Transcriptional and translational regulators: Roles in development and function of innate-like and adaptive regulatory T cells

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Transcriptional and translational regulators: Roles in development and function of innate-like and adaptive regulatory T cells

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Invariant natural killer T (iNKT) cells and regulatory T cells (Tregs) both have roles in maintaining immune homeostasis. However, following disease, these cells often undergo loss of function and reduction in cell frequency. Invariant natural killer T (iNKT) cells are innate-like T lymphocytes capable of rapidly producing cytokines post-stimulation with the potent iNKT cell activator α-Galactosylceramide (α-GalCer). We investigated the function of microRNAs (miRNAs) in iNKT cells, which are translational regulators that can quickly modulate regulation of protein synthesis. While miRNAs have been described in the development of iNKT cell development, no miRNAs had been ascribed to their effector function. We investigated whether microRNA-155 (miR-155) played a role in regulating the effector function of iNKT cells, as it was already shown to do so in CD8+ T cells and NK cells. Although we verified that miR-155 was upregulated in iNKT cells post α-GalCer stimulation, we found that miR-155 deficiency did not have an impact on iNKT cell cytokine production. Considering iNKT cells regulatory functions in maintaining immune homeostasis, miR-155 could be a potential therapeutic target to disrupt effector function in other immune cells while maintaining the effector function in iNKT cells.

We also investigated the role of the transcriptional regulator Id2 in adipose-resident Tregs (aTregs). Inhibitor of DNA binding (Id) proteins are transcriptional regulators that dimerize with E protein transcription factors to inhibit their binding to E box sites and thus, prevent their activity. We verified that Id2 was upregulated in aTregs compared to higher Id3 expression in splenic Tregs. Under standard diet, loss of Id2 in aTregs led to a phenotype seen in high-fat-diet fed mice including a decrease in frequency and canonical aTreg markers including ST2, CCR2, KLRG1, and GATA3. Additionally, Id2 deficiency in Tregs led to increased systemic inflammation and impaired glucose tolerance. In essence Id2 expression was necessary for the aTreg transcriptional program including regulation of aTreg function to mediate tissue homeostasis. In summary, this thesis helps illuminate the unique influence of transcriptional and translational regulation on aTreg and iNKT cells which is critical for immune homeostasis.

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ABBREVIATIONS

α-GalCer: α-Galactosylceramide

ANOVA: analysis of variance

aTreg: adipose-resident regulatory T cell

BMC: bone marrow chimera

BMI: body mass index

CD: control diet

Ctrl: control

DN: double-negative

DP: double-positive

ELISA: enzyme-linked immunosorbent assay

GFP: green fluorescence protein

GTT: glucose tolerance test

HFD: high fat diet

Hif1α: hypoxia inducible factor 1α

HLH: helix-loop-helix

ID: inhibitor of DNA binding

Id2 CKO: Id2 conditional Knockout

IL: Interleukin

ILC2: type 2 innate-lymphoid cell

IR: insulin resistance

ITT: insulin tolerance test

ION: ionomycin

KO: knock-out

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

miR-155: microRNA-155

miRNA: microRNA

iNKT cell: invariant natural killer T cell

NK: natural killer

NOD: non-obese diabetic

PDGFRα: platelet-derived growth factor receptor alpha

PDPN: podoplanin

PPARγ: peroxisome proliferator activated receptor gamma

PLZF: Promyelocytic Leukemia Zinc Finger

PMA: phorbol 12-myristate 13-acetate

RNA-seq: RNA-sequencing

SAT: subcutaneous adipose tissue

ST2: suppression of tumorigenicity 2

SVF: Stromal vascular fraction

T2DM: type II diabetes mellitus

TCR: T Cell Receptor

TPM: transcripts per million kilobase

Treg: regulatory T cell

UTR: untranslated region

VAT: visceral adipose tissue

YFP: yellow fluorescence protein

WT: wild-type

1.0 Tissue Homeostasis

1.1 Introduction

Many studies have detailed the importance of immune regulatory cell populations, responsible for resolution of inflammation, counteracting autoimmunity, and tissue homeostasis where loss of these cell populations, including invariant natural killer T cells (iNKT cells), alternatively activated (M2 macrophages, and, most notably, regulatory T cells (Tregs), can lead to numerous disease states, some of which can be lethal. Regulatory immune cell populations can regulate other cells either directly, through inhibitory receptors, or indirectly, through the release of cytokines or other molecules. However, chronic adverse changes in tissues or even genetic abnormalities can impede the proliferation, survival, or even genesis of regulatory immune cells and lead to the recruitment and expansion of pro-inflammatory cell populations. Loss of these regulatory immune cells leads to complications dependent on factors such as the cell type lost and the tissue affected. These issues can include susceptibility to infection, development of autoimmunity, or loss of metabolic homeostasis.

Here, I will discuss the development, expression profile, and function of two cell populations important for tissue homeostasis, invariant natural killer T (iNKT) cells and adipose-resident Tregs (aTregs). Although both iNKT cells and Tregs are found in various anatomical locations, I will focus on the visceral adipose tissue (VAT) where both populations can be found at elevated frequencies in lean VAT compared to secondary lymphoid organs.

1.2 Invariant Natural Killer T cells

Invariant natural killer T (iNKT) cells are innate-like lymphocytes capable of both pro- and anti-inflammatory roles in autoimmunity, cancer, and infection[1-3]. The population of iNKT cells were first identified in 1995 by their unique expression of NK1.1 on Vα14 restricted T cells in mice and Vα24 in humans, but not on other lymphocytes [4]. The following year, a subsequent study revealed α-Galactosylceramide (α-GalCer) as a potent ligand for the NKT cell semi-invariant TCR through its presentation on the MHCI-like molecule, CD1d [5].

Low iNKT cell numbers are linked to autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus (SLE), and Sjogren's syndrome. In a study by Kojo et al., activation-induced proliferation of iNKT cells using α-GalCer ameliorated disease symptoms in half of all autoimmune patients[6]. In another study by Kawakami et al., it was shown that *Streptococcus pneumoniae* infection was lethal in iNKT cell deficient mice, thus highlighting their crucial involvement in fighting infection[3].

Elucidating the mechanisms behind the vast potential of iNKT cells in numerous diseases remains a challenge due to a number of issues including their low frequency (around 0.5% at steady-state in mouse PBL) and variable but low frequencies in humans[7]. Adding more questions than answers, further studies show that the already rare population of iNKT cells can be further divided into smaller populations defined by unique transcriptional profiles that mimic helper T cell subsets (NKT1, NKT2, and NKT17), as well as follicular helper T cells (NKTfh) and regulatory T cells (NKT10)[8-9].

