was sequenced from WT iNKT1 cells isolated from the thymus, liver and spleen and normalized read counts were compared. (A) Number of genes that were differentially expressed between WT thymus, liver and spleen iNKT1 cells. (B) Hallmark Pathways identified by GSEA as enriched in liver compared to thymus iNKT1 gene sets (C) Reads for 3 replicate RNA-seq samples from WT thymus (T, blue), liver (L, red) and spleen (S, green) for *Socs3*, *Dhfr*, *Cpt1a* and *Decr,* which are representative genes from the pathways identified in (B). (D) Hallmark Pathways identified as enriched in thymus as compared to liver iNKT1 gene sets. (E) Reads from replicate RNA-seq samples showing genes representative the pathways identified in (D). (F) RNA-seq reads for Tgfbr2 and Tbx21. *P<0.05, **<P0.01, ***P<0.005 by ANOVA with multiple comparisons.

gene set at the extreme ends of a differential gene expression analysis; however, DEGs in the leading edge of this analysis may not reach statistical significance on their own based on different statistical methods. Therefore, we examined some of the DEGs in our RNA-seq analysis using EdgeR or Anova. In the IL6-JAK-STAT3 pathways, *Socs3* mRNA, encoding a suppressor of cytokine signaling that binds STAT3, was increased in liver and spleen compared to the thymus (Fig. 4.1C). In the mTORC1 pathway, which was not an enriched pathway in the thymus to spleen comparison, *Dhfr* mRNA was increased in the liver but not the spleen (Fig. 4.1C, 4.S2). Fatty acid metabolism is required for survival of CD8 Trm cells in the skin (Pan et al., 2017), and CD8 Trm cells in different tissues utilize distinct fatty acid binding proteins to uptake fatty acids (Frizzell et al., 2020). We did not observe any differences in mRNA encoding fatty acid binding proteins, however, mRNA encoding enzymes in this pathway, including *Cpt1a* and *Decr1* mRNAs, encoding carnitine palmitoyltransferase 1A and 2,4 dienoyl-CoA reductase respectively, were higher in both the liver and spleen compared to the thymus even though GSEA identified this pathway as enriched only in the liver (Fig. 4.1C, 4.S1). These data suggest that liver and spleen iNKT1 cells have active IL6-JAK-STAT3 signaling and at least the liver may utilize fatty acid metabolism but that these pathways are less active in thymic iNKT1 cells. Interestingly, by GSEA, thymic iNKT1 cells were significantly enriched for only the hallmark MYC-Targets pathway but this gene set included only 10 genes, of which only *Rabepk*, encoding a mannose 6-phosphate receptor transport protein, reached statistical significance by Anova (Fig. 4.1D, E).

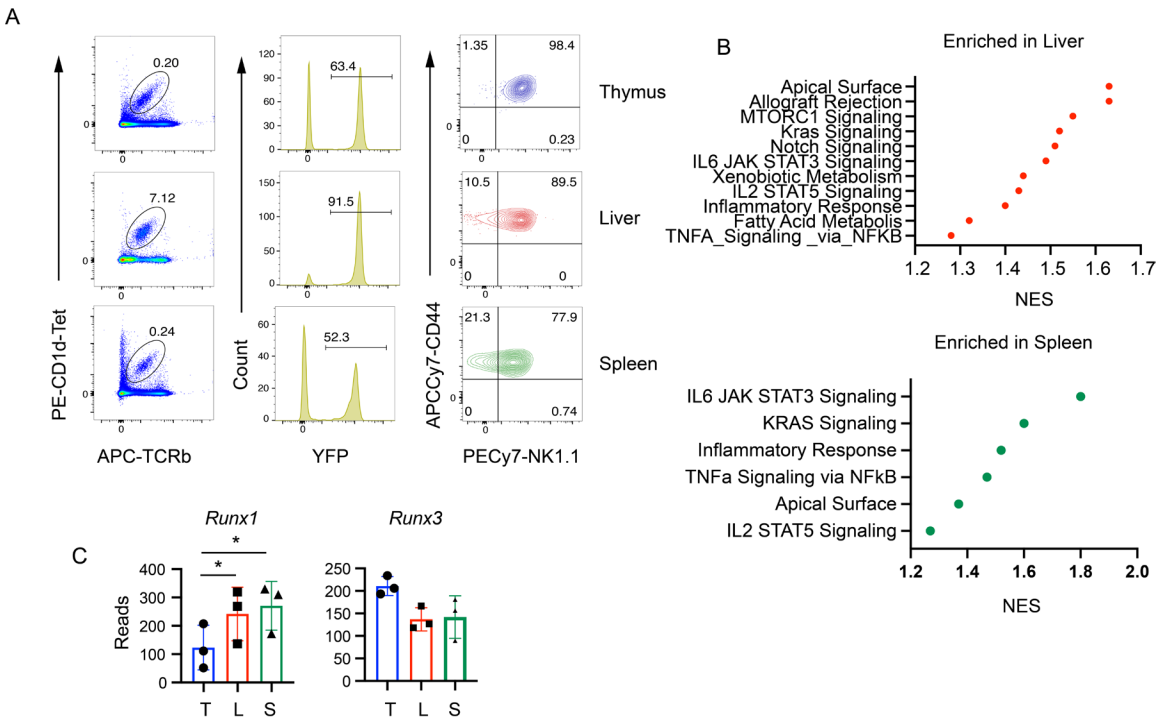

Figure 4.S1: Validation of the Tbx21Cre mouse. Flow cytometry analysis of Tbx21Cre mice by analysis Rosa26-flox-stop-flox-YFP in iNKT cells from the thymus, liver and spleen. iNKT cells were identified as CD1d-Tet+ and TCRb+. YFP+ iNKT cells were then examined for expression of NK1.1 and CD44. Mature iNKT1 cells express NK1.1 and progenitors that are T-BET+ can be found among CD44+NK1.1- cells.

In contrast, the TGF-β signaling pathway did not quite reach statistical significance by GSEA but multiple genes in this pathway were significantly differentially expressed by RNA-seq (Fig. 4.1D, E). These genes include *Cdh1*, encoding Cadherin 1, *Itgae*, encoding the alpha chain of the integrin CD103, *Itga1*, encoding the alpha chain of CD49a, and *Vim*, encoding vimentin (Fig. 4.1D). *Tgfbr2*, encoding a component of the TGF-β receptor, was expressed similarly in thymus, liver, and spleen iNKT1 cells suggesting that this difference in TGF-β signaling-associated genes is not a consequence of differences in TGF-β receptor expression. *Tbx21*, encoding the transcription factor T-BET, which is required for development of iNKT1 cells and CD8 Trm cells but is dampened by TGF-β signaling in many cell types, was not differentially expressed (Crowl et al., 2022; Li et al., 2006b; Mackay et al., 2015; Nath et al., 2019). Taken together, these data indicate that while the transcriptome of iNKT1 cells is very similar in the thymus, liver and spleen, there are DEGs associated with cytokine signaling, metabolism, and TGF-β signaling.

Differential expression of adhesion receptors on thymic and liver iNKT1 cells.

The enrichment of TGF-β signaling associated receptors was of interest given that iNKT1 cells are tissue resident cells with hybrid NK cell, ILC1, and CD4 and CD8 Trm cell characteristics (Verykokakis et al., 2014). Response to TGF-β was recently shown to depend on *Runx3*, which is expressed in CD8 Trm but not CD4 Trm, explaining their differential response to TGF-β (Fonseca et al., 2022). Our RNA-seq data revealed a modest increase in *Runx1* mRNA in liver and spleen as compared to

thymus iNKT1 cells and broad expression of *Runx3* mRNA in all iNKT1 cells, consistent with their ability to respond to TGF-β (Fig. 4.S1). *Itgae* and *Itga1* encode the alpha chains of the integrins CD103 and CD49a, respectively, both of which are induced on subsets of CD8 Trm cells by TGF-β produced from epithelial cells (Fonseca et al., 2022; Hadley et al., 1999; Mokrani et al., 2014; Zhang and Bevan, 2013). By flow cytometry we confirmed that CD103 was expressed on a subset of iNKT1 cells in the thymus but not in the liver (Fig. 4.2A, B). CD49a was expressed on both thymic and liver iNKT1 cells although at a lower frequency and lower MFI on liver iNKT1 cells (Fig. 4.2A, B). In contrast, CD69, a protein expressed on tissue resident cells that functions as an inhibitor of the S1P receptor S1P1 (Shiow et al., 2006; Stein et al., 2021), was highly expressed on both thymic and liver iNKT1 cells with a subtle increase on liver iNKT1 cells (Fig. 4.2A, B). A substantial population of thymic iNKT1 cells expressed CD49a along with CD103 and CD69 although there were populations that expressed only one or none of these receptors (Fig. 4.2A). These receptors are associated with tissue residency of CD8 T cells; however, liver iNKT1 cells are known to require LFA-1 and ICAM-1 to maintain tissue residency (Thomas et al., 2011). Notably, the MFI for ICAM-1 and LFA-1 on liver iNKT1 cells was higher than on thymic iNKT1 cells (Fig. 4.2C). CD44, another adhesion receptor associated with mature iNKT cells (Benlagha et al., 2005), was also expressed at slightly higher levels on liver as compared to thymic iNKT1 cells (Fig. 4.2C). These data reveal striking differences in the tissue residency-associated adhesion programs of thymic and liver iNKT1 cells and are consistent with a TGF-β induced program in the thymus.

TGF-β signaling is required for development of CD49a^{+}CD103^{+} thymic iNKT1 cells.

Previous studies demonstrated a requirement for TGF-β during selection of iNKT cells and a possible role for TGF-β in promoting iNKT17 differentiation while limiting iNKT1 differentiation (Doisne et al., 2009; Havenar-Daughton et al., 2012; Li et al., 2006a). However, in those studies, *Tgfbr2* was inactivated prior to T cell receptor-mediated selection making it difficult to dissociate effects on selection from effects on differentiation. To address the role of TGFβRII on iNKT1 cell development postselection, we crossed *Tbx21^{Cre} Rosa26-StopFlox-YFP* mice to mice with floxed alleles of *Tgfbr2* to create *Tgfbr2$^{F/F}$ Tbx21^{Cre} Rosa26-StopFlox-YFP* (cKO) mice (Srinivas et al., 2001). In these mice, the total number of YFP^{+} iNKT cells (iNKT1) in the thymus was decreased by approximately 30% compared to *Tbx21^{Cre} Rosa26-StopFlox-YFP* (Ctrl) mice (Fig. 4.3A. B). The iNKT1 cells that were present in the cKO thymus lacked expression of CD103 but the number of CD49a^{+}CD103^{-} cells was not affected suggesting that CD49a^{+}CD103^{+} iNKT1 cells failed to develop. The MFI of CD49a on CD49a^{+}CD103^{-} iNKT1 cells was reduced indicating that TGFβRII was also required for proper expression of CD49a (Fig. 4.3C, D). Interestingly, CD69 continued to be expressed on the majority of cKO iNKT1 cells but the number of CD49a^{-}CD69^{-} cells was increased, consistent with the loss of some CD49a^{+}CD69^{+} iNKT1 cells (Fig. 4.3C, D).

In CD8 Trm cells, CD4 Th1 cells and NK cells, TGF-β signaling dampens the expression of the transcription factor T-BET, despite T-BET being required for the development of these cells (Crowl et al., 2022; Li et al., 2006b; Mackay et al., 2015;

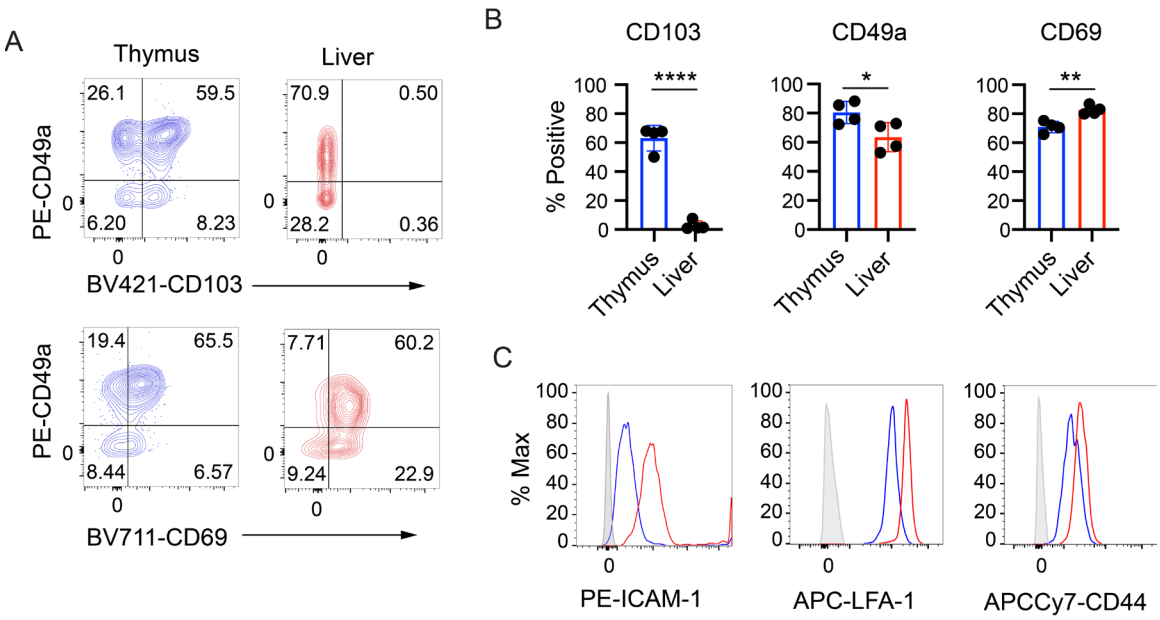

Figure 4.2: Thymic iNKT1 cells uniquely express TGF-β-associated adhesion proteins. (A) Flow cytometry for CD49a versus CD103 (top) or CD69 (bottom) on iNKT1 cells from the thymus (blue) or liver (red). n = 4 (B) Summary of multiple flow cytometry experiments showing the percent of iNKT1 cells that are positive for CD103, CD49a or CD69 in the thymus (blue) or liver (red). Each data point is an independent mouse. *P<0.05, **<P0.01, ***P<0.005 by Student's t-test. (C) Representative flow cytometry histograms for ICAM-1, LFA-1 or CD44 on thymus (blue) or liver (red) iNKT1 cells. Data are representative of 3 experiments.

Viel et al., 2016). T-BET is also a central regulator of iNKT1 cell development and therefore we examined its expression in iNKT cells from Ctrl and cKO mice (Matsuda et al., 2006; Townsend et al., 2004). We examined total thymic iNKT cells for expression of the signature transcription factor PLZF and T-BET, which allowed for resolution of iNKT1 (T-BET^{+}PLZF^{lo}), iNKT2 (T- BET^{-}PLZF^{high}), and iNKT17 (T-BET^{-}PLZF^{lo}) cells (Kovalovsky et al., 2008; Lee et al., 2013; Savage et al., 2008). The frequency of T-BET^{+}PLZF^{lo} iNKT1 cells was lower among cKO iNKT cells than in Ctrl iNKT cells, as expected given that there are fewer iNKT1 cells in cKO mice (Fig. 4.3E, F). However, regardless of whether we gated on all iNKT cells or CD122^{+} T cells, which should enrich for iNKT1 cells, the MFI of T-BET in T-BET expressing cells was not altered indicating that TGF-β was not impacting T-BET expression (Fig. 4.3E, F). Taken together, these data demonstrate that TGFβRII is required for proper development of thymic iNKT1 cells and for their expression of CD103 and CD49a but, unlike other T-BET+ lymphocytes, TGF-β signaling does not affect expression of T-BET.

TGFβRII promotes a gene program associated with tissue residency in thymic iNKT1 cells.

To gain a global view of the gene program promoted by TGF-β signaling in thymic iNKT1 cells we performed RNA-sequencing. We found 110 DEGs (adj. $p < 0.01$) between Ctrl and cKO thymic iNKT1 cells, 68 that were more highly expressed in Ctrl and 42 that were more highly expressed in cKO iNKT1 cells (Fig 4.4A). GSEA revealed

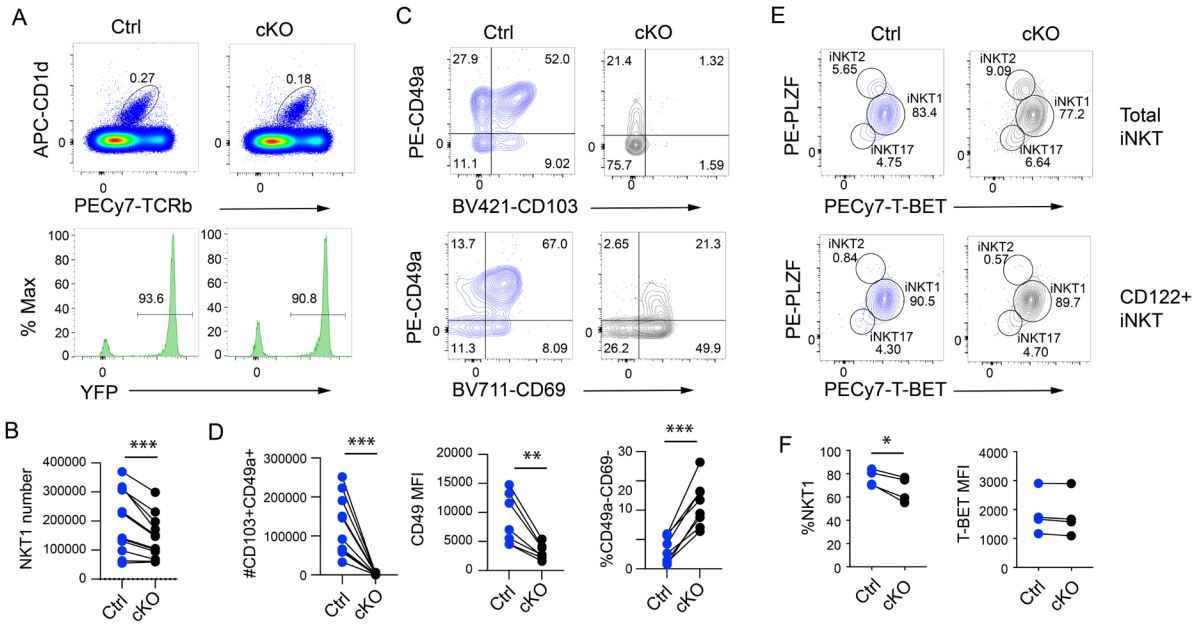

Figure 4.3: TGF-β regulates the development of thymic CD49a+CD103+ iNKT1 cells. (A) Flow cytometry analysis for thymic CD1d-tet^{+}TCRb^{+}YFP^{+} iNKT1 cells in Ctrl and cKO mice. (B) Summary of thymic iNKT1 cell numbers in Ctrl (blue) and cKO (black) mice. (C) Expression of CD49a versus CD103 (top) or CD69 (bottom) on Ctrl (blue) or cKO (black) iNKT1 cells. (D) Number of thymic CD49a+CD103+ iNKT1 cells, CD49a MFI and the percent of CD49a-CD69- iNKT1 cells. (E) Top panels: Intracellular flow cytometry for PLZF and T-BET identifying iNKT1, iNKT2 and iNKT17 cells among total iNKT cells in the thymus of Ctrl (blue) and cKO (black) mice. Bottom panels: PLZF and T-BET on gated CD122+ iNKT cells. (F) Summary of the % of iNKT cells that are iNKT1 and the MFI for T-BET. *P<0.05, **<P0.01, ***P<0.005 by Student's t-test.