Here, I will start with a synopsis on the development of iNKT cells, covering how they are selected on their semi-invariant T cell receptor (TCR), an older paradigm of iNKT

cell maturation, the unique gene expression profiles of iNKT cell subsets, and briefly, mention some less well understood types of iNKT cells. In later sections, I will discuss what is known about microRNAs in iNKT cells, activation of iNKT cells by α-Galactosylceramide and other ligands, and hyporesponsiveness of iNKT cells. Knowledge of the regulatory network in order to manipulate the pro- and anti-inflammatory functions of iNKT cells and utilization of iNKT cell subsets may help improve their therapeutic potential.

1.2.1 Development and Diversity of iNKT Cells

INKT cells are T lymphocytes and, like other T cells, develop in the thymus. With some exceptions, iNKT cells develop through the same CD4 and CD8 double negative (DN) stages of T cell development and eventually reach a CD4+ CD8+ double positive (DP) stage, like conventional T cells (Figure 1A). It is at the DP stage that iNKT cells are selected on their semi-invariant TCR,Vα14-Jα18 in mice, which can be stained using a tetramer of CD1d, the iNKT cell cognate ligand (Figure 2), loaded with a synthetic version of α-GalCer (Figure 1B). From the double positive stage in T cell development, NKT cells proceed to downregulate CD24 ($CD44^{-}$ $NK1.1^{-}$; stage 1) and acquire the memory marker CD44 ($CD44^{+}$ $NK1.1^{-}$; Stage 2). They next acquire the NK surface marker NK1.1 ($CD44^{+}$ $NK1.1^{+}$; Stage 3) [10]. The study of iNKT cells has transitioned from surface markers to characterizing the heterogeneous population based on their unique transcription profiles and cytokine production that mimic Th1, Th2, and Th17 cells [11], although the linear model of maturation (stages) remains a useful tool for understanding development (Figure 1C). In 2008, two independent studies by Bendelac and Sant'Angelo described the transcription factor Promyelocytic Leukemia Zinc Finger (PLZF) and found that it was

necessary for iNKT cell development, differentiation, and localization [12-13]. Interestingly, PLZF is still expressed in progenitor T cells in mice lacking CD1d, which are also deficient in iNKT cells, providing evidence that PLZF expression is induced before positive selection independent of TCR selection in Vα14-Jα18 positive cells[14].

Other studies found that the population of stage 2 iNKT cells comprises two distinct terminally differentiated populations, termed NKT2 (CD4+ $PLZF_{High}$) and NKT17 (CD4+ $PLZF_{Int}$ $ROR\gamma t^{+}$) cells, dependent on IL-17Rb expression, while stage 3 iNKT cells, termed NKT1 (CD4+ PLZF- TBET+), are dependent on IL-15Rb [15]. An important role for E proteins, basic helix-loop-helix transcriptions factors, has also been shown in regulating the differentiation of iNKT cells subsets, specifically in the promotion of NKT2 and NKT17 cells [16].

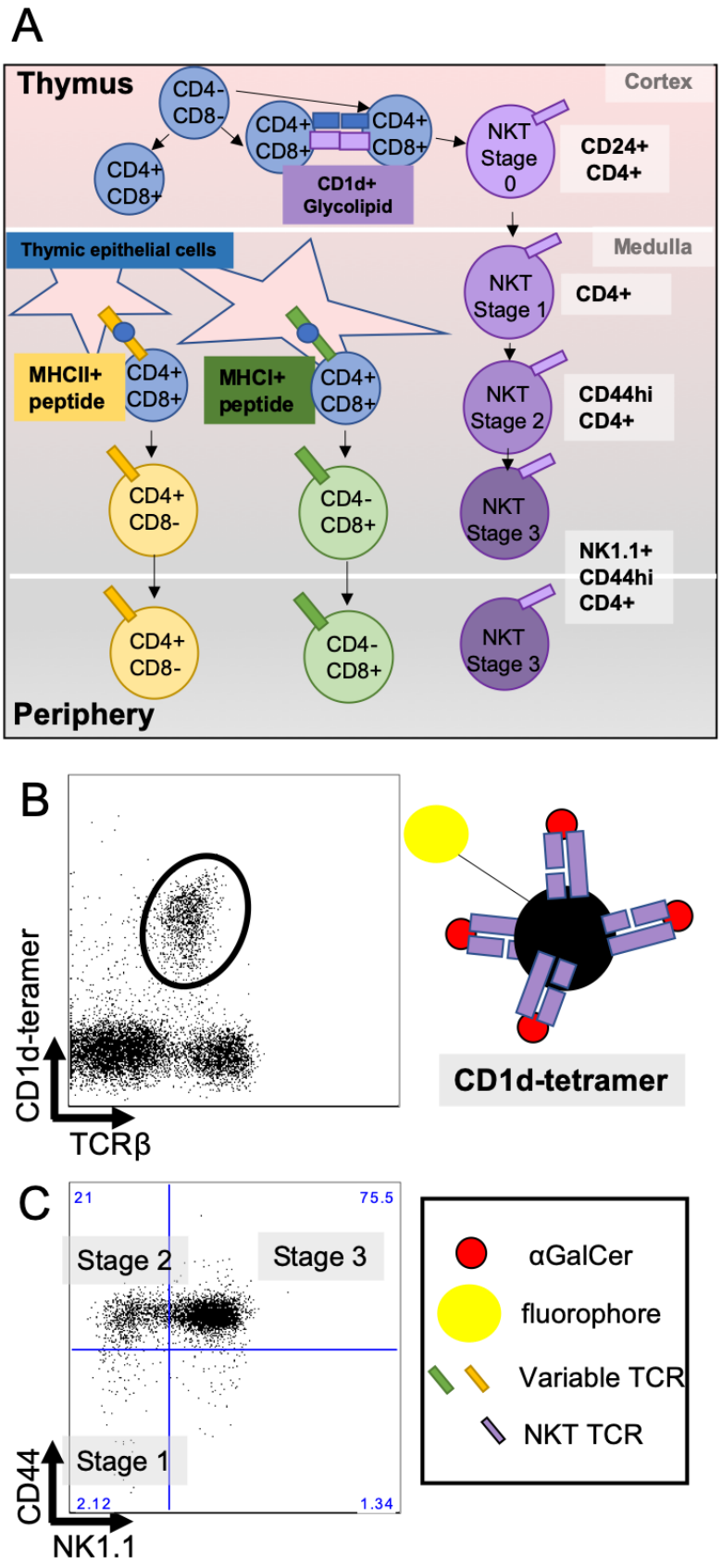
The above mentioned populations of iNKT cells have been verified in the thymus, while additional iNKT cell subsets, such as NKT_{FH} and NKT10 cells, likely mature in the periphery [11]. Follicular helper iNKT cells (NKT_{FH}) express a similar gene expression profile to T_{FH} cells, including expression of CXCR5 and PD-1, and the transcription factor, Bcl-6. One key difference is that NKT_{FH} do not help form long-lived plasma cells [17].

Following an initial rapid cytokine production and proliferation response, iNKT cells treated with α-GalCer were originally believed to undergo anergy[18], a state described as unresponsive to antigen and non-proliferative, but Sag et al. found that these pre-stimulated iNKT cells could be further stimulated to proliferate through TCR or TCR-independent methods [19]. These cells, termed NKT10 cells, produced increased IL-10 relative to naïve and were protective in models of cancer and autoimmunity compared to previously unstimulated iNKT cells. Further research revealed that NKT10 cells do not

express the iNKT cell master transcription factor PLZF, but are dependent on the transcription factor E4BP4, which is also necessary for iNKT cell production of IL-10 [20].

Outside conventional T cell development, there is one final iNKT cell population to mention discovered by Dashtsoodol et al. In their study, the authors used an E8III-Cre+ Rag2 floxed mice to block T cell development from reaching the CD4 and CD8 DP stage and found a population of iNKT cells that do not proceed through the DP stage of T cell development, thus being of DN origin [21]. These iNKT cells displayed characteristics of the cytotoxic cell lineage, including elevated expression of the transcription factor Runx3 and granzyme B compared to their counterparts of DP stage origin. They also preferentially localize to the liver, supporting the idea of specialized roles for different populations of iNKT cells.

Not surprisingly, NKT cell subsets are found at different frequencies in various mouse strains, such as NKT1 cell skewing in C57BL/6 mice, an increase in frequency and cell number of NKT2 cells in BALB/c mice, or even an NKT cell deficiency in non-obese diabetic (NOD) mice [22-23]. The diversity of the iNKT cell population extends into other tissues not mentioned above including spleen, lung, lymph nodes, and bone marrow[24]. There are additional populations of iNKT cells that are well less studied including CD8+ NKT cells that predominately express IFNγ[25], or the CD4+, CD8+, and DN human NKT cell populations[26].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Glycolipid
NKT
Stage
0
CD24+
CD4+
Thymic epithelial cells
Medulla
NKT
Stage 1
CD4+
MHCII+
peptide
CD4+
CD8+
MHCI+
peptide
CD4+
CD8+
NKT
Stage 2
CD44hi
CD4+
CD4+
CD8-
CD4-
CD8+
NKT
Stage 3
NK1.1+
CD44hi
CD4+
CD4+
CD8-
CD4-
CD8+
NKT
Stage 3
Periphery
B
CD1d-teramer
TCRβ
CD1d-tetramer
C
21
75.5
Stage 2
Stage 3
Stage 1
2.12
1.34
CD44
NK1.1
αGalCer
fluorophore
Variable TCR
NKT TCR

Figure 1. invariant Natural Killer T Cell development

(A) Invariant natural killer T (iNKT) cells are T lymphocytes with a semi-invariant Vα-14 TCR in mice (Vα-24 in humans). Unlike conventional CD4+ CD8+ T cells that are selected on thymic epithelial cells expressing either MHCI (selection of CD8+ T Cells [green]) or MHCII (selection of CD4+ T cells [gold]), iNKT cells are selected at the CD4+ CD8+ double positive stage on other CD4+ CD8+ double positive T cells (blue) expressing CD1d plus glycolipid. The positive selected cells then go on to stage 0 in development defined as expression of CD24 and CD4. iNKT cells proceed to stage 1 with the downregulation of the immature marker CD24. In stage 2, iNKT cells obtain one of their unique features – expression the memory marker CD44 usually found on memory T lymphocytes, thus giving them the memory-like phenotype. Lastly, iNKT cells gain expression of the NK marker, NK1.1. Stage 3 iNKT cells leave the thymus and populate the periphery. (B) iNKT cells can be studied by staining for TCRβ and and the iNKT cell TCR using a CD1d (purple) tetramer loaded with the synthetic analog of α-Galactosylceramide (α-GalCer), KRN7000, attached to streptavidin (black) conjugated to a fluorophore (yellow). (C) iNKT cell stages can be studied by staining as mentioned previous (TCRβ and CD1dtetramer+α-GalCer) and gating CD44 against NK1.1. Stage 1 comprises the double negative population. In Stage 2 iNKT cells obtain expression of the memory marker CD44. In stage 3, iNKT cells obtain expression of the NK cell marker, NK1.1.

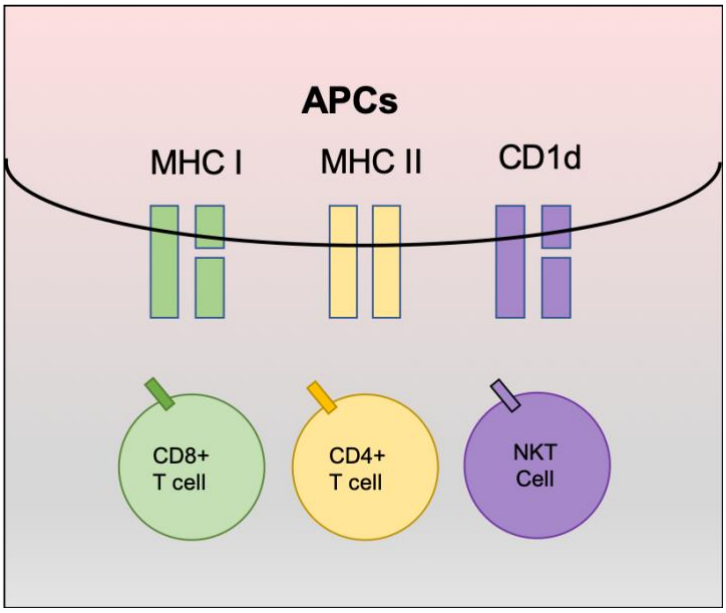

Figure 2. Selection of T cells on antigen presenting molecules

Antigen presentation molecules are expressed by antigen presenting cells (APCs), although they can also be expressed by other cell types. The common antigen-presenting molecules are major histocompatibility complex I (MHC I, green) and II (MHC II, gold). MHC I interacts with CD8+ T cells and MHC II interacts with CD4+ T cells. There is also the MHC I-like molecule CD1d (purple) that present glycolipids instead of protein antigens and interacts with the NKT cell TCR.

1.2.2 The function of microRNAs in iNKT cells

MiRNAs are ~22 bp non-coding RNAs transcribed by RNA polymerase II and capped and polyadenylated in a similar manner to gene transcripts. Immature primary miRNAs (pri-miRNAs) are processed in the nucleus by Drosha, an RNAse III protein. The ~70bp hairpin precursor miRNA (pre-miRNA) is then shuttled out of the nucleus. In the cytoplasm, pre-miRNAs are further processed by a different RNAse III molecule, Dicer. The resulting ~22bp double strand RNA product dissociates with one ‘dominant’ strand, known as the guide strand, coupling with the RNA-induced silencing complex, or RISC. The miRNA then guides RISC to conserved ‘seed’ regions in the 3’ untranslated regions

(UTR) of target mRNAs, which blocks translation and/or leads to degradation of mRNA [27].