that Ctrl cells were enriched for only one pathway (FDR < 25%), the Hallmark TGF-β signaling pathway, whereas cKO cells were enriched multiple pathways, most significantly, the IL-6-JAK-STAT3 pathway genes (Fig. 4.4B, 4.S2). The IL6-JAK-STAT3 pathway included *Socs3* and *Itgb3* mRNAs, which were both significantly higher in cKO as compared to Ctrl cells (Fig. 4.4C). *Tgfbr2* was confirmed to be down in the cKO cells as was *Smad7*, encoding an inhibitor of TGF-β signaling that is induced by TGF-β signaling (Fig. 4.4D). Classic TGF-β signaling associated genes such as *Cdh1*, *Itgae*, *Ski*, *Vim*, and *Itga1* were reduced in cKO iNKT1 cells (Fig. 4.4E). *Inpp4b*, encoding inositol polyphospate-4-phospatase type II, which was recently implicated in TGF-β receptor endocytosis (Aki et al., 2020), was also decreased in cKO cells (Fig. 4.S2). Multiple genes associated with migration were increased in cKO cells including *Mmp9*, *S1pr5*, *Sell* and *Hif1a* (Fig. 4.4F, 4.S2). Interestingly, the gene encoding Hobit, *Zfp683*, a transcription factor that functions redundantly with Blimp1 to enforce tissue residency gene programs, was also decreased (Fig. 4.4F) (Mackay et al., 2016). However, *Prdm1* mRNA, encoding Blimp1, was not decreased. *Tbx21* mRNA was not impacted by deletion of *Tgfbr2,* consistent with our observation that T- BET is expressed appropriately in *Tgfbr2* cKO iNKT1 cells (Fig. 4.S2, 4.3E, F). *Eomes* mRNA was also very low in both Ctrl and cKO thymic iNKT1 cells (Fig. 4.S2). Moreover, in thymic iNKT1 cells, TGF-β signaling was dissociated from regulation of *Zeb2*, a known T-BET target gene, that targets *S1pr5* to antagonize tissue residency (Dominguez et al., 2015; Evrard et al., 2022), despite that TGF-β repressed *S1pr5* (Fig. 4.4, 4.S2). These data implicate TGFβR2 in the regulation of classic TGF-β target genes involved in adhesion and

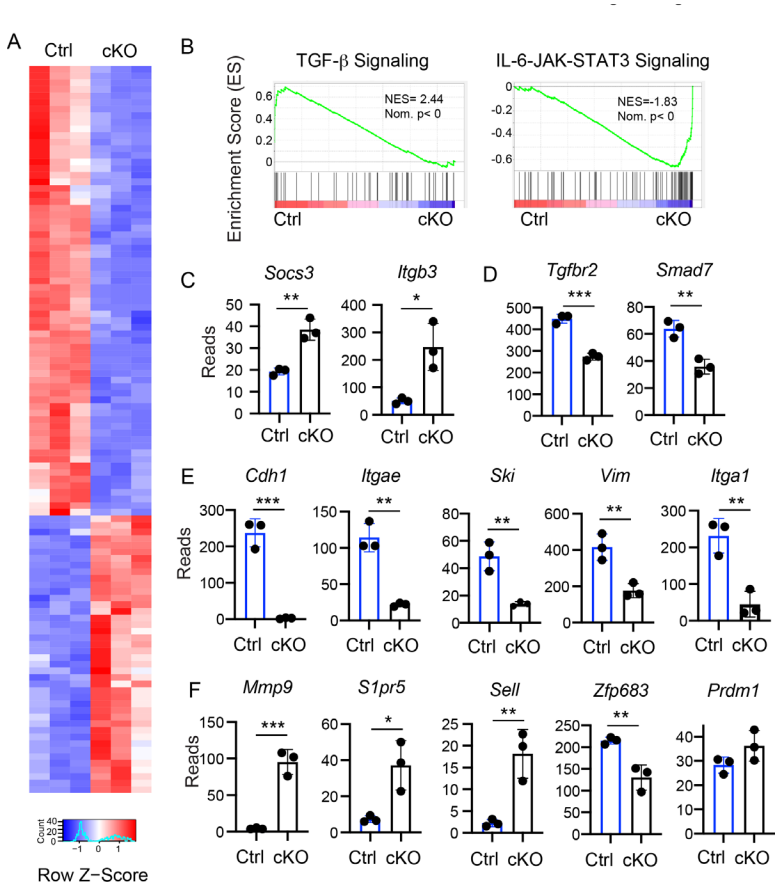

Figure 4.4: TGFβRII promotes a classic TGF-β gene signature and represses an IL6-STAT3 signaling signature in thymic iNKT1 cells. (A) Heat map for differentially expressed genes between Ctrl and cKO thymic iNKT1 by RNA-seq. (B) Top enriched Hallmark Pathway by GSEA in Ctrl and cKO. (C) Normalized reads for replicate RNA-seq samples for representative IL6-STAT3 signaling genes, (D) Tgfbr2 and Smad7, (E) classic TGF-b signaling targets and (F) genes associated with migration. *P<0.05, **<P0.01, ***P<0.005 by Student's t-test.

migration in iNKT1 cells and in the repression of IL6-JAK-STAT3 signaling but not in the regulation of the lineage defining transcription factor T-BET.

The gene program regulated by TGF-β in iNKT1 cells shares significant overlap with that of skin and salivary gland CD8 Trm cells (Christo et al., 2021). However, there were also some notable differences including *Runx3, S1pr1, Junb*, *Cxcr4*, and *Lef1*, which were identified as TGF-β regulated in skin CD8 Trm but were not observed in our dataset (Fig. 4.S2).

Tgfbr2 is required for differentiation but not thymic retention of iNKT1 cells

Thymic iNKT1 cells are tissue resident cells (Berzins et al., 2006); however, the mechanisms controlling tissue residency are not well understood. A previous study identified CD103 on thymic iNKT cells but injection of neutralizing anti-CD103 antibodies did not impact thymic retention (Wang and Hogquist, 2018). Nonetheless, we considered the hypothesis that multiple TGF-β induced genes may contribute to thymic iNKT1 residency. To test whether the decrease in thymic iNKT1 cell numbers in *Tgfbr2* cKO mice was a consequence of increased thymic emigration, we analyzed iNKT1 cell numbers after 10 days of continuous feeding of mice with the S1p lyase inhibitor 4-deoxypyridoxine (DOP) (Schwab et al., 2005). S1P lyase is essential for setting up the gradient of S1P that promotes the chemotaxis of thymocytes into the blood (Schwab et al., 2005). Disrupting this gradient results in thymic retention of cells that would otherwise emigrate including mature CD4 and CD8 T cells. Indeed, treatment of Ctrl or

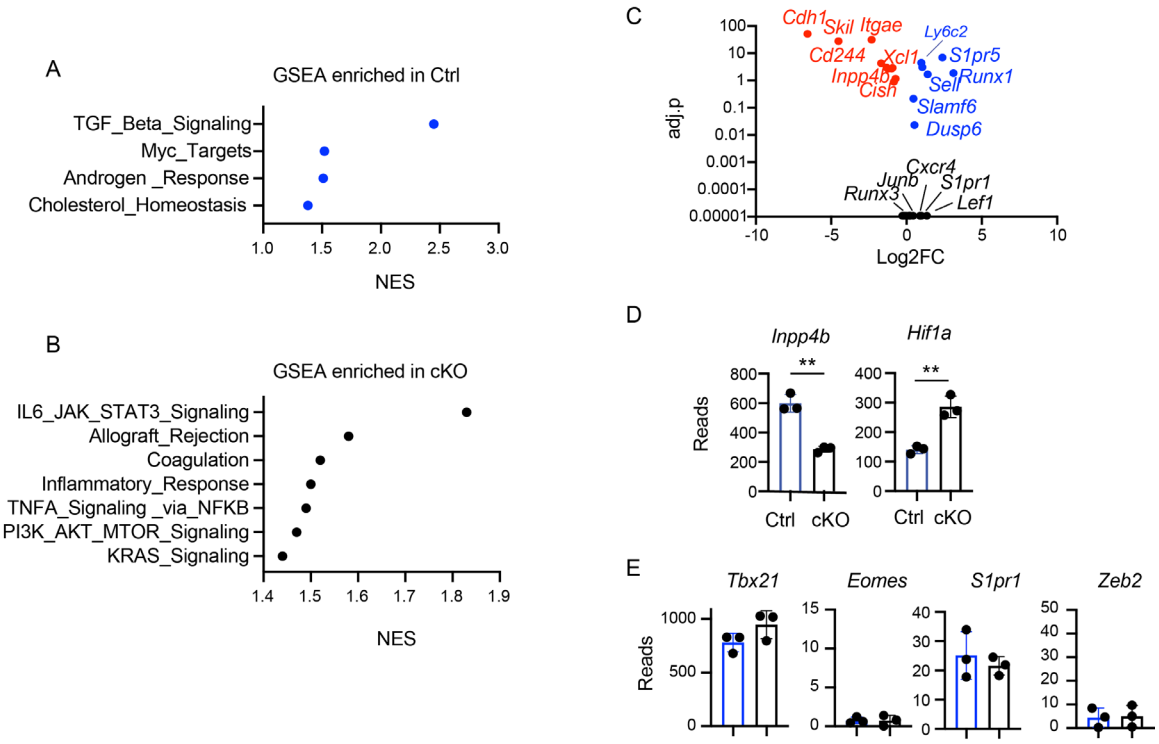

Figure 4.S2: (A) GSEA revealing pathways enriched in Ctrl as compared to cKO thymic iNKT1 cells. (B) GSEA revealing pathways enriched in cKO as compared to Ctrl thymic iNKT1 cells. (C) Graph of Log2FC and adj.p of genes in Ctrl and cKO thymic iNKT1 cells selected for those that are TGF-β regulated in skin CD8 Trm cells. Colors show genes that are up-regulated (blue) or down-regulated (red) or not changed (black) in our dataset. Only a subset of genes are labeled for clarity. (D) Read counts for *Inpp4b* and *Hif1a*, or (E) *Tbx21*, *Eomes*, *S1pr1* and *Zeb2* in Ctrl (blue) and cKO (black) thymic iNKT1 cells.

cKO mice with DOP for 10 days resulted in an accumulation of TCRβ$^+$ T cells in the thymus (Fig. 4.S3A-C). However, thymic iNKT1 cell numbers increased only in the Ctrl mice and not in cKO mice after treatment with DOP (Fig. 4.S3A, B, D). Total iNKT numbers did increase in the thymus of cKO mice but, in contrast to Ctrl mice, in the cKO mice this increase was contributed almost exclusively by Rosa26-YFP negative cells (Fig. 4.S3D). CD49a$^+$CD103$^+$ iNKT1 cells remained low among cKO thymic iNKT1 cells and CD49a MFI continued to be lower in cKO iNKT1 cells but CD69 continued to be expressed on the majority of cells (Fig. 4.S3E, F). In contrast, CD49a$^-$CD103$^-$ (DN) iNKT1 cells accumulated in both Ctrl and cKO mice (Fig. 4.S3G). These data are consistent with the hypothesis that CD49a$^-$CD103$^-$ iNKT1 cells are the most immature iNKT1 cells that either retain the potential to emigrate from the thymus or are the immediate progeny of cells that had emigration potential. However, CD49a$^+$ and CD49a$^+$CD103$^+$ iNKT1 cells in Ctrl or *Tgfbr2* cKO mice do not accumulate after treatment with DOP and therefore iNKT1 cells are not reduced in number due to increased S1p-dependent emigration.

Tgfbr2 is required for optimal expression of CD49a and liver iNKT1 cell function.

CD8 Trm cells in the liver are not dependent on TGF-β but TGF-β signaling does have a subtle impact on their gene expression program suggesting that there is a source of TGF-β in the liver or that these cells are imprinted prior to reaching the liver (Christo et al., 2021). While liver iNKT1 cells do not express many of the genes associated with TGF-β signaling, they do express CD49a and other genes associated with the TGF-b signature such as *Inpp4a*, and low but detectable levels of *Vim* and *Skil*

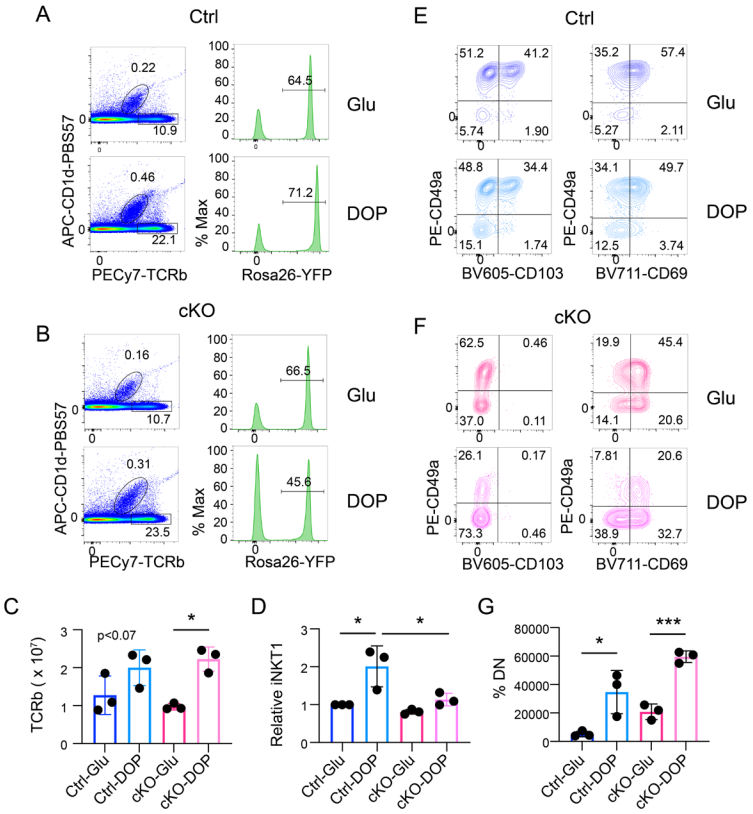

Figure 4.S3: Inhibition of the S1P-gradient reveals that TGF-b does not block thymic emigration. Mice were treated for 10 days with DOP to block the S1P gradient. Flow cytometry to identify TCRb$^+$ T cells or CD1d-tet$^+$TCRb$^+$YFP$^+$ iNKT1 cells in Ctrl (A) and cKO (B) mice fed glucose (Glu) or DPO (DOP) for 10 days. (C) Summary of the number of TCRb+ T cells or the (D) relative number of iNKT1 cells in Ctrl or cKO mice fed Glu or DOP. (E) FACS plots showing expression of CD49a versus CD103 or CD69 on Glu (upper) or DOP (lower) fed Ctrl or (G) cKO mice. (G) The percent of iNKT1 cells that are negative for CD49a and CD49a+ in Ctrl or cKO mice fed Glu or DOP. *P<0.05, **<P0.01, ***P<0.005 by ANOVA with multiple comparisons.

(Fig. 4.S1). Therefore, we examined the consequence of deleting *Tgfbr2* with *Tbx21*^{Cre} on liver iNKT1 cells. In contrast to the thymus, the frequency of iNKT1 cells among liver lymphocytes was not affected in *Tgfbr2* cKO mice (Fig. 4.5A, B); however, the frequency of CD49a^{+} cells and the MFI of CD49a was decreased (Fig. 4.5C, D). CD69 continued to be expressed and the frequency of CD49a^{-}CD69^{-} iNKT1 cells did not increase significantly in the liver of cKO mice (Fig. 4.5C, E), suggesting that CD49a is down regulated but is not completely lost. The observation that TGFβRII-deficiency impacted CD49a expression on liver iNKT1 cells prompted us to test the functional capacity of these cells. TGF-β was shown to impair the functionality of CD8 Trm cells in the skin and salivary gland (Christo et al., 2021). In contrast to this, we found that liver iNKT1 cells from *Tgfbr2* cKO mice were somewhat less capable of producing both IFN-γ and IL-4 in response to an in vivo injection of αGalCer than liver iNKT1 cells from Ctrl mice (Fig. 4.5F, G, H). Taken together, these data demonstrate that despite the absence of a strong TGF-β gene signature in liver iNKT1 cells, TGF-β optimizes their ability to make cytokines in response to antigen stimulation.

Conclusions

These data are consistent with the possibility that TGF-β in the liver directly impacts iNKT1 cells. However, it is possible that TGF-β impacts iNKT1 cell precursors in the thymus that subsequently migrate to the liver after having been imprinted by TGF-β for optimal function. In this regard, it is noteworthy that while the iNKT1 cells that leave the thymus have been proposed to be upstream of effector fate differentiation, when iNKT recent thymic emigrants were examined in the periphery they already express

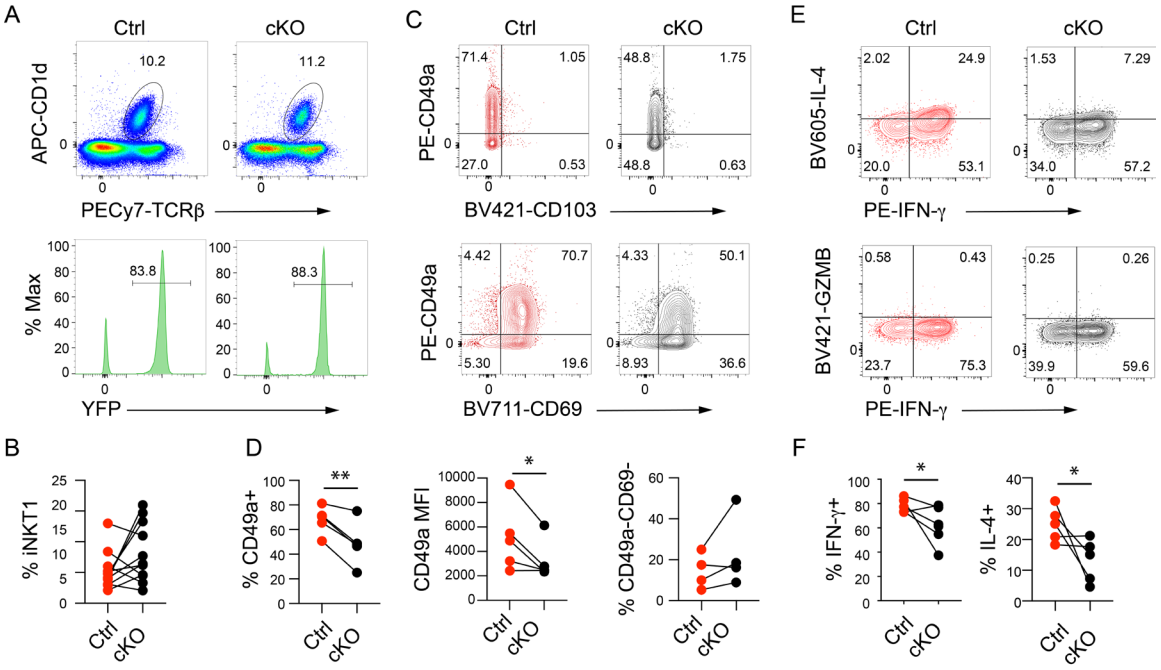

Figure 4.5: TGFβRII regulates CD49a and cytokine production from liver iNKT1 cells. (A) Flow cytometry analysis for liver CD1d-tet^{+}TCRb^{+}YFP^{+} iNKT1 cells in Ctrl and cKO mice. (B) Summary of the percent of iNKT1 cells among liver lymphoid cells in Ctrl (blue) and cKO (black) mice. (C) Expression of CD49a versus CD103 (top) or CD69 (bottom) on Ctrl (red) or cKO (black) liver iNKT1 cells. (D) Summary of the percent of iNKT1 cells that are CD49a^{+}, the MFI of CD49, and the percent that are CD49a^{-}CD69^{-}. (E) Intracellular staining for IL-4 and IFN-γ (top) or GZMB and IFN-γ (bottom) and (F) summary of the percent of liver iNKT1 cells that are IFN-γ^{+} or IL-4^{+} two hours after injection of αGalCer.

detectable T-BET (Wang and Hogquist, 2018). Moreover, recent scRNA-seq studies revealed multiple iNKT cells subsets that may have the potential to emigrate from the thymus, some of which already express *Tbx21* (Harsha Krovi et al., 2020). Our DOP experiments suggest that emigrating thymic iNKT1 cells are CD49a$^-$ CD103$^-$ and raise the possibility that these cells may already express *Tbx21*, since some of these cells are YFP$^+$ in cKO mice. Therefore, it is feasible that emigrating iNKT or iNKT1 progenitors may be imprinted by TGF-β signaling prior to leaving the thymus.