MicroRNAs (miRNAs) were first discovered in the nematode *Caenorhabditis elegans* in 1993 [28]. Since then, miRNAs have been found to be conserved in mammals and to play crucial roles in all areas of cell biology. For example, in immune cells miRNAs are involved in proliferation of B cells, cytokine production in natural killer (NK) cells, and in the differentiation of Th2 cells [29-31]. Numerous studies have identified the importance of miRNAs for the proper maintenance and function of the immune system. Dysregulation of miRNAs can lead to lymphomas, autoimmunity, and other immunopathologies [32-33].

One of the earliest microRNA studies in iNKT cells showed that deletion of the microRNA processing machinery, Dicer/Drosha, precluded the development of iNKT cells in the thymus illustrating the importance of miRNAs in the genesis of this cell population [34]. Other miRNAs, such as miR-150 and miR-181a, have been found to play roles in iNKT cell development and metabolism, respectively [35-36].

In our own studies, we investigated the role of miR-155 in iNKT cells. MiR-155 was upregulated at 3-hours post-α-GalCer stimulation during iNKT cells' rapid cytokine production. However, in miR-155 deficient mice, there was no change in iNKT cell cytokine production at 3 hours, revealing that the rapid effector function of iNKT cells is independent of miR-155[37]. This raises the potential of using miR-155 as a therapeutic target in autoimmunity to target pro-inflammatory cell populations, such as CD8+ T cells and NK cells, or B cell lymphoma in the case of unrestrained proliferative B cells in miR-155 overexpression. Targeting miR-155 for inhibition would negatively impact the function of pro-inflammatory cells, while retaining the regulatory effector functions of iNKT cells.

1.2.3 iNKT cell activation and hyporesponsiveness

iNKT cells can be activated through various methods that can be TCR-dependent or TCR-independent. These include endogenous, microbial, and viral ligands, cytokines alone, and synthetic ligands [38-40]. The most studied and potent ligand is α-GalCer [41]. Classified as a Th0-ligand, α-GalCer induces iNKT cells to rapidly produce cytokines including both IL-4 and IFNγ within hours of activation [42]. An extensive study found that depending on the expression CD4 and NK1.1 (CD4+, NK1.1+, or CD4+ NK1.1+), and tissue location, α-GalCer-stimulated iNKT cells were capable of secreting IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IFNγ, GM-CSF, and TNF[43].

Although it was originally thought that after an initial activation, iNKT cells underwent anergy [18], Wingender et al. discovered that previously stimulated iNKT cells are in fact more proliferative than naïve iNKT cells when stimulated with α-GalCer and thus could not be considered anergic[44]. A subsequent study showed that it was only using Th0/Th1 iNKT cell ligands that could truly induce this long-term unresponsiveness [45]. To demonstrate this, hyporesponsiveness was achieved with the Th1 ligand C-Gly despite being a weaker ligand than the Th2 ligand OCH, an α-GalCer analogue with a truncated sphingosine base. Stimulation of iNKT cells with OCH leads to production of predominantly IL-4 instead of IFNγ, lower proliferation compared to α-GalCer, and does not lead to hyporesponsiveness[46].

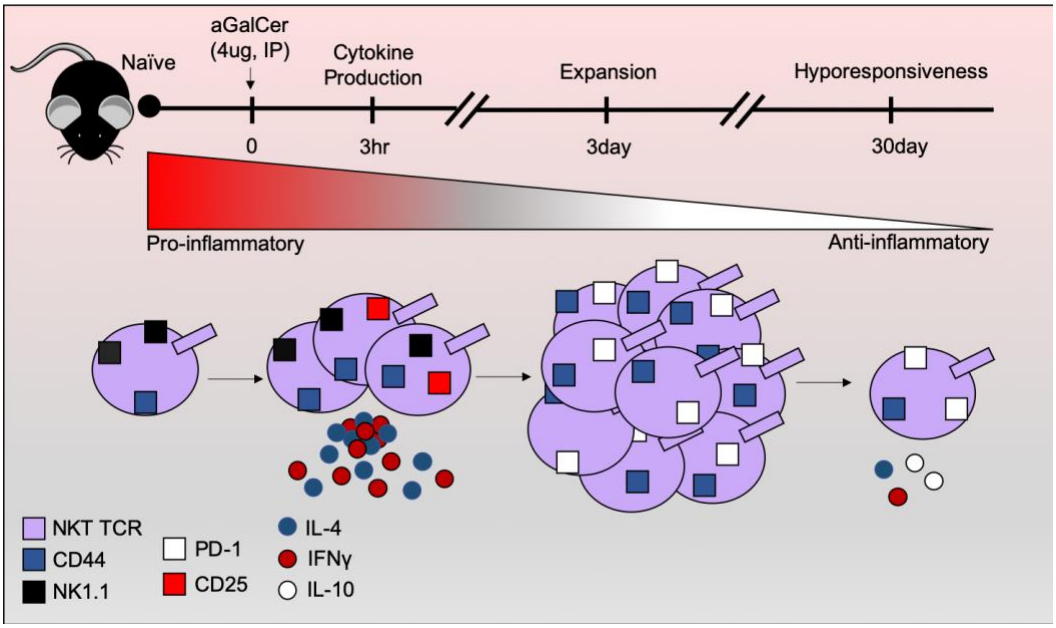

Figure 3. alpha-Galactosylceramide stimulation of iNKT cells

Naïve iNKT cells express the memory-marker CD44 and the NK cell marker NK1.1. Upon α-GalCer stimulation, iNKT cells rapidly produce cytokines including IFNγ and IL-4 and express the activation marker CD25 within 3-hours post stimulation (Cytokine Production). At 3-days post-α-GalCer stimulation, iNKT cells are at peak proliferation (Expansion), have downregulated NK1.1, and start to express the activation/exhaustion marker PD-1. Beyond 30 days post-α-GalCer stimulation iNKT cells contract, express higher levels of PD-1, and have reduced cytokine production, but also acquired production of IL-10, upon re-stimulation with α-GalCer (Hyporesponsiveness).

1.2.4 Conclusion

Despite comprising a small proportion of the immune cells in mice and humans, iNKT cells play important roles in recognizing both self and foreign antigen. Surprisingly, the already rare population of iNKT cells is very heterogenous, with subsets that are analogous to other immune cell types in function, cytokine production, and transcription factor requirements. This alone may explain the broad roles of iNKT cells in various diseases including autoimmunity, infection and cancer. iNKT cell subsets appear to

localize to specific tissues. In the liver and spleen, there is skewing towards NKT1 cells that express IFNγ, while NKT2 cells expressing IL-4 localize in the thymus [47]. Although NKT10 cells can be generated after stimulation, NKT10 cells, or at least an iNKT cell with similar properties, can be observed at higher frequency in the visceral adipose tissue (VAT) without the need for stimulation first.

From what is known about the VAT, the IL-10 production by NKT10 cells is thought to help maintain the homeostatic environment in healthy lean VAT. Other iNKT cell subsets may help maintain tissue homeostasis in other organs, although it has been observed that iNKT cell populations in the tissues remain heterogenous. If different iNKT cell subsets have unique roles in specific tissues, why is it that many tissues house a heterogenous population of iNKT cells? Do these iNKT cell subsets have individual roles, or could there be synergistic role for multiple iNKT cell subsets in tissue homeostasis? Could the ratio of one iNKT cell subset over another be used as a measurement of changes in homeostasis?

While studies have begun to examine the potential of individual iNKT cell subsets, and thus uncovering differences in cytokine production, studying their role *in vivo* may be difficult. However, my own research shows that it may be possible to skew iNKT cells preferentially towards one subset over others. C57B/6 mice already have an enrichment of NKT1 cells relative to other NKT cell subsets, so the further increase in this subset in our miR-155 deficient mice may not have constituted enough of a change to see a change in phenotype at steady-state. For example, miR-155 deficiency may have a noticeable impact on BALB/c mice which are naturally enriched with NKT2 and NKT17 cells [8].

The expansion of iNKT cells has been correlated with disease improvement. Considering that miR-155 is necessary for effector function of CD8+ T cells and NK cells, as well as upregulated in a number of diseases including cancer, targeting miR-155 may be a potential therapy to reduce inflammatory cell populations while retaining the regulatory function iNKT cells.

1.3 Regulatory T cells and Visceral Adipose Tissue

Regulatory T cells (Tregs) are a population of CD4+ T cells critical for peripheral tolerance and tissue homeostasis. This is evident from genetic defects in the Treg transcription factor, Foxp3, in mice and humans, where uncontrolled activation of conventional T cells leads to uncontrolled proliferation of auto-reactive T cells resulting in autoimmunity. With the gene for Foxp3 located on the X chromosome, males with a defect in their single copy of Foxp3 are susceptible to problems while heterozygous females are normal. Foxp3 has been shown to be important for the gene expression profile of Tregs which includes expression of the IL2 receptor, CD25, and suppressive receptors, such as CTLA4 [48]. Additional studies show that expression of Foxp3 by retroviral vector in conventional T cells resulted in a Treg-like phenotype with suppressive functions [49].

To maintain homeostasis, Tregs are equipped with multiple regulatory mechanisms including secretion of anti-inflammatory cytokines and expression of inhibitor receptors. Unfortunately, Tregs ability to tone down inflammatory responses also make them detrimental in the context of cancer, but this does not preclude the necessity of this cell population considering the lethality of Treg deficiency [50].

Considering the role of Tregs in maintaining tissue homeostasis, it is not surprising that they were found in the visceral VAT, a site important in the maintenance of metabolic homeostasis. Surprisingly, Tregs can make up half or more of the CD4+ T cell population in the VAT. In the last few decades, the VAT has been established to be an immune-rich site involved in metabolic homeostasis, in addition to its roles in storage and thermal regulation of the body. Lean VAT maintains an anti-inflammatory environment, but can switches to a chronic low-grade inflammatory environment following obesity[51]. While early studies in the VAT focused on inflammatory M1 macrophages during obesity, in the last decade studies have focused on other cell populations in the lean VAT, including adipose-resident Tregs (aTregs). Here, I will discuss the immune cells and signals in the lean and obese VAT environment, the establishment of aTregs in the VAT, and the factors that aid or inhibit aTreg maintenance and survival. I will begin with an introduction on the issues on obesity and its association with VAT.

1.3.1 Obesity and Visceral Adipose Tissue

Obesity is an increasing problem that plagues more than 2 billion people worldwide and can be attributed to recent changes in human history including increased caloric intake and sedentary lifestyle. Obesity is associated with a number of health issues including most commonly type II diabetes mellitus (T2DM) and cardiovascular disease. In the United States alone, the obesity rates among adults was 39.8% between 2015-2016, a rate that has continued to grow and more than doubled in the last 40 years [52]. Problems related to obesity have long been correlated with increases in VAT, which surrounds the internal organs compared to subcutaneous adipose tissue (SAT) that lies underneath the skin[53]. While it can be difficult to assess VAT without the use of imaging

techniques, a combination of body mass index (BMI) and waist measurement can be used to assess the disease risk associated with VAT, although criteria for disease determination is dependent on several factors including sex, race, and even geographic location [54].

1.3.2 VAT Immune Cell Environment

Studies have shown intricate patterns of cross-talk between immune cells, adipocytes, and other cells found in the stromal vascular fraction (SVF) for maintaining AT homeostasis. Loss of this homeostasis during development of obesity leads to systemic problems, attributed in-part to sterile, chronic low-level inflammation [55]. Lean AT is populated by an array of anti-inflammatory cells that are critical in controlling inflammation. In severely obese individuals, the VAT becomes heavily populated by pro-inflammatory immune cell populations. Thus, it has become more important to understand the factors that contribute to the maintenance and survival of anti-inflammatory cell populations in lean VAT, such as adipose-resident Tregs.

The lean VAT is populated by several types of both innate and adaptive immune cells, including CD4+ T helper (Th2) cells, iNKT cells, type 2 innate lymphoid cells (ILC2s), eosinophils, PLZF+ γδ T cells, alternatively activated (M2) macrophages, and regulatory T cells (Tregs). Although iNKT cells are found at low frequencies in most locations, they are found at significantly higher frequencies in human and mouse VAT[56]. Similarly, although Tregs make up a small percentage of the CD4+ population in the secondary lymphoid organs such as the spleen and LNs, Tregs in the VAT can make up half or more of the CD4+ population[57]. The presence of these two cell populations

involved in self-tolerance lends support to the idea that the VAT is involved in metabolic homeostasis.

The lean VAT environment is maintained in part by the above-mentioned cell populations and their signals. ILC2s and Th2 cells are responsible for producing type 2 cytokine signals in the VAT, including IL-4, IL-5 and IL-13. In particular, IL-5 produced by ILC2s is necessary to recruit eosinophils [58]. Studies using IL4 reporter mice showed that eosinophils are responsible for the majority of IL-4 in the VAT, which is necessary to maintain the M2 macrophage population [59]. In iNKT cell deficiency studies, adoptive transfer of iNKT cells alone increased the levels of the insulin-sensitizing cytokine, adiponectin, decreased adipocyte size, and increased VAT IL-10. The increase in adiponectin in iNKT cell transfer experiments suggests iNKT-adipocyte interactions as adipocytes are the producers of adiponectin in lean VAT. Adiponectin is an important cytokine that induces the expression of IL-10RA and production of IL-10 in immune cells and also supports proliferation of aTregs [60-61]. iNKT cells could also be stimulated with α-GalCer to induce production of IL-2, IL-4 and IL-10, although the identity and effects of a natural occurring VAT ligand on iNKT cells remains to be elucidated [56-62].