In summary, our study revealed that iNKT1 cells in the thymus differ from those in the liver and spleen in being enriched for a TGF-β signaling gene signature while lacking a signature of IL6-STAT3 signaling and fatty acid metabolism. We demonstrated a role for TGF-β in supporting the development of a subset of thymic iNKT1 cells that express CD49a, CD103 and CD69 while being largely dispensable for development of CD49a$^+$CD103$^-$ iNKT1 cells, although TGF-β does impact the MFI of CD49a. The loss of TGF- β signaling did not lead to thymic iNKT1 cell emigration despite the loss of these adhesion receptors and up regulation of genes associated with emigration such as *S1pr5*. However, immature thymic iNKT cells downstream of those with the potential to emigrate fail to differentiate effectively into CD49a$^+$CD103$^+$ cells in the absence of TGFβR2. Our data suggest that a subset of iNKT1 cells are in contact with TGF-β producing cells in the thymus, possibly medullary thymic epithelial cells (mTEC). Indeed, it is known that LTβR-dependent CCL21$^+$ CD104$^+$ MHCII^{low} mTECs impact iNKT1 cells through provision of IL-15Rα-IL-15 (Lucas et al., 2020; White et al.,

2014). Moreover, Aire+ mTEC numbers are dependent on CD1d, suggesting a direct role for iNKT cells, likely through provision of RANKL (White et al., 2018; White and Anderson, 2014). Therefore, while the $CD49a^{+}CD103^{+}$ subset of thymic iNKT1 cells may depend on mTECs it is also possible that they impact mTEC survival or function. Taken together, our data reveal a role for TGF- β signaling in guiding the development of a subset of thymic iNKT1 cells and for optimal peripheral iNKT1 cell function.

Chapter Five

Discussion

Natural killer T cells represent part of a subset of innate-like lymphocytes, lymphocytes that are able to mature into effector subsets, with striking similarity to conventional T_H cells, without requiring direct activation. Rapid activation by broad recognition of glycolipid paired with the ability to produce a broad range of cytokines puts NKT cells (and other ILLs) in a unique position to direct the type of immune response within a tissue. Furthermore, these cells are able to be activated via cytokine receptors, strength of the TCR signal or a combination of the two, allowing for additional modulation of their cytokine production (Brennan et al., 2013; Kumar et al., 2017). As such, extensive efforts have been made to understand how NKT cells develop, circulate and localize to target tissues, and help guide the immune response in a tissue-specific manner.

In this thesis, I have investigated the roles of the transcription factor Ets1 and TGF-β signaling in the maturation and function of NKT cells within the thymus and liver. Using a conditional deletion model driven by *Tbx21*^{Cre}, we were able to specifically delete *Ets1* in NKT cells that have committed to maturation into NKT1 effector cells. As germline deletion of *Ets1* results in perinatal mortality and an aberrant T cell phenotype in mice, it has been difficult to characterize the role of Ets1 in post-selection NKT cells. However, some studies have been able to circumvent this by targeting protein

interaction domains of Ets1 (Tai et al., 2020) or introduction of a *Vα14* transgene (Chuang et al., 2021). We have shown that Ets1 has distinct functions in the thymus and liver. In the thymus, Ets1 is important for the development of NKT1 cells while in the liver, Ets1 acts to regulate an NKT-like adhesion program and enforce tissue retention. A previous study demonstrated that T-cell specific deletion of TGF-β signaling resulted in a failure of NKT cells to develop properly (Marie et al., 2006). We also show that NKT1 cells in the thymus, but not spleen or liver, bear a TGF-β signaling associated gene signature. This signaling was required for the suppression of migratory genes and expression of adhesion receptors typically associated with tissue retention. Our data highlights a role for TGF-β signaling in facilitating the maturation of NKT1 cells that remain in the thymus while also contributing to proper activation-induced production of cytokines in peripheral NKT1 subsets.

Ets1 in iNKT cell effector maturation

In later stages of development, expression of Ets1 is primarily restricted to lymphoid cells (Chen, 1995). Moreover, Ets1 remains highly expressed in NKT cells (Anderson et al., 1999), highlighting a poorly understood role for Ets1 following V(D)J recombination and selection. My analysis of Ets1 represents the first characterization of *Ets1* deletion specifically in post-selected NKT cells. Focusing on the liver, we report that Ets1 acts as a regulator of a broad range of adhesion-associated genes in NKT1 cells. Indeed, *Ets1*-deficient NKT1 cells in the liver had reduced expression of LFA-1 and ICAM-1; which are essential for liver retention, CD44; an adhesion and activation receptor, and CD69; typically associated with negative regulation of S1PR1-mediated

migration in T_{RM} cells. When treating Ets1 cKO mice with antibodies against LFA-1 and ICAM-1, these adhesion receptors were suddenly dispensable for retention in the liver. In the wild-type context, LFA-1 and ICAM-1 are necessary and sufficient for liver retention. It is unlikely that CD69 is compensating for reduced LFA-1 and ICAM-1 expression as we see the emergence of both a $CD69^{lo}$ and $CD69^{bright}$ population of NKT cells in the liver. Despite this, parabiosis and S1PR blocking studies in the liver revealed that Ets1 cKO NKTs are still tissue resident. We conclude that Ets1 is essential to enforcing LFA-1/ICAM-1-mediated retention of NKT1 cells in the liver. In the absence of Ets1, hepatic NKT cells establish new adhesion contacts to facilitate their retention. The presence of two distinct CD69 populations could indicate that localization of NKT1 cells within the liver has been altered, exposing them to new cell contacts within the tissue microenvironment, but this remains to be determined. If re-localization is occurring, it would be pertinent to determine which alternative adhesion receptors are being utilized by cKO NKT cells. One potential area of focus could be alternative receptor formation using the integrin beta chain CD18, which is a component of the LFA-1 receptor. By introducing a heterozygous deletion of *Tbx21*, encoding T-bet, we found that expression of ICAM-1 and CD44 were restored to WT levels, with CD69 being expressed at higher levels than in WT. Additionally, reduced expression of T-bet restored LFA-1/ICAM-1 reliance in cKO/Het hepatic NKT1 cells. Taken together, we demonstrate a previously unknown role for Ets1 in enforcing LFA-1/ICAM-1-mediated retention of NKT1 cells in the liver by way of modulating expression of T-bet, a signature T_H1-associated transcription factor (Fig. 5.1).

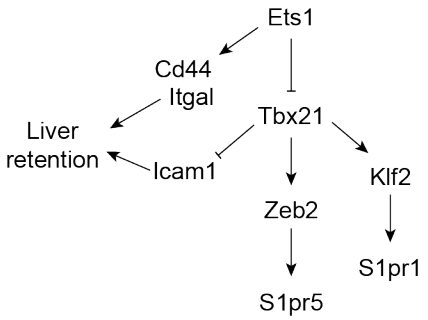

Figure 5.1: Ets1 regulates expression of migration and adhesion genes in NKT1 cells via regulation of T-bet. One mechanism by which Ets1 regulates the development and function of NKT1 cells is via modulation of T-bet expression. T-bet regulates the expression of *Icam1* and supresses the requirement for classic liver retention interactions. Additionally, T-bet drives the expression of *S1pr5*, *S1pr1*, and *Klf2*, which are essential to the lymphocyte migratory program.

In addition to regulating adhesion genes, we also see an upregulation of many migration genes including *S1pr1*, *S1pr5*, *Klf2* and *Sell*. Through RT-qPCR analysis of the liver, we were able to determine that *S1pr5* mRNA in cKO/Het NKT1 cells returned to WT levels. *S1pr1* and *Klf2* mRNA also appear to return to WT expression levels, though this result did not reach significance. Reduced expression of T-bet only partially reduced mRNA of *Zeb2*, a direct target of T-bet, suggesting that Ets1 or other DEGs also contribute to Zeb2 activity, consistent with previous reports (Bar Yaacov et al., 2019). Klf2 has been shown to be a regulator of S1PR1 (Carlson et al., 2006), both of which are important for migration of conventional T cells and NKT cells (Allende et al., 2008; Wang and Hogquist, 2018). Current knowledge of tissue egress mechanisms suggests that Klf2 drives the expression of *S1pr1*. Our preliminary data supports the conclusion that in NKT1 cells, T-bet acts as a regulator of Klf2 and, consequently, S1PR1 expression (Fig. 5.1). Our findings are also consistent with studies in T_{RM} cells showing that T-bet and Zeb2 promote the expression of S1PR5 (Evrard et al., 2021). Additionally, T-bet-dependent expression of S1PR5 is required for migration of NK cells (Walzer et al., 2007; Jenne et al., 2009). We hypothesize that T-bet promotes the expression of S1pr1 in a Klf2-dependent manner while promoting expression of S1pr5 via Zeb2. Whether NKT1 cells also utilize *S1pr5* for migration, similarly to $CD8^+$ T_{RM} cells or NK cells, remains to be determined. In summary, this data highlights an important role for T-bet in driving the migratory gene program in NKT1 cells.

A recent study (Zhong et al., 2022) did ATAC-seq analysis of naïve and LCMV-activated CD4 and CD8 T cells in the spleen to gain a better understanding of the major

epigenomic changes that during T cell activation. Zhong and colleagues report that the broadest changes in accessibility in T cells were associated with Ets, Runx and bZIP/IRF motifs while some motifs, including T-box, were more accessible in activated T cells. Subsequent analysis revealed that Ets1, Runx1 and Tcf1 were bound to a majority of regions. Interestingly the authors conclude that as chromatin regions bound by Ets1 undergo little change following activation of CD4 and CD8 T cells that Ets1 primarily acts as a regulator of housekeeping genes. However, there were a small set of Ets1 binding regions that had increased accessibility in activated T cells (Zhong et al., 2022). Compared to our ATAC-seq in NKT1 cells, we find notable similarities. ATAC-seq analysis of WT and cKO liver NKT1 cells revealed 3,660 differentially accessible chromatin regions. Motif enrichment analysis of these regions showed a strong enrichment of bZIP, Runx and T-bet binding motifs in regions of increased accessibility in cKO NKT1 cells, consistent with our findings that T-bet regulates the migration gene program and essential adhesion genes in NKT cells. Regions of reduced accessibility were enriched for multiple Ets binding motifs. The broad association with Ets motifs in differentially accessible regions could suggest that Ets1 regulates the accessibility of many chromatin regions in NKT1 cells. Given that NKT cells have already differentiated into their effector states, it is likely that more activation-associated motifs like T-box would be enriched in NKT1s at steady state. Moreover, as T-bet is critical for the development and function of NKT1 cells, this enrichment of T-box motifs in cKO NKT1 cells could be characteristic of other T_H1-associated ILLs.

In the thymus, Ets1 contributes to proper development of NKT1 cells. Initially, increased expression of migratory genes paired with the decrease in NKT1 cells present in cKO mice led us to consider that thymic NKT1 cells were migrating earlier than intended. However, DOP assays revealed that the decrease in NKT1 cell number was a consequence of defects in maturation. It is possible that maturation defects observed in the thymus are a result of changes in the adhesion profile of NKT1 cells. As the thymus is the primary site of T cell development, any alterations in localization could also influence the types of signals received. For example, cKO thymic NKT1 cells have impaired expression of *Tgfbr2*, a component of the TGF-β receptor. TGF-β signaling is important for the expression of the residency-associated adhesion receptors CD49a and CD103 and will be addressed in the following section.

TGF-β signaling in iNKT1 maturation and function

Unique phenotypes in Ets1 cKO thymic and hepatic NKT1 cells support the idea that while core commonalities exist between all NKT1 cells, Ets1 controls NKT1 effector maturation and function in a tissue-specific manner. Consistent with this, wild-type thymic NKT1 cells were found to share fewer transcriptional similarities compared to splenic and hepatic NKT1 cells in the periphery. GSEA of all three WT tissues revealed that only thymic NKT1 cells were enriched for the TGF-β signaling pathway. Outside of its roles in immune suppression, TGF-β signaling plays a key role in activation of NK cells (Viel et al., 2016), NKT cell selection (Marie et al., 2006) and initiating the tissue residency gene program of $CD8^+$ T_{RM} cells after infection (Mackay et at., 2013; Zhang and Bevan, 2013; Nath et al., 2019). Given that thymic NKT1 cells are tissue-resident

and express CD49a and CD103, we investigated the role of TGF-β signaling in NKT1 cells using a conditional deletion of the *Tgfbr2* gene (*Tgfbr2* cKO). Our data revealed that TGF-β signaling is required for development of CD49^{+} CD103^{+} NKT1 cells in the thymus. RNA-seq analysis of *Tgbr2* WT and cKO thymi showed that cKO NKT1 cells had increased expression of a number of genes associated with migration including *S1pr5*. However, TGF-β signaling did not block thymic egress of NKT1 cells. We also observed a reduction in the expression of *Zfp683*, encoding Hobit. Hobit, alongside Blimp-1, are consistently expressed across multiple tissue-resident cell types and are believed to act as universal regulators of tissue residency in lymphocytes (Mackay et al., 2016). Consistent with observations in other cell types, TGF-β signaling promotes the expression of a common residency-associated gene program in thymic NKT1 cells. Despite TGF-β signaling repressing *S1pr5*, we did not observe changes in expression of either T-bet or Zeb2, suggesting that *S1pr5* can be suppressed by TGF-β signaling independently of the T-bet-Zeb2 axis in NKT1 cells.

While NKT1 cells in the periphery do not possess the same TGF-β gene signature as thymic NKT1 cells, they still produce CD49a. As such, we sought to investigate a role for TGF-β signaling in hepatic NKT1 cells. *Tgfbr2* cKO hepatic NKT1s had reduced expression compared to WT and were impaired in their ability to produce cytokines upon αGalCer-mediated activation, most notably the IFNγ^{+} IL-4^{+} double-producing population of NKT1 cells saw the greatest reduction in frequency. Together, the data indicates that TGF-β signaling is important for the development of NKT1 cells in thymus, by promoting expression of CD49a and CD103, while contributing to proper

cytokine production in liver NKT1 cells. While the transcriptional similarities between thymic NKT1 cells and T_{RM} cells are apparent, our analysis highlights the need for continued investigation into the different ways that tissue-resident cells uniquely utilize common residency gene programs.

The Ets1-TGF-β axis in thymic iNKT1 cell development

Based on RNA-seq data from thymic NKT1 cells in both the *Ets1* cKO and *Tgfbr2* cKO models, we observed that Ets1 and TGF-β signaling both work to enforce expression of key adhesion receptors and repress expression of migratory genes. Among the DEGs analyzed in *Ets1* cKO thymic NKT1 cells, we found that *Tgfbr2* was among the list of downregulated genes. Furthermore, when characterizing the *Ets1* cKO phenotype, we found that $CD49^+$ $CD103^+$ NKT1 cells were nearly absent, a phenotype resembling our findings in the *Tgfbr2* cKO thymus (Fig. 5.2A). We also identified a subset of adhesion genes with similar changes in expression between the *Ets1* cKO and *Tgfbr2* cKO RNA-seq datasets (Fig. 5.2B) Based on preliminary data, it is possible that loss of TGF-β signaling is contributing to the *Ets1* cKO phenotype. In turn, Ets1 could act to regulate the development of thymic NKT1 cells, in part, by positively regulating expression of *Tgfbr2* and facilitating TGF-β signaling (Fig. 5.3). If true, it would then be interesting to tease out the contributions of T-bet/Zeb2 and TGF-β in coordination of *S1pr5* expression. However, it is important to note that we have only uncovered a part of the *Ets1* cKO phenotype.

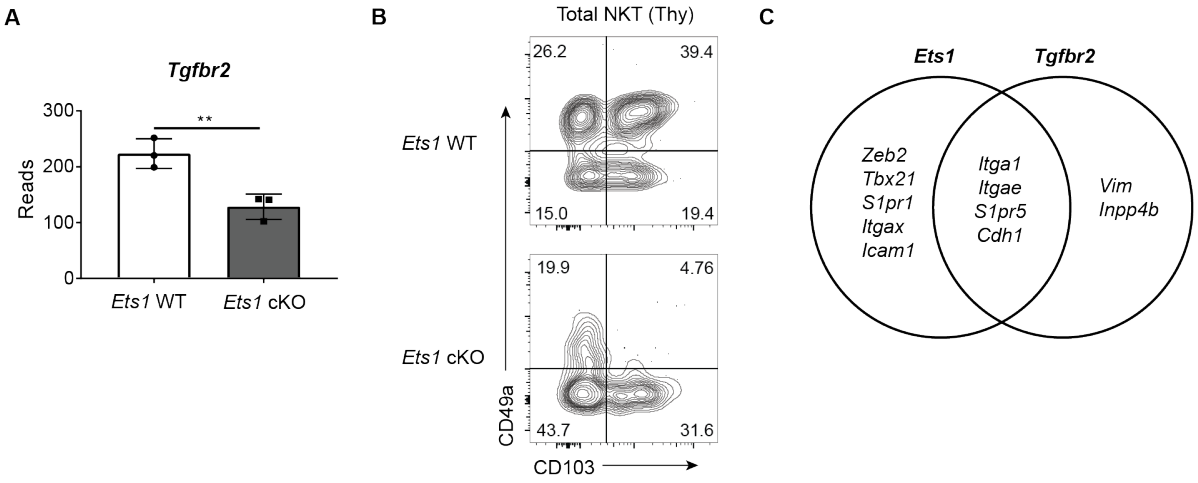

Figure 5.2: Similar phenotypes and gene expression changes in *Ets1* and *Tgfbr2* cKO NKT1 cells. (A) Normalized read counts of *Tgfbr2* mRNA in *Ets1* WT and cKO NKT1 cells. (B) Representative flow cytometry analysis of CD49a and CD103 expression in *Ets1* cKO NKT cells (N = 3). (C) Comparison of DEGs from RNA-seq analysis of thymic NKT1 cells in either *Ets1* cKO (left) or *Tgfbr2* cKO (right) mice.