More recently, IL-17 producing PLZF+ γδ T cells were found to be important for inducing PDPN+ PDGFRα+ VAT stromal cells to produce IL-33, an alarmin important in maintaining insulin sensitivity[63]. Another study further probed the PDPN+ PDGFRα+ stromal cells and identified three subsets from CD45- CD31- PDPN+ PDGFRα+ Sca-1+ sorted stromal cells, termed VAT mesenchymal stromal cells 1-3, as the major producers of IL-33 [64]. M2 macrophages and aTregs are tightly maintained by the lean VAT environment, where both are critical sources of the anti-inflammatory cytokine IL-10 [65].

M2 macrophages have additional protective functions that differentiate them from their M1 counterparts such as lower expression of pro-inflammatory cytokines, and expression of anti-inflammatory and tissue repair cytokines including arginase 1, protectin, and TGFβ. aTregs will be covered in more detail later on in their own section.

Habitual increased caloric intake can lead to loss of homeostasis in VAT. Diet-induced obesity (DIO) is associated with increased adipocyte necrosis, which drives macrophage secretion of chemokines, including CCL2, which lead to the recruitment of CD11c+ M1 macrophages. M1 macrophages produce pro-inflammatory cytokines TNFα, IL-6, and IL-1β, as well as iNOS2 [66-67]. Adaptive CD8+ and CD4+ Th1 T cells are also recruited to obese VAT suggesting the presence of a VAT-specific antigen involved [68]. In addition, during caloric intake, the satiety hormone leptin is released by adipocytes and is further elevated in obesity, which is thought to be due to a buildup of leptin resistance [69]. In contrast to adiponectin, leptin is known to inhibit the proliferation of aTregs and further promote type 1 immunity [70-71]. Ongoing low-level chronic inflammation eventually leads to a severe reduction in the anti-inflammatory cell populations, including iNKT cells, Tregs, and ILCs and skews the M2/M1 macrophage ratio in favor of M1 macrophages.

While the list of cells and signals described here is not comprehensive, it details a tight working environment. The lean VAT environment relies on constant signaling to maintain or expand anti-inflammatory populations. Before I move on to the establishment of Tregs in the VAT, I will discuss Id2 and its class of transcriptional regulators that are highly expressed in aTregs, but not lymphoid Tregs.

1.3.3 Inhibitor of DNA binding protein expression in T lymphocytes

Inhibitor of DNA binding (Id) proteins are a class of helix-loop-helix (HLH) proteins involved in various cellular processes including cell development and differentiation and whose dysregulation is linked to tumorigenesis. Id proteins regulate transcription by heterodimerizing with other classes of HLH proteins, such as the E proteins, and blocking their partner's binding to DNA, as Id protein themselves lack a DNA binding domain. E proteins contain a basic binding domain that allows them to bind to E box sites on DNA with the canonical sequence CANNTG. E protein binding can have both a positive and negative influence on gene transcription [72].

Over the years the study of E and Id proteins have extended into immunology with many studies focused on T lymphocytes and, even more specifically, Tregs. Out of the four known Id proteins (Id1-4) in mammals, many of the studies on T cells have focused on the first three Id proteins, and, particularly, on the dissimilar roles of Id2 and Id3. In T cells, overexpression of Id2 under the Lck promoter interrupted T cell development as evident by the increase of CD4- CD8+ TCR- thymocytes [73]. Although found to play minor compensatory roles, studies have demonstrated Id2 and Id3 expression to define separate branches or differentiated T cell populations. In the case of CD8+ T cells, higher Id2 expression led to increased effector KLRG1+ CD8 T cells and higher Id3 expression is required for long lived memory CD8 T cells (Yang, 2011). In iNKT cells, Id2 and Id3 determine the NKT1 and NKT2 cell subsets, respectively [74].

In Tregs, similar and unique roles for E and Id proteins have been found. In studies with E2A and HEB deficient Tregs, these cells exhibited increased frequency and cell number, enhanced differentiation, and improved suppressive function [75-76]. A study by

Miyazaki et al. found that Id2/Id3 double knockout mice had a reduction in LN and splenic Tregs and interfered with their localization, although they saw no major phenotype in the Id2 or Id3 single knockout Tregs [77]. However, microarray data from the first study describing aTregs found that Id2 was highly expressed in this adipose-resident tissue population of Tregs [57].

Above, I discussed the importance of E and Id proteins in relation to Tregs. From the studies mentioned, it can be conceived that Id proteins can be turned on and off as necessary by a T cell as it undergoes development or differentiation. In the following section, I will cover in more depth the establishment of Tregs in the VAT.

1.3.4 Establishment of Tregs in the VAT

In mice, the population of aTregs is seeded from the thymus early in life. Mice at 5 weeks of age have an aTreg population, albeit smaller than that found in lymphoid tissues. Around 10 weeks, this population of aTregs is further expanded and aTregs continue to expand, reaching a peak frequency around 25-30 weeks, followed by an age-dependent dramatic decrease around 40 weeks attributed to phosphorylation of a serine residue of PPARγ . At their peak, aTregs can make up half or more of the VAT CD4+ population [57-78-79] (Figure 4).

TCR sequencing of aTregs found that there were multiple clonal populations. From these clonal populations, Vα2 and Vβ4 was chosen for the purposes of making a TCR transgenic (Tg) mouse, because it was highly represented at 10% of all TCR sequences in one mouse. The Tg mouse line was named vTreg53. aTregs in vTreg53 Tg mice enriched the VAT much sooner, had elevated aTreg markers (discussed next), and the Tg mice had improved insulin sensitivity and glucose tolerance [80].

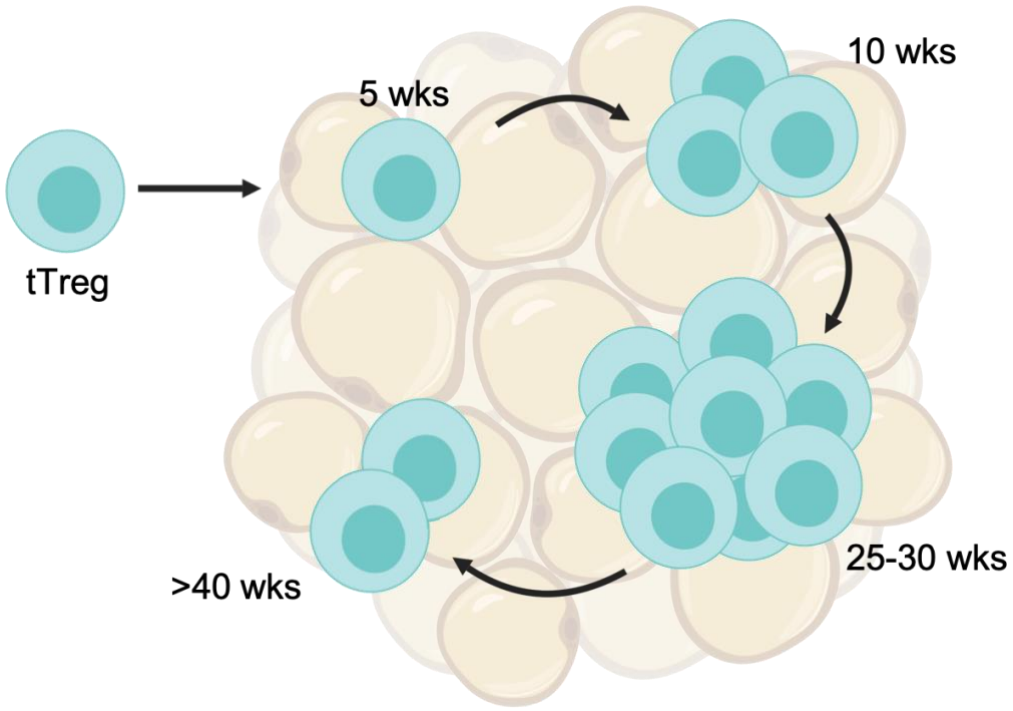

Figure 4 Adipose Treg age-dependent increase in mice

Thymic derived Tregs (tTregs) seed the adipose tissue early on and are detectable at five weeks of age. aTregs continue to proliferate reaching a frequency similar to other peripheral Treg populations at 10 weeks of age. aTregs further proliferate reaching a peak around 25-30 weeks. Around 40 weeks there is an age-dependent decrease in the aTreg frequency.

Apart from the canonical Treg gene expression profile, aTregs have a unique gene expression profile that differs from that of lymphoid Tregs. Among enriched genes in aTregs are the adipocyte master transcriptional regulator PPARγ. Many studies have described anti-inflammatory properties for PPARγ, especially in cases with the PPARγ agonist thiazolidinedione (e.g. pioglitazone). Loss of PPARγ in Foxp3+ cells affected Tregs in the VAT but not in the secondary lymphoid tissues and also led to a decrease in other aTreg over-expressed markers such as the chemokine receptor CCR2, the Th2

transcription factor GATA3, a marker for T cell maturation KLRG1, and the activation marker CD69.

Further studies have discovered other gene requirements for aTreg development and function. Vasanthakumar et al. revealed that IRF4 is required for expression of PPARγ, while both transcription factors IRF4 and BATF are required for expression of ST2, the receptor for IL-33, and that the ST2/IL-33 signaling axis is important for expansion of aTregs. Our own studies verified the upregulation of Id2 in aTregs relative to splenic Tregs. We found that Id2 deficiency in aTregs led to a decreased frequency of these cells. In addition, the remaining aTregs had decreased expression of ST2, CCR2, KLRG1, GATA3, increased expression of cell death markers including Fas, and decreased cytokine expression, which also translated to impaired glucose tolerance [81].

Above, I discussed a number of aTreg intrinsic factors important for their establishment in the VAT, but there still remain questions regarding what factors are involved in recruitment and retention of Tregs in the VAT. aTregs express several other chemokine receptors besides CCR2 that make it difficult to assess whether any single chemokine plays a major role in aTreg recruitment to the adipose tissue [57]. In terms of an adipose-specific aTreg TCR, the Vα2 and Vβ4 variables were chosen due to their high occurrence (10%) in one mouse. Would other TCRs also result in a highly enriched Treg frequency in the VAT and are there conditions in the VAT that favor one TCR over another? Is there one or multiple ligands that select for the different aTreg clonal types? What are the differences in signal strengths of the aTreg clonal types and do they all serve similar or different purposes (self-tolerance, infection, cancer, etc)? In this burgeoning field, there are still many questions left to answer.

1.3.5 Adipose-resident Tregs are receptive to cytokines and adipokines in the VAT

Early in life it is difficult to detect immune cells in mouse VAT in part because there is very little tissue to examine. Despite their slow expansion, aTregs have been shown to be receptive to expansion signals (Figure 5). One study of iNKT cells in the VAT showed that aTregs are dependent on IL-2 produced by iNKT cells and that α-GalCer stimulation of iNKT cells led to an increase in the aTreg frequency [62]. Administration of recombinant IL-2 can rescue and expand the frequency of aTregs in HFD studies [57]. Conversely, iNKT cell deficiency, using CD1d KO mice, stunts the frequency of aTregs. Another more recent study revealed that a population of PLZF+ γδ T cells expands with similar kinetics as aTregs and that they are necessary to maintain and expand the aTreg population in adult mice, in an IL-17 dependent manner [63].

Apart from cytokines produced by immune cells, aTregs also respond to IL-33, which is primarily produced by adipocytes. Indeed, aTreg expansion requires expression of the Il-33R chain ST2. In addition, IL-33 alone is sufficient to reverse diet-induced obesity (DIO) reduction of and also to restore insulin sensitivity.

Surprisingly, levels of IL-33 in the VAT continue to increase during obesity [82-83]. The explanation for this IL-33 accumulation remains unclear but could be due to the diminished receptive population of aTregs, as IL-33 is also elevated in ST2 KO mice and in obesity, where the remaining aTregs have decreased ST2 expression. Another potential explanation for the elevated IL-33 levels in obesity dependent on the IL-33/ST2 signaling axis could be a negative feedback regulatory mechanism of ST2 in aTregs in response to constant IL-33 signaling [64-84].

Adiponectin and leptin are adipocyte-derived hormones, also known as adipokines, with anti-inflammatory and pro-inflammatory properties, respectively. Both adipokines have an inverse relationship with elevated leptin and lower adiponectin levels in obesity. The receptors for both adipokines are expressed on aTregs with decreased expression of adiponectin receptor 1 (adipoR1) during obesity [85]. In addition, adiponectin increases expression of IL-10 and the IL-10R1A, and reduces the production of pro-inflammatory IFNγ in human leukocytes[60]. It has yet to be established what direct effect adiponectin has on aTregs.

Leptin is an important factor for type 1 immunity, with mice deficient in leptin being more susceptible to infection but also protected from models of autoimmunity. Prior to the discovery of aTregs, leptin and its receptor were mainly studied in isolated human CD4+ Foxp3+ Tregs from healthy donor PBMCs [70]. These human Tregs secrete leptin and express high levels of the leptin receptor. Ultimately, it was found that blocking leptin signaling with an antibody led to increased proliferation of Tregs, suggesting that leptin negatively regulates Treg proliferation.