Moving towards a greater understanding of iNKT cell development

Since their discovery, NKT cells (and other ILLs) have been highly interesting immune cells to study; not only to interrogate the transcriptional and epigenetic mechanisms that underlie their development and maturation but also due to their central role in directing cytokine profile of the immune response and regulation of other immune cells (Matsuda et al., 2008). In fact, NKT cells play an important role in the attenuation of graft-versus-host disease (Kuwatani et al., 2006; Yang et al., 2010; Kuns et al., 2009; Coman et al., 2018). NKT cells also have potent antitumor capabilities (Krijgsman et al., 2018) and have been shown to be a strong candidate for immunotherapeutic approaches using CAR NKT cells (Heczey et al., 2014; Heczey et al., 2020, Karadimitris et al., 2019). As such, expanding our understanding of how NKT cells develop, mature into effector subsets and retain themselves in secondary lymphoid organs represents an important step in optimizing their therapeutic efficiency.

With the development of single-cell next-generation sequencing approaches, we have been able to gain valuable insight into the development of NKT cells across various tissues in mice and humans (Baranek et al., 2020; Shen et al., 2020; Zhou et al., 2020). In the thymus, Baranek and colleagues report a previously unrecognized degree of heterogeneity in developing NKT cells. Pseudo-time analysis of scRNA-seq data in wild-type mice suggests that after selection, NKT0 cells transition into a multipotent NKT2 state. These multipotent NKT2 cells are then able to continue differentiation into NKT1 cells, NKT17 cells or remain in a plastic NKT2 state (Baranek et al., 2020). Interestingly, in contrast with studies showing that $CCR7^{+}$ NKTp cells are

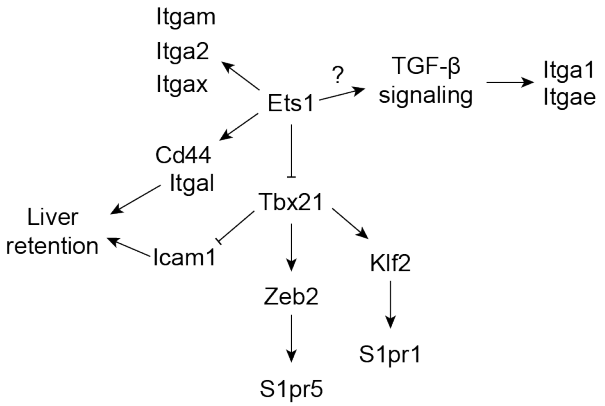

Figure 5.3: Model for Ets1 regulation of iNKT1 cell development and function. Ets1 regulates development using multiple potential mechanisms: (1) modulation of T-bet expression to suppress migration-associated gene programs and promote retention in the liver, (2) enforcing optimal thymic iNKT1 cell maturation by promoting TGF-β signaling, and subsequently CD49a and CD103 and (3) possibly maintaing an adhesion gene program that facilitates proper localization of NKT1 cells in the thymus and liver.

the primary emigrating NKT population in the thymus (Wang and Hogquist, 2018), the authors report that almost every NKT effector is capable of thymic egress (Baranek et al., 2020). In the liver, NKT1 cells are the predominant subset but other effector subsets also exist. scRNA-seq analysis by Shen et al (Shen et al., 2020) identified four clusters of $NK1.1^{+}$ $CD3e^{+}$ NKT cells in the liver; two CD1d-dependent clusters representing 88% of total hepatic NKT cells (type I and type II NKT cells) and two distinct clusters that were CD1d-independent. These CD1d-independent clusters were able to be further differentiated by expression on Sca-1 and CD62L. Functional analysis of CD1d-dependent NKT cells revealed that $Sca\text{-}1^{-}$ $CD62L^{+}$ NKT cells were able to produce IFNγ following cytokine-mediated stimulation (Shen et al., 2020). Owing to their increased frequency in mice, the molecular phenotype of NKT cells has not been as well characterized in humans. To this end, Zhou et al (Zhou et al., 2020) were able to leverage single-cell sequencing to characterize the molecular identities of human peripheral blood NKT cells, opening up the possibility of more refined analysis of human NKTs in the future.

Concluding Remarks

As advancements in immunological technologies and techniques continue to be made, we will be able to gain a more refined understanding of how cells of the immune system develop and function. Within an already complex developmental system lies innate-like lymphocytes, immune cells bearing characteristics of multiple cell types, adding another layer of intricacy and raising a multitude of question regarding their

origins. My analysis of Ets1 has provided insight into the some of the mechanisms guiding development of retention of thymus and liver NKT1 cells, respectively. As Ets1 is capable of binding many genomic regions, either on its own or paired with transcriptional co-activator, extensive analysis will be required to dissect all of its roles in NKT1 cells. These results also contribute to an ever-growing knowledge base of tissue-resident immune cells and the nuances in their transcriptional profiles. Indeed, understanding tissue residency will require continued analysis across multiple cell types. Knowing what signals facilitate homing of NKT cells and their retention in a given tissue will be important in the development of NKT cell-based immunotherapies.

Appendix

Distinct functional requirements for Ets1 across peripheral iNKT1 cells

Introduction

NKT cells represent a subset of innate-like T lymphocytes that are able to differentiate into their effector states without antigen activation. Given their phenotypic similarity to NK cells, helper T cells, ILCs and γδ T cells, several studies have interrogated the transcriptome and epigenetic landscape of these subsets to better characterize the nuances between their development and function. Bulk RNA-seq of NKT effectors, ILCs, NK cells, T_H cells and γδ T cells revealed that despite their differences in antigen recognition, NKT1 cells were transcriptionally similar to other T_H1-like cells including T_H1 cells, NK cells, ILC1s, and intraepithelial γδ T cells. These similarities also extended to NKT2/NKT17, ILC2/3 and γδ T cells, but not T_H2/T_H17 cells (YJ Lee et al., 2016), implicating a conserved effector gene program across these immune cells. Within NKT cells specifically, single-cell RNA-seq analysis of thymic NKT precursors and effector cells demonstrated that developing NKT cells undergo significant alterations in the transcriptome as they differentiate into mature effector subsets. Furthermore, these transcriptional shifts may work to imprint NKT cells in the thymus so they can express the necessary adhesion and chemokine receptors required for homing to target tissues (Engel et al., 2016; Salou et al., 2019). Transcriptomic analysis of MAIT cells and NKT cells in the periphery revealed that MAIT and NKT cells expressed highly similar residency gene programs in the spleen, liver and lungs and

that MAIT and NKT cells in the spleen and liver shared a similar reliance on LFA-1 and ICAM-1 for tissue retention (Salou et al., 2019).

Recently, RNA- and ATAC-seq analysis of NKT effector subsets revealed that the transcriptome and epigenetic landscape of NKT1s, NKT2s and NKT17s are nearly identical in the thymus, spleen and liver, with some divergence observed in the lungs (Murray et al., 2021). During the course of our investigation into the role of Ets1 in NKT1 cells, we found unique requirements for Ets1 between the thymus and liver; where Ets1 facilitates the proper development and maturation of NKTs in the thymus and represses T-bet to enforce the expression of LFA-1 and ICAM-1 and suppress the migratory gene program in the liver. In addition to our analysis of NKT1s in the thymus and liver, we also investigated the role of Ets1 in splenic NKT1s, as the spleen is predominantly NKT1 cells. Here, we show that while Ets1 WT and cKO NKT1s in the spleen and liver are highly similar transcriptionally, Ets1 acts to modulate the proliferation of NKT1s in the spleen. Flow cytometry analysis revealed that splenic and hepatic NKT1 cells have variable expression of adhesion receptors essential for liver retention and that splenic NKT1 cells do not require LFA-1 or ICAM-1 for retention. Taken together, our data suggests that despite transcriptional similarities between NKT1s across different tissues, they exhibit distinct developmental and phenotypic characteristics dependent on the organs they reside in.

Results

Transcriptional and phenotypic divergence between peripheral NKT1 cells

Alongside the RNA-seq analysis in the liver described earlier (see Chapter 3), we also sequenced Ets1 WT and cKO NKT1s sorted from the spleen. Analysis of splenic NKT1s revealed 295 DEGs (FDR<0.05), 164 of which were upregulated and 131 were downregulated in Ets1 cKO mice (Fig A.1A). Principal component analysis (PCA) of the transcriptome of NKT1s in the thymus, spleen and liver showed strong clustering of splenic and hepatic NKT1s, consistent with previous data (Murray et al., 2021) and indicative of highly similar transcriptomes. However, we did observe distinct clustering of thymic NKT1s, which may be indicative of heterogeneity in developmental progression (Fig. A.1B). Gene ontology analysis of all DEGs in the spleen showed similarities to the liver, being enriched for genes associated withmigration, locomotion and adhesion. Interestingly, splenic NKT1s were enriched for genes associated with NK cell mediated cytotoxicity while liver NKT1s were enriched for inflammatory response genes (Fig. A.1C). GSEA of the spleen and liver for hallmark pathways showed that cKO NKT1 cells in both the spleen and liver were enriched for many of the same gene sets including EMT, fatty acid metabolism and targets of the E2F family of cell cycle regulators (Fig. A.1D). When comparing the adhesion, migration and inflammatory genes of interest in the spleen, we found that splenic NKTs had similar upregulation of migration and adhesion genes including *S1pr1*, *S1pr5*, *Klf2*, *Sell* and various NK cell associated integrins (Fig. A.1D). We then performed flow cytometry analysis of

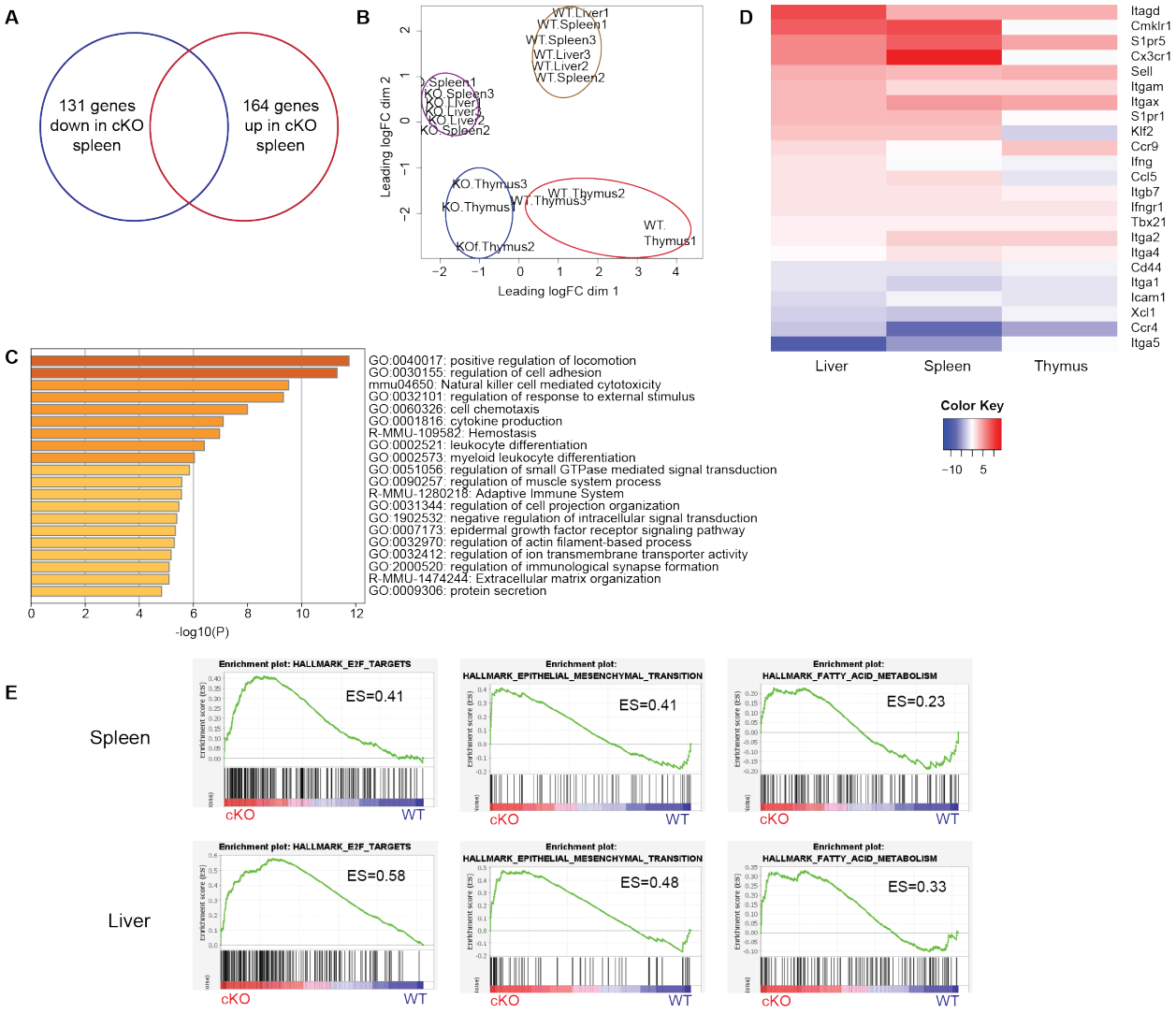

Figure A.1: Transcriptional divergence between peripheral iNKT1 cells. (A) Venn diagram representation of the number of differentially expressed genes (DEG) in sorted Ets1 cKO splenic NKT1 cells (FDR<0.05). (B) Multidimensional scaling analysis of gene expression data from RNA-seq of WT and cKO iNKT1 cells from the thymus, spleen and liver. Similarity is measured by the distance between samples. (C) Gene ontology analysis of all DEG in the WT and cKO spleen. (D) Unscaled heatmap of the log fold-change of selected genes associated with the inflammatory response or adhesion in cKO splenic iNKT1 cells. (E) GSEA hallmark pathway analysis of RNA-seq data from WT and cKO iNKT1 cells in the spleen and liver. Enrichment score correlates with the number of genes found to be upregulated in the described gene sets.

adhesion receptors typically associated with tissue-residency of liver NKTs. Similarly to the liver, splenic NKT1s were found to express high levels of LFA-1. However, in contrast to liver NKT1 cells, there was no significant change in LFA-1 expression in splenic NKT1 cells. We also observed an increase in the percentage of cKO NKT1s in the spleen with low expression of the adhesion marker ICAM-1 (Fig. A.2C). *Ets1*-deficient splenic NKTs also had reduced expression of CD69 and CD44, receptors associated with activation and adhesion/retention, as there was a ~40% increase in the frequency of cKO NKTs that had little to no expression of both receptors (Fig. A.2C).

Studies from the Bendelac and Lantz labs (Thomas et al., 2011; Salou et al., 2019), in addition to data presented earlier, have shown that NKT cells in the liver require LFA-1 and ICAM-1 interactions to be retained in the liver. To assess reliance of splenic NKTs on LFA-1 and ICAM-1, we intravenously injected WT and cKO mice with neutralizing antibodies and analyzed the spleen one hour post-injection. Treatment with anti-LFA-1 had no significant effect on the frequency of total NKTs in the spleen compared to control in either the WT or cKO mice. Interestingly, anti-ICAM-1 administration led to a ~1.5x increase in the frequency of NKTs present in the spleen of WT mice. In contrast, in cKO mice NKTs were present at a slightly lower percentage compared to control (Fig. A.2D). This result directly contrasts observations in the liver, where Ets1 promotes LFA-1/ICAM-1-independent retention of NKTs (Chapter 3, Fig. 3C). Our data suggests distinct requirements for LFA-1 and ICAM-1 in the spleen and liver despite similarities in their transcriptional programs. Furthermore in the wild-type

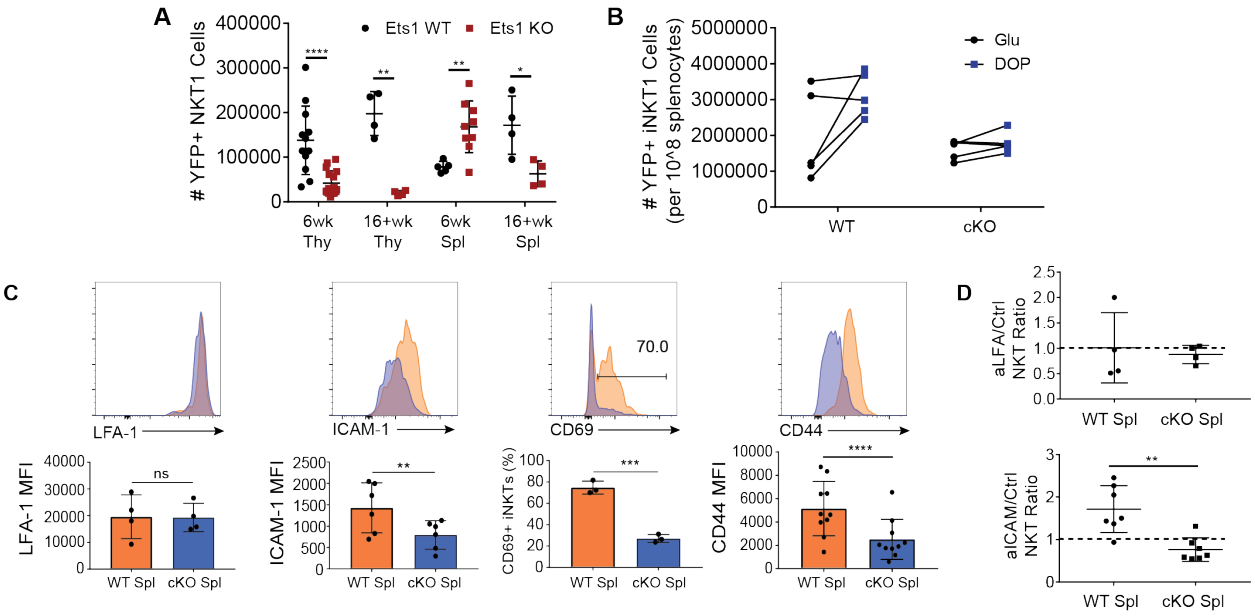

Figure A.2: Ets1 restricts the number of NKT1 cells in the spleen. (A) Number of NKT1 cells present in Ets1 WT and cKO mice at 6 weeks and 16+ weeks of age. (B) 6-8 week old WT and cKO mice were administered Glu or Glu + 4-deoxypyridoxine (DOP) via drinking water for 10 days. Mice we analyzed on day 11. (C) Flow cytometry analysis of adhesion receptors LFA-1, ICAM-1, CD69 and CD44 in 6-8 week old WT and cKO spleen. (D) WT and cKO mice ranging from 8-10 weeks were injected intravenously with 150µg of either anti-LFA-1 or anti-ICAM-1 antibodies. Spleens were analyzed 1 hour post-injection. Data is represented as the percentage of total NKTs in the spleen of anti-LFA-1/ICAM-1 treated mice compared to PBS/IgG2a isotype controls.

context, we report that LFA-1 and ICAM-1 interactions are not required for retention in the spleen, although how ICAM-1 restricts NKT cell access to the spleen remains to be determined.

Ets1 restricts the number of NKT1s in the spleen

During the characterization of the Ets1 cKO phenotype in splenic NKT cells, we found that 6-week-old cKO mice had an increased number of NKT1 cells in the spleen (Fig. A.2A). Initially, we hypothesized that the increase of NKT1s in the spleen could be a result of increased egress of NKTs from the thymus. However, treatment of *Ets1* WT and cKO mice with DOP revealed that there were no significant changes in the number of NKT cells being retained in the thymus of DOP-treated cKO mice (Chapter 3, Fig. 3F), suggesting that the loss of NKT1s in the thymus is the result of a tissue-specific developmental defect. Indeed, DOP treatment had no significant impact on the number of NKT1s in the spleen in WT or cKO mice (Fig. A.2B). To see whether this increased number of NKTs was consistent over time, we looked at the number of NKT1 cells present in the thymus and spleen of WT and cKO mice between 16 and 20 weeks. While we did not observe a difference in the number of NKT1s in the cKO thymus at 6 or 16+ weeks of age, we observed a reduction in the number of NKT1s in the spleen on cKO mice at 16+ weeks compared to 6-week-old mice (Fig. A.2A). This data could suggest that in the spleen, Ets1 acts to restrict the proliferation of NKT1 cells and that over time, hyperproliferation of these effector cells could lead to their exhaustion and an eventual crash of the population.

Ets1 regulates expression of adhesion receptors uniquely across peripheral tissues

We found that Ets1 regulated the expression of CD44, ICAM-1 and CD69 in hepatic NKT1 cells by limiting expression of the transcription factor T-bet. Given that we saw changes in the expression of these adhesion receptors in *Ets1*-deficient splenic NKT1s as well, we sought to investigate whether T-bet negatively regulated their expression in the spleen. Analysis of cKO/Het mice showed no change in the frequency of $CD44^{lo}$ NKT1 cells compared to cKO. While we observed a slight reduction in the frequency of ICAM-1^{lo} splenic NKT1s, this result was not significant (Fig. A.3A). However, we did find that reduced T-bet expression restored the percentage of $CD69^{lo}$ NKT1s in the spleen back to that observed in WT. Restored expression of CD69 could likely be a result of reduced expression of the antagonist S1pr1, consistent with our findings in the liver. We also sought to determine whether reduced expression of T-bet altered the loss of NKTs in cKO spleen treated with anti-ICAM antibodies. Ultimately, we saw no change in the frequency of splenic NKTs in anti-ICAM-1 cKO/Het mice compared to isotype control treated mice, indicating that T-bet is not responsible for the altered retention of anti-ICAM-treated cKO NKTs in the spleen (Fig. A.3B).

Conclusions

Taken together, we provide data highlighting tissue-specific roles for the transcription factor Ets1 in the development and maintenance of NKT1 cells in the spleen. Initial characterization of the phenotype revealed an increase in the number of

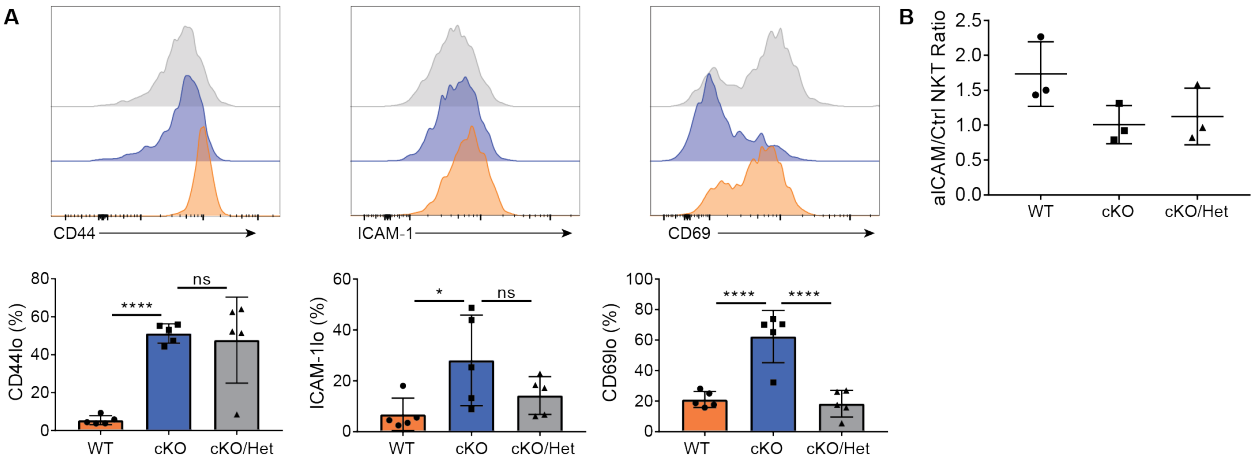

Figure A.3: Ets1 regulates expression of adhesion receptors across peripheral tissues. Representative flow cytometry analysis of *Ets1* WT, cKO and *Ets1* cKO *Tbx21*$^{f/+}$ (cKO/Het) spleens for expression of adhesion markers CD44, ICAM-1 and CD69. Summary data for all experiments is included below. (B) *Ets1* WT, cKO and cKO/Het mice were injected i.v. with 150µg anti-ICAM-1. Mice were sacrificed and spleens were analyzed onehour post-injection.

NKT1 cells present in the spleen of Ets cKO mice. This was not due to increased emigration from the thymus as demonstrated by experiments blocking S1P receptor-mediated migration in the thymus and spleen. This suggests that Ets1-deficient splenic NKTs may be hyperproliferative, which could explain the decreased number of NKT1s in the spleen as the mice age. To properly assess this observation, it will be important to stain for Ki67, a marker of cell cycling, or BrdU, which acts as a proxy for DNA replication, to determine whether cKO NKT1s in the spleen are proliferative paired with FLICA/Annexin V assays to identify apoptotic and dying cells in older NKT subsets. Previous reports identified a role for adhesion receptors LFA-1 and ICAM-1 in retention of splenic NKT cells (Salou et al 2019). We find that there was no change in NKT cell frequency when we treated WT or cKO mice with LFA-1 neutralizing antibodies. Interestingly, anti-ICAM-1 treatment resulted in an increased frequency of NKT cell in the spleen compared to isotype control. The differences in our data could be attributed to a number of factors including the duration of the antibody treatment (1h vs 24h) and the injection of LFA-1 and ICAM-1 antibodies independently (our study) or simultaneously (Salou et al., 2019). Considering that anti-ICAM treatment results in a loss of NKT cells in the liver, it may be possible that NKTs escaping the liver may take up residence in the spleen, or merely pass through the spleen early after treatment, thereby raising splenic NKT1 cell numbers. As for Ets1 cKO mice, anti-ICAM-1 treatment may have the opposite effect in the spleen compared to liver, with splenic NKTs gaining a mild reliance on ICAM-1 for retention. Overall, even though LFA-1, ICAM-1 and CD69 are highly expressed in the spleen, regulation of their expression may vary in the spleen and liver. This is supported by our cKO/Het analysis in the two

tissues. While reduced T-bet expression restored expression of the aforementioned receptors in the liver, NKT1 cells in the cKO/Het spleen only saw restored expression of CD69, likely as a result of decreased S1PR1 expression.

RNA-seq analysis of Ets1 WT and cKO NKT1s in the spleen and liver revealed a similar number of DEGs in Ets1-deficient NKT1s. Furthermore, PCA showed that WT and cKO NKT1s from both tissues had nearly identical transcriptomes. However, the spleen and liver do possess unique DEGs and these genes represent an important next step in dissecting the differences between splenic and hepatic NKT1 cells. As most of the preliminary analysis was focused on adhesion markers typically associated with liver NKT cells, it will be relevant to investigate surface receptors such as CXCR5, which has been associated with mobilization of splenic NKT cells to B cell zones. By obtaining a broader characterization of adhesion receptors on NKT1 we may be able to learn more about what facilitates their localization within tissues, most notably how hepatic NKTs cells are primarily located in the sinusoids while splenic NKTs are more broadly distributed under steady-state conditions.

References

Adler, M., Taylor, S., Okebugwu, K., Yee, H., Fielding, C., Fielding, G., & Poles, M. (2011). Intrahepatic natural killer t cell populations are increased in human hepatic steatosis. *World Journal of Gastroenterology*, *17*(13).

Aki, S., Yoshioka, K., Takuwa, N., & Takuwa, Y. (2020). TGFβ receptor endocytosis and Smad signaling require synaptojanin1, PI3K-C2α-, and INPP4B-mediated phosphoinositide conversions. *Molecular Biology of the Cell*, *31*(5).

Alberola-Ila, J., Hogquist, K. A., Swan, K. A., Bevan, M. J., & Perlmutter, R. M. (1996). Positive and negative selection invoke distinct signaling pathways. *Journal of Experimental Medicine*, *184*(1).

Allende, M. L., Zhou, D., Kalkofen, D. N., Benhamed, S., Tuymetova, G., Borowski, C., Bendelac, A., & Proia, R. L. (2008). S1P 1 receptor expression regulates emergence of NKT cells in peripheral tissues. *The FASEB Journal*, *22*(1).

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2).

Anderson, M. K., Hernandez-Hoyos, G., Diamond, R. A., & Rothenberg, E. V. (1999). Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development*, *126*(14).

Bai, A., Hu, H., Yeung, M., & Chen, J. (2007). Krüppel-Like Factor 2 Controls T Cell Trafficking by Activating L-Selectin (CD62L) and Sphingosine-1-Phosphate Receptor 1 Transcription. *The Journal of Immunology*, *178*(12).

Bar Yaacov, R., Eshel, R., Farhi, E., Shemuluvich, F., Kaplan, T., & Birnbaum, R. Y. (2019). Functional characterization of the ZEB2 regulatory landscape. *Human Molecular Genetics*, *28*(9).

Baranek, T., Lebrigand, K., de Amat Herbozo, C., Gonzalez, L., Bogard, G., Dietrich, C., Magnone, V., Boisseau, C., Jouan, Y., Trottein, F., Si-Tahar, M., Leite-de-Moraes, M., Mallevaey, T., & Paget, C. (2020). High Dimensional Single-Cell Analysis Reveals iNKT Cell Developmental Trajectories and Effector Fate Decision. *Cell Reports*, *32*(10).

Barral, P., Sénchez-Nieo, M. D., Van Rooijen, N., Cerundolo, V., & Batista, F. D. (2012). The location of splenic NKT cells favours their rapid activation by blood-borne antigen. *EMBO Journal*, *31*(10).

Barton, K., Muthusamy, N., Fischer, C., Ting, C. N., Walunas, T. L., Lanier, L. L., & Leiden, J. M. (1998). The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity*, *9*(4).

Benlagha, K., Kyin, T., Beavis, A., Teyton, L., & Bendelac, A. (2002). A thymic precursor to the NK T cell lineage. *Science*, *296*(5567).

Benlagha, K., Wei, D. G., Veiga, J., Teyton, L., & Bendelac, A. (2005). Characterization of the early stages of thymic NKT cell development. *Journal of Experimental Medicine*, *202*(4).

Berga-Bolaños, R., Zhu, W. S., Steinke, F. C., Xue, H. H., & Sen, J. M. (2015). Cell-autonomous requirement for TCF1 and LEF1 in the development of Natural Killer T cells. *Molecular Immunology*, *68*(2).

Berzins, S. P., McNab, F. W., Jones, C. M., Smyth, M. J., & Godfrey, D. I. (2006). Long-Term Retention of Mature NK1.1 + NKT Cells in the Thymus. *The Journal of Immunology*, *176*(7).

Beyaz, S., Kim, J. H., Pinello, L., Xifaras, M. E., Hu, Y., Huang, J., Kerenyi, M. A., Das, P. P., Barnitz, R. A., Herault, A., Dogum, R., Haining, W. N., Yilmaz, Ö. H., Passegue, E., Yuan, G. C., Orkin, S. H., & Winau, F. (2017). The histone demethylase UTX regulates the lineage-specific epigenetic program of invariant natural killer T cells. *Nature Immunology*, *18*(2).

Beyer, M., Wang, H., Peters, N., Doths, S., Koerner-Rettberg, C., Openshaw, P. J. M., & Schwarze, J. (2005). The beta2 integrin CD11c distinguishes a subset of cytotoxic pulmonary T cells with potent antiviral effects in vitro and in vivo. *Respiratory Research*, *6*.

Bikoff, E. K., Morgan, M. A., & Robertson, E. J. (2009). An expanding job description for Blimp-1/PRDM1. *Current Opinion in Genetics and Development*, *19*(4).

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15).

Bories, J. C., Willerford, D. M., Grévin, D., Davidson, L., Camus, A., Martin, P., Stéhelin, D., & Alt, F. W. (1995). Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. *Nature*, *377*(6550).

Borowski, C., & Bendelac, A. (2005). Signaling for NKT cell development: The SAP-FynT connection. *Journal of Experimental Medicine*, *201*(6).

Breed, E. R., Vobořil, M., Ashby, K. M., Martinez, R. J., Qian, L., Wang, H., Salgado, O. C., O'Connor, C. H., & Hogquist, K. A. (2022). Type 2 cytokines in the thymus activate Sirpα+ dendritic cells to promote clonal deletion. *Nature Immunology*, *23*(7), 1042–1051.

Brennan, P. J., Brigl, M., & Brenner, M. B. (2013). Invariant natural killer T cells: An innate activation scheme linked to diverse effector functions. *Nature Reviews Immunology*, *13*(2).

Buggy, Y., Maguire, T. M., McGreal, G., McDermott, E., Hill, A. D. K., O'Higgins, N., & Duffy, M. J. (2004). Overexpression of the Ets-1 transcription factor in human breast cancer. *British Journal of Cancer*, *91*(7).

Cannarile, M. A., Lind, N. A., Rivera, R., Sheridan, A. D., Camfield, K. A., Wu, B. B., Cheung, K. P., Ding, Z., & Goldrath, A. W. (2006). Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nature Immunology*, *7*(12).

Carlson, C. M., Endrizzi, B. T., Wu, J., Ding, X., Weinreich, M. A., Walsh, E. R., Wani, M. A., Lingrel, J. B., Hogquist, K. A., & Jameson, S. C. (2006). Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature*, *442*(7100).

Carr, T., Krishnamoorthy, V., Yu, S., Xue, H. H., Kee, B. L., & Verykokakis, M. (2015). The transcription factor lymphoid enhancer factor 1 controls Invariant natural killer T cell expansion and Th2-type effector differentiation. *Journal of Experimental Medicine*, *212*(5).

Casey, K. A., Fraser, K. A., Schenkel, J. M., Moran, A., Abt, M. C., Beura, L. K., Lucas, P. J., Artis, D., Wherry, E. J., Hogquist, K., Vezys, V., & Masopust, D. (2012). Antigen-independent differentiation and maintenance of effector-like resident memory t cells in tissues. *The Journal of Immunology*, *188*(10), 4866–4875.

Chan, B., Lanyi, A., Song, H. K., Griesbach, J., Simarro-Grande, M., Poy, F., Howie, D., Sumegi, J., Terhorst, C., & Eck, M. J. (2003). SAP couples Fyn to SLAM immune receptors. *Nature Cell Biology*, *5*(2).

Chen, J. H. (1985). The proto-oncogene c-ets is preferentially expressed in lymphoid cells. *Molecular and Cellular Biology*, *5*(11).

Chen, S., Cai, C., Li, Z., Liu, G., Wang, Y., Blonska, M., Li, D., Du, J., Lin, X., Yang, M., & Dong, Z. (2017). Dissection of SAP-dependent and SAP-independent SLAM family signaling in NKT cell development and humoral immunity. *Journal of Experimental Medicine*, *214*(2).

Chen, Y., & Tian, Z. (2020). Roles of Hepatic Innate and Innate-Like Lymphocytes in Nonalcoholic Steatohepatitis. *Frontiers in Immunology*, *11*.

Chiba, K., Yanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., & Hoshino, Y. (1998). FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *The Journal of Immunology*, 160 (10), pp. 5037–5044.

Christo, S. N., Evrard, M., Park, S. L., Gandolfo, L. C., Burn, T. N., Fonseca, R., Newman, D. M., Alexandre, Y. O., Collins, N., Zamudio, N. M., Souza-Fonseca-Guimaraes, F., Pellicci, D. G., Chisanga, D., Shi, W., Bartholin, L., Belz, G. T., Huntington, N. D., Lucas, A., Lucas, M., … Mackay, L. K. (2021). Discrete tissue microenvironments instruct diversity in resident memory T cell function and plasticity. *Nature Immunology*, *22*(9).

Chu, C. F., Feng, H. K., Sun, K. H., Hsu, C. L., & Dzhagalov, I. L. (2020). Examination of Fas-Induced Apoptosis of Murine Thymocytes in Thymic Tissue Slices Reveals That Fas Is Dispensable for Negative Selection. *Frontiers in Cell and Developmental Biology*, *8*.

Chuang, Y. T., Chuang, W. C., Liu, C. C., Liu, C. W., Huang, Y. W., Yang, H. Y., Ho, I. C., & Tai, T. S. (2021). Ets1 promotes the differentiation of post-selected iNKT cells through regulation of the expression of Vα14Jα18 t cell receptor and PLZF. *International Journal of Molecular Sciences*, *22*(22).

Clements, J. L., John, S. A., & Garrett-Sinha, L. A. (2006). Impaired Generation of CD8 + Thymocytes in Ets-1-Deficient Mice. *The Journal of Immunology*, *177*(2).

Coman, T., Rossignol, J., D'Aveni, M., Fabiani, B., Dussiot, M., Rignault, R., Babdor, J., Bouillé, M., Herbelin, A., Coté, F., Moura, I. C., Hermine, O., & Rubio, M. T. (2018). Human CD4- invariant NKT lymphocytes regulate graft versus host disease. *OncoImmunology*, *7*(11).

Corces, M. R., Trevino, A. E., Hamilton, E. G., Greenside, P. G., Sinnott-Armstrong, N. A., Vesuna, S., Satpathy, A. T., Rubin, A. J., Montine, K. S., Wu, B., Kathiria, A., Cho, S. W., Mumbach, M. R., Carter, A. C., Kasowski, M., Orloff, L. A., Risca, V. I., Kundaje, A., Khavari, P. A., … Chang, H. Y. (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nature Methods*, *14*(10).

Crosby, C. M., & Kronenberg, M. (2018). Tissue-specific functions of invariant natural killer T cells. *Nature Reviews Immunology*, *18*(9).

Crowl, J. T., Heeg, M., Ferry, A., Milner, J. J., Omilusik, K. D., Toma, C., He, Z., Chang, J. T., & Goldrath, A. W. (2022). Tissue-resident memory CD8+ T cells possess unique transcriptional, epigenetic and functional adaptations to different tissue environments. *Nature Immunology*, *23*(7), 1121–1131.

Danzer, C., Koller, A., Baier, J., Arnold, H., Giessler, C., Opoka, R., Schmidt, S., Willers, M., Mihai, S., Parsch, H., Wirtz, S., Daniel, C., Reinhold, A., Engelmann, S., Kliche, S., Bogdan, C., Hoebe, K., & Mattner, J. (2016). A mutation within the SH2 domain of slp-76 regulates the tissue distribution and cytokine production of iNKT cells in mice. *European Journal of Immunology*, *46*(9).

Dashtsoodol, N., Bortoluzzi, S., & Schmidt-Supprian, M. (2019). T cell receptor expression timing and signal strength in the functional differentiation of invariant natural killer T cells. *Frontiers in Immunology*, *10*(APR).

D'Cruz, L. M., Stradner, M. H., Yang, C. Y., & Goldrath, A. W. (2014). E and Id Proteins Influence Invariant NKT Cell Sublineage Differentiation and Proliferation. *The Journal of Immunology*, *192*(5).

De Calisto, J., Wang, N., Wang, G., Yigit, B., Engel, P., & Terhorst, C. (2014). SAP-dependent and -independent regulation of innate T cell development involving SLAMF receptors. *Frontiers in Immunology*, *5*(APR).

Deignan, T., Curry, M. P., Doherty, D. G., Golden-Mason, L., Volkov, Y., Norris, S., Nolan, N., Traynor, O., McEntee, G., Hegarty, J. E., & O'Farrelly, C. (2002). Decrease in hepatic CD56+ T cells and Vα24+ natural killer T cells in chronic hepatitis C viral infection. *Journal of Hepatology*, *37*(1).

Delconte, R. B., Shi, W., Sathe, P., Ushiki, T., Seillet, C., Minnich, M., Kolesnik, T. B., Rankin, L. C., Mielke, L. A., Zhang, J. G., Busslinger, M., Smyth, M. J., Hutchinson, D. S., Nutt, S. L., Nicholson, S. E., Alexander, W. S., Corcoran, L. M., Vivier, E., Belz, G. T., … Huntington, N. D. (2016). The Helix-Loop-Helix Protein ID2 Governs NK Cell Fate by Tuning Their Sensitivity to Interleukin-15. *Immunity*, *44*(1).

Delfanti, G., Cortesi, F., Perini, A., Antonini, G., Azzimonti, L., de Lalla, C., Garavaglia, C., Squadrito, M. L., Fedeli, M., Consonni, M., Sesana, S., Re, F., Shen, H., Dellabona, P., & Casorati, G. (2022). TCR-engineered iNKT cells induce robust antitumor response by dual targeting cancer and suppressive myeloid cells. In *Sci. Immunol* (Vol. 7, p. 6563).

Della Chiara, V., Daxinger, L., & Staal, F. J. T. (2021). The route of early T cell development: Crosstalk between epigenetic and transcription factors. *Cells*, *10*(5).

Dhume, K., Finn, C. M., Strutt, T. M., Sell, S., & McKinstry, K. K. (2019). T-bet optimizes CD4 T-cell responses against influenza through CXCR3-dependent lung trafficking but not functional programming. *Mucosal Immunology*, *12*(5).

Dittmer, J. (2015). The role of the transcription factor Ets1 in carcinoma. *Seminars in Cancer Biology*, *35*.

Dobenecker, M. W., Kim, J. K., Marcello, J., Fang, T. C., Prinjha, R., Bosselut, R., & Tarakhovsky, A. (2015). Coupling of T cell receptor specificity to natural killer T cell development by bivalent histone H3 methylation. *Journal of Experimental Medicine*, *212*(3).

Doherty, D. G. (2016). Immunity, tolerance and autoimmunity in the liver: A comprehensive review. *Journal of Autoimmunity*, *66*.

Doisne, J. M., Bartholin, L., Yan, K. P., Garcia, C. N., Duarte, N., Le Luduec, J. B., Vincent, D., Cyprian, F., Horvat, B., Martel, S., Rimokh, R., Losson, R., Benlagha, K., & Marie, J. C. (2009). INKT cell development is orchestrated by different branches of TGF-β signaling. *Journal of Experimental Medicine*, *206*(6).

Doisne, J.-M., Becourt, C., Amniai, L., Duarte, N., Le Luduec, J.-B., Eberl, G., & Benlagha, K. (2009). Skin and Peripheral Lymph Node Invariant NKT Cells Are Mainly Retinoic Acid Receptor-Related Orphan Receptor γt + and Respond Preferentially under Inflammatory Conditions. *The Journal of Immunology*, *183*(3).

Dominguez, C. X., Amezquita, R. A., Guan, T., Marshall, H. D., Joshi, N. S., Kleinstein, S. H., & Kaech, S. M. (2015). The transcription factors ZEB2 and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection. *Journal of Experimental Medicine*, *212*(12).

Dong, Z., Zhang, J., Sun, R., Wei, H., & Tian, Z. (2007). Impairment of liver regeneration correlates with activated hepatic NKT cells in HBV transgenic mice. *Hepatology*, *45*(6).

Drennan, M. B., Franki, A.-S., Dewint, P., Van Beneden, K., Seeuws, S., van de Pavert, S. A., Reilly, E. C., Verbruggen, G., Lane, T. E., Mebius, R. E., Deforce, D., & Elewaut, D. (2009). Cutting Edge: The Chemokine Receptor CXCR3 Retains Invariant NK T Cells in the Thymus. *The Journal of Immunology*, *183*(4).

Emoto, M., Mittrücker, H. W., Schmits, R., Mak, T. W., & Kaufmann, S. H. (1999). Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *162*(9).

Emoto, M., Miyamoto, M., Namba, K., Schmits, R., Van Rooijen, N., Kita, E., & Kaufmann, S. H. E. (2000). Participation of leukocyte function-associated antigen-1 and NK cells in the homing of thymic CD8+NKT cells to the liver. *European Journal of Immunology*, *30*(10).

Engel, I., Seumois, G., Chavez, L., Samaniego-Castruita, D., White, B., Chawla, A., Mock, D., Vijayanand, P., & Kronenberg, M. (2016). Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nature Immunology*, *17*(6).

Eun, Y. C., Kyeong, C. J., Hyo, J. P., Doo, H. C., Jin, S. S., Seung, D. Y., Simpson, E., & Seong, H. P. (2005). Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity*, *23*(4).

Evrard, M., Wynne-Jones, E., Peng, C., Kato, Y., Christo, S. N., Fonseca, R., Park, S. L., Burn, T. N., Osman, M., Devi, S., Chun, J., Mueller, S. N., Kannourakis, G., Berzins, S. P., Pellicci, D. G., Heath, W. R., Jameson, S. C., & Mackay, L. K. (2021). Sphingosine 1-phosphate receptor 5 (S1PR5) regulates the peripheral retention of tissue-resident lymphocytes. *Journal of Experimental Medicine*, *219*(1).

Eyquem, S., Chemin, K., Fasseu, M., Chopin, M., Sigaux, F., Cumano, A., & Bories, J. C. (2004). The development of early and mature B cells is impaired in mice deficient for the Ets-1 transcription factor. *European Journal of Immunology*, *34*(11).

Fabre, S., Carrette, F., Chen, J., Lang, V., Semichon, M., Denoyelle, C., Lazar, V., Cagnard, N., Dubart-Kupperschmitt, A., Mangeney, M., Fruman, D. A., & Bismuth, G. (2008). FOXO1 Regulates L-Selectin and a Network of Human T Cell Homing Molecules Downstream of Phosphatidylinositol 3-Kinase. *The Journal of Immunology*, *181*(5).

Faust, E. A., Saffran, D. C., Toksoz, D., Williams, D. A., & Witte, O. N. (1993). Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from Pre-B cells. *Journal of Experimental Medicine*, *177*(4).

Fonseca, R., Burn, T. N., Gandolfo, L. C., Devi, S., Park, S. L., Obers, A., Evrard, M., Christo, S. N., Buquicchio, F. A., Lareau, C. A., McDonald, K. M., Sandford, S. K., Zamudio, N. M., Zanluqui, N. G., Zaid, A., Speed, T. P., Satpathy, A. T., Mueller, S. N., Carbone, F. R., & Mackay, L. K. (2022). Runx3 drives a CD8+ T cell tissue residency program that is absent in CD4+ T cells. *Nature Immunology*, *23*(8), 1236–1245.

Frizzell, H., Fonseca, R., Christo, S. N., Evrard, M., Cruz-Gomez, S., Zanluqui, N. G., von Scheidt, B., Freestone, D., Park, S. L., McWilliam, H. E. G., Villadangos, J. A., Carbone, F. R., & Mackay, L. K. (2020). Organ-specific isoform selection of fatty acid-binding proteins in tissue-resident lymphocytes. *Science Immunology*, *5*(46).

Garrett-Sinha, L. A. (2013). Review of Ets1 structure, function, and roles in immunity. *Cellular and Molecular Life Sciences*, *70*(18), 3375–3390.

Gebhardt, T., Palendira, U., Tscharke, D. C., & Bedoui, S. (2018). Tissue-resident memory T cells in tissue homeostasis, persistent infection, and cancer surveillance. *Immunological Reviews*, *283*(1).

Geissmann, F., Cameron, T. O., Sidobre, S., Manlongat, N., Kronenberg, M., Briskin, M. J., Dustin, M. L., & Littman, D. R. (2005). Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biology*, *3*(4).

Germanov, E., Veinotte, L., Cullen, R., Chamberlain, E., Butcher, E. C., & Johnston, B. (2008). Critical Role for the Chemokine Receptor CXCR6 in Homeostasis and Activation of CD1d-Restricted NKT Cells. *The Journal of Immunology*, *181*(1).

Gerth, E., & Mattner, J. (2019). The role of adaptor proteins in the biology of natural killer T (NKT) cells. *Frontiers in Immunology*, *10*(JUN).

Gioulbasani, M., Galaras, A., Grammenoudi, S., Moulos, P., Dent, A. L., Sigvardsson, M., Hatzis, P., Kee, B. L., & Verykokakis, M. (2020). The transcription factor BCL-6 controls early development of innate-like T cells. *Nature Immunology*, *21*(9).

Gleimer, M., von Boehmer, H., & Kreslavsky, T. (2012). PLZF controls the expression of a limited number of genes essential for NKT cell function. *Frontiers in Immunology*, *3*(DEC).

Godfrey, D. I., MacDonald, H. R., Kronenberg, M., Smyth, M. J., & Van Kaer, L. (2004). NKT cells: What's in a name? *Nature Reviews Immunology*, *4*(3).

Graham, D. B., Bell, M. P., McCausland, M. M., Huntoon, C. J., van Deursen, J., Faubion, W. A., Crotty, S., & McKean, D. J. (2006). Ly9 (CD229)-deficient mice exhibit T cell defects yet do not share several phenotypic characteristics associated with SLAM- and SAP-deficient mice. *The Journal of Immunology*, *176*(6).

Grenningloh, R., Bok, Y. K., & Ho, I. C. (2005). Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *Journal of Experimental Medicine*, *201*(4).

Griewank, K., Borowski, C., Rietdijk, S., Wang, N., Julien, A., Wei, D. G., Mamchak, A. A., Terhorst, C., & Bendelac, A. (2007). Homotypic Interactions Mediated by Slamf1 and Slamf6 Receptors Control NKT Cell Lineage Development. *Immunity*, *27*(5).

Guidez, F., Howell, L., Isalan, M., Cebrat, M., Alani, R. M., Ivins, S., Hormaeche, I., McConnell, M. J., Pierce, S., Cole, P. A., Licht, J., & Zelent, A. (2005). Histone Acetyltransferase Activity of p300 Is Required for Transcriptional Repression by the Promyelocytic Leukemia Zinc Finger Protein. *Molecular and Cellular Biology*, *25*(13).

Haddad, R., Lanjuin, A., Madisen, L., Zeng, H., Murthy, V. N., & Uchida, N. (2013). Olfactory cortical neurons read out a relative time code in the olfactory bulb. *Nature Neuroscience*, *16*(7).

Hadley, G. A., Rostapshova, E. A., Gomolka, D. M., Taylor, B. M., Bartlett, S. T., Drachenberg, C. I., & Weir, M. R. (1999). Regulation of the epithelial cell-specific integrin, CD103, by human CD8+ cytolytic T lymphocytes. *Transplantation*, *67*(11).

Harsha Krovi, S., Zhang, J., Michaels-Foster, M. J., Brunetti, T., Loh, L., Scott-Browne, J., & Gapin, L. (2020). Thymic iNKT single cell analyses unmask the common developmental program of mouse innate T cells. *Nature Communications*, *11*(1).

Havenar-Daughton, C., Li, S., Benlagha, K., & Marie, J. C. (2012). Development and function of murine RORγt+ iNKT cells are under TGF-β signaling control. *Blood*, *119*(15).

Heczey, A., Courtney, A. N., Montalbano, A., Robinson, S., Liu, K., Li, M., Ghatwai, N., Dakhova, O., Liu, B., Raveh-Sadka, T., Chauvin-Fleurence, C. N., Xu, X., Ngai, H., Di Pierro, E. J., Savoldo, B., Dotti, G., & Metelitsa, L. S. (2020). Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: An interim analysis. *Nature Medicine*, *26*(11).

Heczey, A., Liu, D., Tian, G., Courtney, A. N., Wei, J., Marinova, E., Gao, X., Guo, L., Yvon, E., Hicks, J., Liu, H., Dotti, G., & Metelitsa, L. S. (2014). Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood*, *124*(18).

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H., & Glass, C. K. (2010). Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Molecular Cell*, *38*(4).

Hinks, T. S. C., & Zhang, X. W. (2020). MAIT Cell Activation and Functions. *Frontiers in Immunology*, *11*.

Hollenhorst, P. C., McIntosh, L. P., & Graves, B. J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. *Annual Review of Biochemistry*, *80*.

Hosokawa, H., & Rothenberg, E. V. (2021). How transcription factors drive choice of the T cell fate. *Nature Reviews Immunology*, *21*(3).

Howie, D., Laroux, F. S., Morra, M., Satoskar, A. R., Rosas, L. E., Faubion, W. A., Julien, A., Rietdijk, S., Coyle, A. J., Fraser, C., & Terhorst, C. (2005). Cutting Edge: The SLAM Family Receptor Ly108 Controls T Cell and Neutrophil Functions. *The Journal of Immunology*, *174*(10).

Hu, J. K., Crampton, J. C., Locci, M., & Crotty, S. (2016). CRISPR-mediated Slamf1Δ/Δ Slamf5Δ/Δ Slamf6Δ/Δ triple gene disruption reveals NKT cell defects but not T follicular helper cell defects. *PLoS ONE*, *11*(5).

Hu, T., Gimferrer, I., & Alberola-Ila, J. (2011). Control of early stages in invariant natural killer T-cell development. *Immunology*, *134*(1).

Hu, T., Gimferrer, I., Simmons, A., Wiest, D., & Alberola-Ila, J. (2011). The Ras/MAPK pathway is required for generation of iNKT cells. *PLoS ONE*, *6*(5).

Hwang, J. R., Byeon, Y., Kim, D., & Park, S. G. (2020). Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. *Experimental and Molecular Medicine*, *52*(5).

Ito, H., Ando, K., Nakayama, T., Taniguchi, M., Ezaki, T., Saito, K., Takemura, M., Sekikawa, K., Imawari, M., Seishima, M., & Moriwaki, H. (2003). Role of Vα 14 NKT Cells in the Development of Impaired Liver Regeneration In Vivo. *Hepatology*, *38*(5).

Jenne, C. N., Enders, A., Rivera, R., Watson, S. R., Bankovich, A. J., Pereira, J. P., Xu, Y., Roots, C. M., Beilke, J. N., Banerjee, A., Reiner, S. L., Miller, S. A., Weinmann, A. S., Goodnow, C. C., Lanier, L. L., Cyster, J. G., & Chun, J. (2009). T-bet-dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow. *Journal of Experimental Medicine*, *206*(11).

John, S. A., Clements, J. L., Russell, L. M., & Garrett-Sinha, L. A. (2008). Ets-1 regulates plasma cell differentiation by interfering with the activity of the transcription factor blimp-1. *Journal of Biological Chemistry*, *283*(2).

Johnston, B., Kim, C. H., Soler, D., Emoto, M., & Butcher, E. C. (2003). Differential Chemokine Responses and Homing Patterns of Murine TCRαβ NKT Cell Subsets. *The Journal of Immunology*, *171*(6).

Jones, M. E., & Zhuang, Y. (2007). Acquisition of a Functional T Cell Receptor during T Lymphocyte Development Is Enforced by HEB and E2A Transcription Factors. *Immunity*, *27*(6).

Kakimi, K., Guidotti, L. G., Koezuka, Y., & Chisari, F. V. (2000). Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *Journal of Experimental Medicine*, *192*(7).

Karadimitris, A., Ripoll-Fiol, C., & Guerra, J. C. (2019). Invariant NKT cells as a platform for CAR immunotherapy and prevention of acute Graft-versus-Host Disease. *HemaSphere*, *3*(S2).

Kee, B. L. (2009). E and ID proteins branch out. *Nature Reviews Immunology*, *9*(3).

Khan, M. A., & Khan, A. (2021). Role of NKT cells during viral infection and the development of NKT cell-based nanovaccines. *Vaccines*, *9*(9).

King, I. L., Amiel, E., Tighe, M., Mohrs, K., Veerapen, N., Besra, G., Mohrs, M., & Leadbetter, E. A. (2013). The Mechanism of Splenic Invariant NKT Cell Activation Dictates Localization In Vivo. *The Journal of Immunology*, *191*(2).

Kinsella, R. J., Kähäri, A., Haider, S., Zamora, J., Proctor, G., Spudich, G., Almeida-King, J., Staines, D., Derwent, P., Kerhornou, A., Kersey, P., & Flicek, P. (2011). Ensembl BioMarts: A hub for data retrieval across taxonomic space. *Database*, *2011*.

Klibi, J., Joseph, C., Delord, M., Teissandier, A., Lucas, B., Chomienne, C., Toubert, A., Bourc'his, D., Guidez, F., & Benlagha, K. (2021). PLZF Acetylation Levels Regulate NKT Cell Differentiation. *The Journal of Immunology*, *207*(3).

Kobayashi, E., Motoki, K., Uchida, T., Fukushima, H., & Koezuka, Y. (1995). KRN7000, a novel immunomodulator, and its antitumor activities. *Oncology Research*, *7*(10–11).

Kola, I., Brookes, S., Green, A. R., Garber, R., Tymms, M., Papas, T. S., & Seth, A. (1993). The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(16).

Koubi, M., Poplineau, M., Vernerey, J., N'Guyen, L., Tiberi, G., Garciaz, S., El-Kaoutari, A., Maqbool, M. A., Andrau, J. C., Guillouf, C., Saurin, A. J., & Duprez, E. (2018). Regulation of the positive transcriptional effect of PLZF through a non-canonical EZH2 activity. *Nucleic Acids Research*, *46*(7).

Kovalovsky, D., Alonzo, E. S., Uche, O. U., Eidson, M., Nichols, K. E., & Sant'Angelo, D. B. (2010). PLZF Induces the Spontaneous Acquisition of Memory/Effector Functions in T Cells Independently of NKT Cell-Related Signals. *The Journal of Immunology*, *184*(12).

Kovalovsky, D., Uche, O. U., Eladad, S., Hobbs, R. M., Yi, W., Alonzo, E., Chua, K., Eidson, M., Kim, H. J., Im, J. S., Pandolfi, P. P., & Sant'Angelo, D. B. (2008). The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature Immunology*, *9*(9).

Krijgsman, D., Hokland, M., & Kuppen, P. J. K. (2018). The role of natural killer T cells in cancer-A phenotypical and functional approach. *Frontiers in Immunology*, *9*(FEB).

Kumar, A., Zamora-Pineda, J., Degagné, E., & Saba, J. D. (2017). S1P Lyase Regulation of Thymic Egress and Oncogenic Inflammatory Signaling. *Mediators of Inflammation*, *2017*.

Kuns, R. D., Morris, E. S., MacDonald, K. P. A., Markey, K. A., Morris, H. M., Raffelt, N. C., Banovic, T., Don, A. L. J., Rowe, V., Burman, A. C., Clouston, A. D., Farah, C., Besra, G. S., Illarionov, P. A., Smyth, M. J., Porcelli, S. A., & Hill, G. R. (2009). Invariant natural killer T cell-natural killer cell interactions dictate transplantation outcome after α-galactosylceramide administration. *Blood*, *113*(23).

Kurobe, H., Liu, C., Ueno, T., Saito, F., Ohigashi, I., Seach, N., Arakaki, R., Hayashi, Y., Kitagawa, T., Lipp, M., Boyd, R. L., & Takahama, Y. (2006). CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity*, *24*(2).

Kuwatani, M., Ikarashi, Y., Iizuka, A., Kawakami, C., Quinn, G., Heike, Y., Yoshida, M., Asaka, M., Takaue, Y., & Wakasugi, H. (2006). Modulation of acute graft-versus-host disease and chimerism after adoptive transfer of in vitro-expanded invariant Vα14 natural killer T cells. *Immunology Letters*, *106*(1).

Kwan, J., & Killeen, N. (2004). CCR7 Directs the Migration of Thymocytes into the Thymic Medulla. *The Journal of Immunology*, *172*(7).

Latour, S., Roncagalli, R., Chen, R., Bakinowski, M., Shi, X., Schwartzberg, P. L., Davidson, D., & Veillette, A. (2003). Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nature Cell Biology*, *5*(2).

Lazarevic, V., Zullo, A. J., Schweitzer, M. N., Staton, T. L., Gallo, E. M., Crabtree, G. R., & Glimcher, L. H. (2009). The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nature Immunology*, *10*(3).

Lee, C.-G., Kwon, H.-K., Sahoo, A., Hwang, W., So, J.-S., Hwang, J.-S., Chae, C.-S., Kim, G.-C., Kim, J.-E., So, H.-S., Hwang, E. S., Grenningloh, R., Ho, I.-C., & Im, S.-H. (2012). Interaction of Ets-1 with HDAC1 Represses IL-10 Expression in Th1 Cells. *The Journal of Immunology*, *188*(5).

Lee, W. Y., Moriarty, T. J., Wong, C. H. Y., Zhou, H., Strieter, R. M., Van Rooijen, N., Chaconas, G., & Kubes, P. (2010). An intravascular immune response to Borrelia burgdorferi involves Kupffer cells and iNKT cells. *Nature Immunology*, *11*(4).

Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C., & Hogquist, K. A. (2013). Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature Immunology*, *14*(11).

Lee, Y. J., Starrett, G. J., Lee, S. T., Yang, R., Henzler, C. M., Jameson, S. C., & Hogquist, K. A. (2016). Lineage-Specific Effector Signatures of Invariant NKT Cells

Are Shared amongst γδ T, Innate Lymphoid, and Th Cells. *The Journal of Immunology*, *197*(4).

Lee, Y. J., Wang, H., Starrett, G. J., Phuong, V., Jameson, S. C., & Hogquist, K. A. (2015). Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. *Immunity*, *43*(3).

Levéen, P., Larsson, J., Ehinger, M., Cilio, C. M., Sundler, M., Sjöstrand, L. J., Holmdahl, R., & Karlsson, S. (2002). Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood*, *100*(2).

Levings, M. K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal Malefyt, R., & Roncarolo, M.-G. (2001). IFN-α and IL-10 Induce the Differentiation of Human Type 1 T Regulatory Cells. *The Journal of Immunology*, *166*(9).

Li, J., Wu, D., Jiang, N., & Zhuang, Y. (2013). Combined Deletion of Id2 and Id3 Genes Reveals Multiple Roles for E Proteins in Invariant NKT Cell Development and Expansion. *The Journal of Immunology*, *191*(10).

Li, M. O., Sanjabi, S., & Flavell, R. A. (2006). Transforming Growth Factor-β Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms. *Immunity*, *25*(3).

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. L., & Flavell, R. A. (2006). Transforming growth factor-β regulation of immune responses. *Annual Review of Immunology*, *24*.

Li, Z. Y., Morman, R. E., Hegermiller, E., Sun, M., Bartom, E. T., Maienschein-Cline, M., Sigvardsson, M., & Kee, B. L. (2021). The transcriptional repressor ID2 supports natural killer cell maturation by controlling TCF1 amplitude. *Journal of Experimental Medicine*, *218*(6).

Liu, X., Li, L., Si, F., Huang, L., Zhao, Y., Zhang, C., Hoft, D. F., & Peng, G. (2021). NK and NKT cells have distinct properties and functions in cancer. *Oncogene*, *40*(27).

Loh, L., Wang, Z., Sant, S., Koutsakos, M., Jegaskanda, S., Corbett, A. J., Liu, L., Fairlie, D. P., Crowe, J., Rossjohn, J., Xu, J., Doherty, P. C., McCluskey, J., & Kedzierska, K. (2016). Human mucosal-associated invariant T cells contribute to

antiviral influenza immunity via IL-18-dependent activation. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(36).

Lu, Y., Zhong, M. C., Qian, J., Calderon, V., Cruz Tleugabulova, M., Mallevaey, T., & Veillette, A. (2019). SLAM receptors foster iNKT cell development by reducing TCR signal strength after positive selection. *Nature Immunology*, *20*(4).

Lucas, B., White, A. J., Cosway, E. J., Parnell, S. M., James, K. D., Jones, N. D., Ohigashi, I., Takahama, Y., Jenkinson, W. E., & Anderson, G. (2020). Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells. *Nature Communications*, *11*(1).

Lucas, M., Gadola, S., Meier, U., Young, N. T., Harcourt, G., Karadimitris, A., Coumi, N., Brown, D., Dusheiko, G., Cerundolo, V., & Klenerman, P. (2003). Frequency and Phenotype of Circulating Vα24/Vβ11 Double-Positive Natural Killer T Cells during Hepatitis C Virus Infection. *Journal of Virology*, *77*(3).

Mackay, L. K., Minnich, M., Kragten, N. A. M., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F. M., Stark, R., Pellicci, D. G., Godfrey, D. I., Belz, G. T., Pellegrini, M., Gebhardt, T., … Van Gisbergen, K. P. J. M. (2016). Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science*, *352*(6284).

Mackay, L. K., Rahimpour, A., Ma, J. Z., Collins, N., Stock, A. T., Hafon, M. L., Vega-Ramos, J., Lauzurica, P., Mueller, S. N., Stefanovic, T., Tscharke, D. C., Heath, W. R., Inouye, M., Carbone, F. R., & Gebhardt, T. (2013). The developmental pathway for CD103+ CD8+ tissue-resident memory T cells of skin. *Nature Immunology*, *14*(12).

Mackay, L. K., Wynne-Jones, E., Freestone, D., Pellicci, D. G., Mielke, L. A., Newman, D. M., Braun, A., Masson, F., Kallies, A., Belz, G. T., & Carbone, F. R. (2015). T-box Transcription Factors Combine with the Cytokines TGF-β and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity*, *43*(6).

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S., & Zeng, H. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience*, *13*(1).

Manne, V., Handa, P., & Kowdley, K. V. (2018). Pathophysiology of Nonalcoholic Fatty Liver Disease/Nonalcoholic Steatohepatitis. *Clinics in Liver Disease*, *22*(1).

Mao, A. P., Constantinides, M. G., Mathew, R., Zuo, Z., Chen, X., Weirauch, M. T., & Bendelac, A. (2016). Multiple layers of transcriptional regulation by PLZF in NKT-cell development. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(27).

Marie, J. C., Liggitt, D., & Rudensky, A. Y. (2006). Cellular Mechanisms of Fatal Early-Onset Autoimmunity in Mice with the T Cell-Specific Targeting of Transforming Growth Factor-β Receptor. *Immunity*, *25*(3).

Maroulakou, I. G., Papas, T. S., & Green, J. E. (1994). Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. *Oncogene*, *9*(6).

Martin, E., Treiner, E., Duban, L., Guerri, L., Laude, H., Toly, C., Premel, V., Devys, A., Moura, I. C., Tilloy, F., Cherif, S., Vera, G., Latour, S., Soudais, C., & Lantz, O. (2009). Stepwise development of mait cells in mouse and human. *PLoS Biology*, *7*(3).

Masopust, D., Choo, D., Vezys, V., Wherry, E. J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K. A., Barber, D. L., Kawamura, K. S., Fraser, K. A., Webby, R. J., Brinkmann, V., Butcher, E. C., Newell, K. A., & Ahmed, R. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. *Journal of Experimental Medicine*, *207*(3), 553–564.

Masson, F., Minnich, M., Olshansky, M., Bilic, I., Mount, A. M., Kallies, A., Speed, T. P., Busslinger, M., Nutt, S. L., & Belz, G. T. (2013). Id2-Mediated Inhibition of E2A Represses Memory CD8 + T Cell Differentiation. *The Journal of Immunology*, *190*(9).

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., & Cyster, J. G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*, *427*(6972).

Matsuda, J. L., Mallevaey, T., Scott-Browne, J., & Gapin, L. (2008). CD1d-restricted iNKT cells, the “Swiss-Army knife” of the immune system. *Current Opinion in Immunology*, *20*(3).

Matsuda, J. L., Zhang, Q., Ndonye, R., Richardson, S. K., Howell, A. R., & Gapin, L. (2006). T-bet concomitantly controls migration, survival, and effector functions during the development of Vα14i NKT cells. *Blood*, *107*(7).

McCaughtry, T. M., Wilken, M. S., & Hogquist, K. A. (2007). Thymic emigration revisited. *Journal of Experimental Medicine*, *204*(11).

Meierovics, A., Yankelevich, W. J. C., & Cowley, S. C. (2013). MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(33).

Miyaki, E., Hiraga, N., Imamura, M., Uchida, T., Kan, H., Tsuge, M., Abe-Chayama, H., Hayes, C. N., Makokha, G. N., Serikawa, M., Aikata, H., Ochi, H., Ishida, Y., Tateno, C., Ohdan, H., & Chayama, K. (2017). Interferon alpha treatment stimulates interferon gamma expression in type I NKT cells and enhances their antiviral effect against hepatitis C virus. *PLoS ONE*, *12*(3).

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews*, *22*(2).

Moisan, J., Grenningloh, R., Bettelli, E., Oukka, M., & Ho, I. C. (2007). Ets-1 is a negative regulator of Th17 differentiation. *Journal of Experimental Medicine*, *204*(12).

Mokrani, M., Klibi, J., Bluteau, D., Bismuth, G., & Mami-Chouaib, F. (2014). Smad and NFAT Pathways Cooperate to Induce CD103 Expression in Human CD8 T Lymphocytes. *The Journal of Immunology*, *192*(5).

Monticelli, L. A., Yang, Y., Knell, J., D'Cruz, L. M., Cannarile, M. A., Engel, I., Kronenberg, M., & Goldrath, A. W. (2009). Transcriptional regulator Id2 controls survival of hepatic NKT cells. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(46).

Morita, M., Motoki, K., Akimoto, K., Natori, T., Sakai, T., Sawa, E., Yamaji, K., Koezuka, Y., Kobayashi, E., & Fukushima, H. (1995). Structure-Activity Relationship of-Galactosylceramides against B16-Bearing Mice. In *J. Med. Chem* (Vol. 38, pp. 2176–2187).

Motoki, K., Morita, M., Kobayashi, E., Uchida, T., Akimoto, K., Fukushima, H., & Koezuka, Y. (1995). Immunostimulatory and Antitumor Activities of Monoglycosylceramides Having Various Sugar Moieties. *Biological and Pharmaceutical Bulletin*, *18*(11).

Mouly, E., Chemin, K., Nguyen, H. V., Chopin, M., Mesnard, L., Leite-de-Moraes, M., Burlen-defranoux, O., Bandeira, A., & Bories, J. C. (2010). The Ets-1 transcription factor controls the development and function of natural regulatory T cells. *Journal of Experimental Medicine*, *207*(10).

Muñoz-Ruiz, M., Sumaria, N., Pennington, D. J., & Silva-Santos, B. (2017). Thymic Determinants of γδ T Cell Differentiation. *Trends in Immunology*, *38*(5).

Murray, M. P., Engel, I., Seumois, G., Herrera-De la Mata, S., Rosales, S. L., Sethi, A., Logandha Ramamoorthy Premlal, A., Seo, G. Y., Greenbaum, J., Vijayanand, P., Scott-Browne, J. P., & Kronenberg, M. (2021). Transcriptome and chromatin landscape of iNKT cells are shaped by subset differentiation and antigen exposure. *Nature Communications*, *12*(1).

Muthusamy, N., Barton, K., & Leiden, J. M. (1995). Defective activation and survival of T cells lacking the Ets-1 transcription factor. *Nature*, *377*(6550).

Nakui, M., Ohta, A., Sekimoto, M., Sato, M., Iwakabe, K., Yahata, T., Kitamura, H., Koda, T., Kawano, T., Makuuchi, H., Taniguchi, M., & Nishimura, T. (2000). Potentiation of antitumor effect of NKT cell ligand, α-galactosylceramide by combination with IL-12 on lung metastasis of malignant melanoma cells. *Clinical and Experimental Metastasis*, *18*(2).

Nan, Y., Wu, C., & Zhang, Y. J. (2017). Interplay between Janus kinase/signal transducer and activator of transcription signaling activated by type I interferons and viral antagonism. *Frontiers in Immunology*, *8*(DEC).

Nath, A. P., Braun, A., Ritchie, S. C., Carbone, F. R., Mackay, L. K., Gebhardt, T., & Inouye, M. (2019). Comparative analysis reveals a role for TGF-β in shaping the residency-related transcriptional signature in tissue-resident memory CD8 + T cells. *PLoS ONE*, *14*(2).

Nguyen, H. V., Mouly, E., Chemin, K., Luinaud, R., Despres, R., Fermand, J. P., Arnulf, B., & Bories, J. C. (2012). The Ets-1 transcription factor is required for Stat1-

mediated T-bet expression and IgG2a class switching in mouse B cells. *Blood*, *119*(18).

Nichols, K. E., Hom, J., Gong, S. Y., Ganguly, A., Ma, C. S., Cannons, J. L., Tangye, S. G., Schwartzberg, P. L., Koretzky, G. A., & Stein, P. L. (2005). Regulation of NKT cell development by SAP, the protein defective in XLP. *Nature Medicine*, *11*(3).

Nitta, T., Nitta, S., Yu, L., Lipp, M., & Takahama, Y. (2009). CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(40).

Northrup, D., Yagi, R., Cui, K., Proctor, W. R., Wang, C., Placek, K., Pohl, L. R., Wang, R., Ge, K., Zhu, J., & Zhao, K. (2017). Histone demethylases UTX and JMJD3 are required for NKT cell development in mice. *Cell and Bioscience*, *7*(1).

Nutt, S. L., Fairfax, K. A., & Kallies, A. (2007). BLIMP1 guides the fate of effector B and T cells. *Nature Reviews Immunology*, *7*(12).

O'Konek, J. J., Illarionov, P., Khursigara, D. S., Ambrosino, E., Izhak, L., Castillo, B. F., Raju, R., Khalili, M., Kim, H. Y., Howell, A. R., Besra, G. S., Porcelli, S. A., Berzofsky, J. A., & Terabe, M. (2011). Mouse and human iNKT cell agonist β-mannosylceramide reveals a distinct mechanism of tumor immunity. *Journal of Clinical Investigation*, *121*(2).

Olson, C. M., Bates, T. C., Izadi, H., Radolf, J. D., Huber, S. A., Boyson, J. E., & Anguita, J. (2009). Local Production of IFN-γ by Invariant NKT Cells Modulates Acute Lyme Carditis. *The Journal of Immunology*, *182*(6).

Pan, Y., Tian, T., Park, C. O., Lofftus, S. Y., Mei, S., Liu, X., Luo, C., O'Malley, J. T., Gehad, A., Teague, J. E., Divito, S. J., Fuhlbrigge, R., Puigserver, P., Krueger, J. G., Hotamisligil, G. S., Clark, R. A., & Kupper, T. S. (2017). Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature*, *543*(7644).

Pasquier, B., Yin, L., Fondanèche, M. C., Relouzat, F., Bloch-Queyrat, C., Lambert, N., Fischer, A., De Saint-Basile, G., & Latour, S. (2005). Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked

lymphoproliferative syndrome gene product. *Journal of Experimental Medicine*, *201*(5).

Pellicci, D. G., Hammond, K. J. L., Uldrich, A. P., Baxter, A. G., Smyth, M. J., & Godfrey, D. I. (2002). A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1-CD4+ CD1d-dependent precursor stage. *Journal of Experimental Medicine*, *195*(7).

Pereira, R. M., Martinez, G. J., Engel, I., Cruz-Guilloty, F., Barboza, B. A., Tsagaratou, A., Lio, C. W. J., Berg, L. J., Lee, Y., Kronenberg, M., Bandukwala, H. S., & Rao, A. (2014). Jarid2 is induced by TCR signalling and controls iNKT cell maturation. *Nature Communications*, *5*.

Punt, J. A., Havran, W., Abe, R., Sarin, A., & Singer, A. (1997). T cell receptor (TCR)-induced death of immature CD4+CD8+ thymocytes by two distinct mechanisms differing in their requirement for CD28 costimulation: Implications for negative selection in the thymus. *Journal of Experimental Medicine*, *186*(11).

Qualai, J., Li, L. X., Cantero, J., Tarrats, A., Fernández, M. A., Sumoy, L., Rodolosse, A., McSorley, S. J., & Genescà, M. (2016). Expression of CD11c is associated with unconventional activated T cell subsets with high migratory potential. *PLoS ONE*, *11*(4).

Racanelli, V., & Rehermann, B. (2006). The liver as an immunological organ. *Hepatology*, *43*(2 SUPPL. 1).

Rahimpour, A., Koay, H. F., Enders, A., Clanchy, R., Eckle, S. B. G., Meehan, B., Chen, Z., Whittle, B., Liu, L., Fairlie, D. P., Goodnow, C. C., McCluskey, J., Rossjohn, J., Uldrich, A. P., Pellicci, D. G., & Godfrey, D. I. (2015). Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *Journal of Experimental Medicine*, *212*(7).

Ramirez, K., Chandler, K. J., Spaulding, C., Zandi, S., Sigvardsson, M., Graves, B. J., & Kee, B. L. (2012). Gene Deregulation and Chronic Activation in Natural Killer Cells Deficient in the Transcription Factor ETS1. *Immunity*, *36*(6).

Rengarajan, J., Mittelstadt, P. R., Mages, H. W., Gerth, A. J., Kroczek, R. A., Ashwell, J. D., & Glimcher, L. H. (2000). Sequential involvement of NFAT and Egr transcription factors in FasL regulation. *Immunity*, *12*(3).

Ribot, J. C., Lopes, N., & Silva-Santos, B. (2021). γδ T cells in tissue physiology and surveillance. *Nature Reviews Immunology*, *21*(4).

Robertson, F. C., Berzofsky, J. A., & Terabe, M. (2014). NKT cell networks in the regulation of tumor immunity. *Frontiers in Immunology*, *5*(OCT).

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011). Integrative genomics viewer. *Nature Biotechnology*, *29*(1).

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). EdgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1).

Rosen, H., Alfonso, C., Surh, C. D., & McHeyzer-Williams, M. G. (2003). Rapid induction of medullary thymocyte phenotypic maturation and egress inhibition by nanomolar sphingosine 1-phosphate receptor agonist. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(19).

Russell, L., & Garrett-Sinha, L. A. (2010). Transcription factor Ets-1 in cytokine and chemokine gene regulation. *Cytokine*, *51*(3), 217–226.

Sadler, A. J., Suliman, B. A., Yu, L., Yuan, X., Wang, D., Irving, A. T., Sarvestani, S. T., Banerjee, A., Mansell, A. S., Liu, J. P., Gerondakis, S., Williams, B. R. G., & Xu, D. (2015). The acetyltransferase HAT1 moderates the NF-κB response by regulating the transcription factor PLZF. *Nature Communications*, *6*.

Salou, M., Legoux, F., Gilet, J., Darbois, A., Du Halgouet, A., Alonso, R., Richer, W., Goubet, A. G., Daviaud, C., Menger, L., Procopio, E., Premel, V., & Lantz, O. (2019). A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *Journal of Experimental Medicine*, *216*(1), 133–151.

Salou, M., Legoux, F., & Lantz, O. (2021). MAIT cell development in mice and humans. *Molecular Immunology*, *130*.

Savage, A. K., Constantinides, M. G., & Bendelac, A. (2011). Promyelocytic Leukemia Zinc Finger Turns on the Effector T Cell Program without Requirement for Agonist TCR Signaling. *The Journal of Immunology*, *186*(10).

Savage, A. K., Constantinides, M. G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O., & Bendelac, A. (2008). The Transcription Factor PLZF Directs the Effector Program of the NKT Cell Lineage. *Immunity*, *29*(3).

Schmidt-Supprian, M., Tian, J., Grant, E. P., Pasparakis, M., Maehr, R., Ovaa, H., Ploegh, H. L., Coyle, A. J., & Rajewsky, K. (2004). Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-κB activation. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(13).

Schmieg, J., Yang, G., Franck, R. W., & Tsuji, M. (2003). Superior Protection against Malaria and Melanoma Metastases by a C-glycoside Analogue of the Natural Killer T Cell Ligand α -Galactosylceramide. *Journal of Experimental Medicine*, *198*(11).

Schwab, S. R., Pereira, J. P., Matloubian, M., Xu, Y., Huang, Y., & Cyster, J. G. (2005). Immunology: Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science*, *309*(5741).

Shen, H., Gu, C., Liang, T., Liu, H., Guo, F., & Liu, X. (2020). Unveiling the heterogeneity of NKT cells in the liver through single cell RNA sequencing. *Scientific Reports*, *10*(1).

Shiow, L. R., Rosen, D. B., Brdičková, N., Xu, Y., An, J., Lanier, L. L., Cyster, J. G., & Matloubian, M. (2006). CD69 acts downstream of interferon-α/β to inhibit S1P 1 and lymphocyte egress from lymphoid organs. *Nature*, *440*(7083).

Shissler, S. C., & Webb, T. J. (2019). The ins and outs of type I iNKT cell development. *Molecular Immunology*, *105*.

Slauenwhite, D., & Johnston, B. (2015). Regulation of NKT cell localization in homeostasis and infection. *Frontiers in Immunology*, *6*(MAY).

Smyth, M. J., Crowe, N. Y., Pellicci, D. G., Kyparissoudis, K., Kelly, J. M., Takeda, K., Yagita, H., & Godfrey, D. I. (2002). Sequential production of interferon-by NK1.1 T cells and natural killer cells is essential for the antimetastatic effect of-galactosylceramide.

Sojka, D. K., Plougastel-Douglas, B., Yang, L., Pak-Wittel, M. A., Artyomov, M. N., Ivanova, Y., Zhong, C., Chase, J. M., Rothman, P. B., Yu, J., Riley, J. K., Zhu, J., Tian, Z., & Yokoyama, W. M. (2014). Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *ELife*, *2014*(3).

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., & Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Developmental Biology*, *1*.

Stein, J. V., Ruef, N., & Wissmann, S. (2021). Organ-Specific Surveillance and Long-Term Residency Strategies Adapted by Tissue-Resident Memory CD8+ T Cells. *Frontiers in Immunology*, *12*.

Su, I. H., Dobenecker, M. W., Dickinson, E., Oser, M., Basavaraj, A., Marqueron, R., Viale, A., Reinberg, D., Wülfing, C., & Tarakhovsky, A. (2005). Polycomb group protein Ezh2 controls actin polymerization and cell signaling. *Cell*, *121*(3).

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., & Mesirov, J. P. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43).

Sunshine, A., Goich, D., Stith, A., Sortino, K., Dalton, J., Metcalfe, S., Svensson, E. C., & Garrett-Sinha, L. A. (2019). Ets1 Controls the Development of B Cell Autoimmune Responses in a Cell-Intrinsic Manner. *ImmunoHorizons*, *3*(7).

Syn, W. K., Agboola, K. M., Swiderska, M., Michelotti, G. A., Liaskou, E., Pang, H., Xie, G., Philips, G., Chan, I. S., Karaca, G. F., Pereira, T. D. A., Chen, Y., Mi, Z., Kuo, P. C., Choi, S. S., Guy, C. D., Abdelmalek, M. F., & Diehl, A. M. (2012). NKT-associated hedgehog and osteopontin drive fibrogenesis in non-alcoholic fatty liver disease. *Gut*, *61*(9).

Syn, W. K., Oo, Y. H., Pereira, T. A., Karaca, G. F., Jung, Y., Omenetti, A., Witek, R. P., Choi, S. S., Guy, C. D., Fearing, C. M., Teaberry, V., Pereira, F. E. L., Adams, D. H., & Diehl, A. M. (2010). Accumulation of natural killer T cells in progressive nonalcoholic fatty liver disease. *Hepatology*, *51*(6).

Tai, T. S., Tsao, H. W., Chuang, W. C., Liu, C. C., Huang, Y. W., Oettgen, P., Chuang, Y. T., & Ho, I. C. (2020). Ets1 regulates the differentiation and function of iNKT cells through both Pointed domain-dependent and domain-independent mechanisms. *Cellular and Molecular Immunology*, *17*(11).

Takanami, I., Takeuchi, K., & Karuke, M. (2001). Expression of Ets-1 is correlated with urokinase-type plasminogen activator and poor prognosis in pulmonary adenocarcinoma. *Tumor Biology*, *22*(4).

Takeda, K., Hayakawa, Y., Van Kaer, L., Matsuda, H., Yagita, H., & Okumura, K. (2000). Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(10).

Thomas, S. Y., Scanlon, S. T., Griewank, K. G., Constantinides, M. G., Savage, A. K., Barr, K. A., Meng, F., Luster, A. D., & Bendelac, A. (2011). PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *Journal of Experimental Medicine*, *208*(6).

Townsend, M. J., Weinmann, A. S., Matsuda, J. L., Salomon, R., Farnham, P. J., Biron, C. A., Gapin, L., & Glimcher, L. H. (2004). T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. *Immunity*, *20*(4).

Trapnell, C., Pachter, L., & Salzberg, S. L. (2009). TopHat: Discovering splice junctions with RNA-Seq. *Bioinformatics*, *25*(9).

Tupin, E., Benhnia, M. R. E. I., Kinjo, Y., Patsey, R., Lena, C. J., Haller, M. C., Caimano, M. J., Imamura, M., Wong, C. H., Crotty, S., Radolf, J. D., Sellati, T. J., & Kronenberg, M. (2008). NKT cells prevent chronic joint inflammation after infection with Borrelia burgdorferi. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(50).

Tyznik, A. J., Verma, S., Wang, Q., Kronenberg, M., & Benedict, C. A. (2014). Distinct Requirements for Activation of NKT and NK Cells during Viral Infection. *The Journal of Immunology*, *192*(8).

Ueno, T., Saito, F., Gray, D. H. D., Kuse, S., Hieshima, K., Nakano, H., Kakiuchi, T., Lipp, M., Boyd, R. L., & Takahama, Y. (2004). CCR7 signals are essential for

cortex-medulla migration of developing thymocytes. *Journal of Experimental Medicine*, *200*(4).

Umeshappa, C. S., Solé, P., Yamanouchi, J., Mohapatra, S., Surewaard, B. G. J., Garnica, J., Singha, S., Mondal, D., Cortés-Vicente, E., D'Mello, C., Mason, A., Kubes, P., Serra, P., Yang, Y., & Santamaria, P. (2022). Re-programming mouse liver-resident invariant natural killer T cells for suppressing hepatic and diabetogenic autoimmunity. *Nature Communications*, *13*(1).

Van Gisbergen, K. P. J. M., Kragten, N. A. M., Hertoghs, K. M. L., Wensveen, F. M., Jonjic, S., Hamann, J., Nolte, M. A., & Van Lier, R. A. W. (2012). Mouse Hobit is a homolog of the transcriptional repressor Blimp-1 that regulates NKT cell effector differentiation. *Nature Immunology*, *13*(9).

Vasanthakumar, A., Xu, D., Lun, A. T., Kueh, A. J., Gisbergen, K. P., Iannarella, N., Li, X., Yu, L., Wang, D., Williams, B. R., Lee, S. C., Majewski, I. J., Godfrey, D. I., Smyth, G. K., Alexander, W. S., Herold, M. J., Kallies, A., Nutt, S. L., & Allan, R. S. (2017). A non-canonical function of Ezh2 preserves immune homeostasis. *EMBO Reports*, *18*(4).

Verykokakis, M., Boos, M. D., Bendelac, A., & Kee, B. L. (2010). SAP Protein-Dependent Natural Killer T-like Cells Regulate the Development of CD8+ T Cells with Innate Lymphocyte Characteristics. *Immunity*, *33*(2).

Verykokakis, M., Krishnamoorthy, V., Iavarone, A., Lasorella, A., Sigvardsson, M., & Kee, B. L. (2013). Essential Functions for ID Proteins at Multiple Checkpoints in Invariant NKT Cell Development. *The Journal of Immunology*, *191*(12).

Verykokakis, M., Zook, E. C., & Kee, B. L. (2014). ID'ing innate and innate-like lymphoid cells. *Immunological Reviews*, *261*(1).

Viel, S., Marçais, A., Guimaraes, F. S. F., Loftus, R., Rabilloud, J., Grau, M., Degouve, S., Djebali, S., Sanlaville, A., Charrier, E., Bienvenu, J., Marie, J. C., Caux, C., Marvel, J., Town, L., Huntington, N. D., Bartholin, L., Finlay, D., Smyth, M. J., & Walzer, T. (2016). TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Science Signaling*, *9*(415).

Vogt, S., & Mattner, J. (2021). NKT Cells Contribute to the Control of Microbial Infections. *Frontiers in Cellular and Infection Microbiology*, *11*.

Volpe, E., Sambucci, M., Battistini, L., & Borsellino, G. (2016). Fas-fas ligand: Checkpoint of t cell functions in multiple sclerosis. *Frontiers in Immunology*, *7*(SEP).

Vong, Q. P., Leung, W. H., Houston, J., Li, Y., Rooney, B., Holladay, M., Oostendorp, R. A. J., & Leung, W. (2014). TOX2 regulates human natural killer cell development by controlling T-BET expression. *Blood*, *124*(26).

Walsh, D. A., Borges da Silva, H., Beura, L. K., Peng, C., Hamilton, S. E., Masopust, D., & Jameson, S. C. (2019). The Functional Requirement for CD69 in Establishment of Resident Memory CD8 + T Cells Varies with Tissue Location. *The Journal of Immunology*, *203*(4), 946–955.

Walunas, T. L., Wang, B., Wang, C.-R., & Leiden, J. M. (2000). Cutting Edge: The Ets1 Transcription Factor Is Required for the Development of NK T Cells in Mice. *The Journal of Immunology*, *164*(6).

Walzer, T., Chiossone, L., Chaix, J., Calver, A., Carozzo, C., Garrigue-Antar, L., Jacques, Y., Baratin, M., Tomasello, E., & Vivier, E. (2007). Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nature Immunology*, *8*(12).

Wang, H., D'Souza, C., Lim, X. Y., Kostenko, L., Pediongco, T. J., Eckle, S. B. G., Meehan, B. S., Shi, M., Wang, N., Li, S., Liu, L., Mak, J. Y. W., Fairlie, D. P., Iwakura, Y., Gunnersen, J. M., Stent, A. W., Godfrey, D. I., Rossjohn, J., Westall, G. P., … Chen, Z. (2018). MAIT cells protect against pulmonary Legionella longbeachae infection. *Nature Communications*, *9*(1).

Wang, N., Satoskar, A., Faubion, W., Howie, D., Okamoto, S., Feske, S., Gullo, C., Clarke, K., Sosa, M. R., Sharpe, A. H., & Terhorst, C. (2004). The Cell Surface Receptor SLAM Controls T Cell and Macrophage Functions. *Journal of Experimental Medicine*, *199*(9).

Wang, Y., Yun, C., Gao, B., Xu, Y., Zhang, Y., Wang, Y., Kong, Q., Zhao, F., Wang, C. R., Dent, S. Y. R., Wang, J., Xu, X., Li, H. B., & Fang, D. (2017). The Lysine Acetyltransferase GCN5 Is Required for iNKT Cell Development through EGR2 Acetylation. *Cell Reports*, *20*(3).

Wasylyk, C., Bradford, A. P., Gutierrez-Hartmann, A., & Wasylyk, B. (1997). Conserved mechanisms of Ras regulation of evolutionary related transcription factors, Ets1 and pointed P2. *Oncogene*, *14*(8).

Weinreich, M. A., & Hogquist, K. A. (2008). Thymic Emigration: When and How T Cells Leave Home. *The Journal of Immunology*, *181*(4).

Weinreich, M. A., Odumade, O. A., Jameson, S. C., & Hogquist, K. A. (2010). T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. *Nature Immunology*, *11*(8).

White, A. J., Jenkinson, W. E., Cowan, J. E., Parnell, S. M., Bacon, A., Jones, N. D., Jenkinson, E. J., & Anderson, G. (2014). An Essential Role for Medullary Thymic Epithelial Cells during the Intrathymic Development of Invariant NKT Cells. *The Journal of Immunology*, *192*(6).

White, A. J., Lucas, B., Jenkinson, W. E., & Anderson, G. (2018). Invariant NKT Cells and Control of the Thymus Medulla. *The Journal of Immunology*, *200*(10).

Xu, X., Huang, W., Heczey, A., Liu, D., Guo, L., Wood, M., Jin, J., Courtney, A. N., Liu, B., Di Pierro, E. J., Hicks, J., Barragan, G. A., Ngai, H., Chen, Y., Savoldo, B., Dotti, G., & Metelitsa, L. S. (2019). NKT cells coexpressing a GD2-specific chimeric antigen receptor and IL15 show enhanced in vivo persistence and antitumor activity against neuroblastoma. *Clinical Cancer Research*, *25*(23).

Yagi, H., Kamba, R., Chiba, K., Soga, H., Yaguchi, K., Nakamura, M., & Itoh, T. (2000). Immunosuppressant FTY720 inhibits thymocyte emigration. *European Journal of Immunology*, *30*(5).

Yalim-Camci, I., Balcik-Ercin, P., Cetin, M., Odabas, G., Tokay, N., Sayan, A. E., & Yagci, T. (2019). ETS1 is coexpressed with ZEB2 and mediates ZEB2-induced epithelial-mesenchymal transition in human tumors. *Molecular Carcinogenesis*, *58*(6).

Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., & Ostrowski, M. C. (1996). Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Molecular and Cellular Biology*, *16*(2).

Yang, J., Gao, L., Liu, Y., Ren, Y., Xie, R., Fan, H., & Qian, K. (2010). Adoptive therapy by transfusing expanded donor murine natural killer T cells can suppress acute graft-versus-host disease in allogeneic bone marrow transplantation. *Transfusion*, *50*(2).

Yu, J. S., Hamada, M., Ohtsuka, S., Yoh, K., Takahashi, S., & Miaw, S. C. (2017). Differentiation of IL-17-producing invariant natural killer T cells requires expression of the transcription factor c-Maf. *Frontiers in Immunology*, *8*(OCT).

Zamisch, M., Tian, L., Grenningloh, R., Xiong, Y., Wildt, K. F., Ehlers, M., Ho, I. C., & Bosselut, R. (2009). The transcription factor Ets1 is important for CD4 repression and Runx3 up-regulation during CD8 T cell differentiation in the thymus. *Journal of Experimental Medicine*, *206*(12).

Zhang, N., & Bevan, M. J. (2013). Transforming growth factor-β signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. *Immunity*, *39*(4).

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., & Samelson, L. E. (1998). LAT: The ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*, *92*(1).

Zhang, Y., Liu, T., Meyer, C. A., Eeckhoute, J., Johnson, D. S., Bernstein, B. E., Nussbaum, C., Myers, R. M., Brown, M., Li, W., & Shirley, X. S. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biology*, *9*(9).

Zhong, Y., Walker, S. K., Pritykin, Y., Leslie, C. S., Rudensky, A. Y., & van der Veeken, J. (2022). Hierarchical regulation of the resting and activated T cell epigenome by major transcription factor families. *Nature Immunology*, *23*(1).

Zhou, L., Adrianto, I., Wang, J., Wu, X., Datta, I., & Mi, Q. S. (2020). Single-Cell RNA-Seq Analysis Uncovers Distinct Functional Human NKT Cell Sub-Populations in Peripheral Blood. *Frontiers in Cell and Developmental Biology*, *8*.

Zook, E. C., Li, Z. Y., Xu, Y., de Pooter, R. F., Verykokakis, M., Beaulieu, A., Lasorella, A., Maienschein-Cline, M., Sun, J. C., Sigvardsson, M., & Kee, B. L. (2018). Transcription factor ID2 prevents E proteins from enforcing a naïve T lymphocyte gene program during NK cell development. *Science Immunology*, *3*(22).

Zook, E. C., Ramirez, K., Guo, X., van der Voort, G., Sigvardsson, M., Svensson, E. C., Fu, Y. X., & Kee, B. L. (2016). The ETS1 transcription factor is required for the development and cytokine-induced expansion of ILC2. *Journal of Experimental Medicine*, *213*(5).