Here I have discussed cytokines and adipokines likely to promote Treg maintenance and expansion in the VAT. Some of these signals continue to be expressed in obesity, indicating there are others regulatory mechanisms that dictate survival of aTregs in the VAT. In this section, I also included the pro-inflammatory adipokine, leptin, as its role in human Treg regulation has been studied directly. In the final section, I will list examples that support a role for aTregs in maintaining glucose tolerance and insulin sensitivity.

1.3.6 The role of aTregs in glucose tolerance and insulin sensitivity

Obesity is linked to a number of health complications including T2DM. The physiological changes associated with T2DM include increased glucose intolerance and insulin resistance. The same study that first described Tregs in the VAT also examined the role of aTregs on metabolic parameters, including glucose tolerance and insulin sensitivity. Using DEREG mice, where a diphtheria toxin receptor-eGFP fusion protein is expressed under the Foxp3 promoter, Tregs were found to be depleted more efficiently in AT compared to other tissues, following administration of diphtheria toxin. The decrease in aTregs correlated with a decrease in signaling of the insulin receptor despite an increase in fasting blood insulin, but no changes in fasting blood glucose.

In the same study, mice were placed on HFD, resulting in reduced frequency of aTregs and increased glucose intolerance in the mice. IL-2-Ab complexes helped to increase the number of aTregs in HFD-fed mice, as mentioned above, but also significantly improved glucose tolerance over PBS treated controls [57]. In a subsequent study, an aTreg-PPARγ CKO model revealed that PPARγ expression in aTregs was necessary for the maintenance of glucose tolerance and pioglitazone's ability to improve glucose tolerance [86]. The same group examined mice around aTreg peak frequency (25 weeks) at an age where the aTreg frequency sharply declines (40 weeks) and found that the older mice had a significant increase in insulin resistance compared to the younger mice, as measured by HOMA-IR[79]. In our own studies, Id2 CKO mice displayed increased glucose intolerance and elevated blood insulin, a sign of insulin resistance, compared to WT controls following a glucose tolerance test (GTT) [81].

GTTs in vTreg53 transgenic mice, which have higher levels of aTregs, showed an increase in glucose tolerance, as the transgenic mice returned to basal glucose levels faster than non-transgenic controls [80]. In an insulin tolerance test (ITT) which tests the body's response to insulin, in vTreg53 Tg mice also revealed an increase in insulin sensitivity, as the Tg mice had much lower blood glucose levels and took longer to return to basal glucose levels after insulin administration, compared to non-transgenic controls, reflecting an increased sensitivity to insulin [80].

In the above examples, aTregs were always necessary for proper, or improved, glucose tolerance or insulin sensitivity compared to mice with decrease aTreg frequency and cell numbers. The data currently suggest that aTreg frequency, the aTreg TCR (Vα2, Vβ4), and proper expression of the aTreg transcriptional profile are important for VAT homeostasis. However, it isn't clear through what mechanism, or combination of mechanisms, aTregs act to maintain proper VAT homeostasis. aTregs are known to express other canonical Treg markers, such as CTLA4 and IL-10, at increased levels relative to lymphoid Tregs. In our own studies we saw a decrease in expression of IL-10 and IL-13 in Id2 CKO aTregs, but we have not assessed the expression of Treg inhibitory receptors. Nonetheless, it is clear that glucose tolerance and insulin sensitivity require maintenance of the aTreg population.

1.3.7 Conclusion

The VAT is a dynamic environment, with age-dependent increases and decreases in aTregs, but also changes in other cell populations. While aTregs are protective in obesity-related inflammation, there appears to be a threshold that results in a sharp

decrease of aTregs and other anti-inflammatory populations during chronic inflammation in obesity.

Studying the IL-33/ST2 signaling axis in aTregs has revealed important cross-talk between immune cells and stromal cells. One question remains why IL-33 can rescue aTregs during HFD, but at the same time IL-33 is upregulated during obesity. In HFD, the remaining aTregs have reduced ST2. One possibility could be that ST2 is downregulated on aTregs following chronic IL-33 stimulation. Another is that ST2 transcription switches to its soluble isoform, which is secreted and acts as a decoy receptor. Considering the role of soluble ST2 in cardiovascular disease, which is associated with obesity, soluble ST2 may also play a role in obese VAT [87]. Constitutive expression of cell-surface ST2 on aTregs may have the potential to maintain the population of aTregs, considering that IL-33 is elevated in obesity.

Potentially, IL-33 may rescue aTregs under mild obese conditions, but not severe obesity. The study by Han et al. placed mice on a HFD for 14-16 weeks, which led to a decrease in aTregs and glucose intolerance, but was reversed with IL-33 treatment. It may be necessary to test IL-33 in a morbid obese mouse model. Other studies that found elevated IL-33 involved samples from severely obese patients. This could be due to the severity of metabolic disease, or may reflect specific aspects of to human fat [84].

Unfortunately, not many studies have assessed aTreg function in models of obesity such as leptin-deficient mice or agouti yellow mice. In both of these models, mice have severe insulin resistance and develop T2DM. Feuerer et al. saw that leptin deficient mice originally held a higher frequency of aTregs at 5 weeks of age, which would be expected considering leptin's inhibitory role on Tregs, followed by a decline. However, no

study has investigated whether aTregs can be rescued in these models of severe obesity using methods described above, such crossing to vTreg53 transgenic mice, administration of IL-2 Ab complexes or IL-33, or thiazolidinedione-drugs like pioglitazone. While rescue of aTregs in these models might be due to the severity of the genetic abnormalities, these models may help demarcate the difference between pre-metabolic/metabolic syndrome.

There is limited information on the transcriptional profile of aTregs, including expression of Foxp3, GATA3, IRF4, BATF and PPARγ. From this list, GATA3 is probably the most under-studied with respect to the anti-inflammatory functions of aTregs, considering its known regulatory role in barrier tissue Tregs. Besides the production of IL-10, most studies have focused on changes on unique markers of Tregs during HFD or other experimental conditions. Considering the high expression of canonical Treg markers in the adipose, such as the inhibitory receptor CTLA4, it would be interesting to investigate whether other markers play a role in the VAT.

Ultimately, it may be necessary to clearly define markers that differentiate between mild and severe obesity, or even pre-metabolic/metabolic syndrome. Unfortunately, the development of metabolic syndrome is complex and not just dependent on a single factor such as weight gain. This problem is highlighted in studies that show that not every obese individual goes on to develop metabolic syndrome. It may be important in future studies to investigate whether aTregs are important in preventing other diseases associated with obesity such as cardiovascular disease and certain cancers. These studies could provide other useful biomarkers to assess the progression of disease.

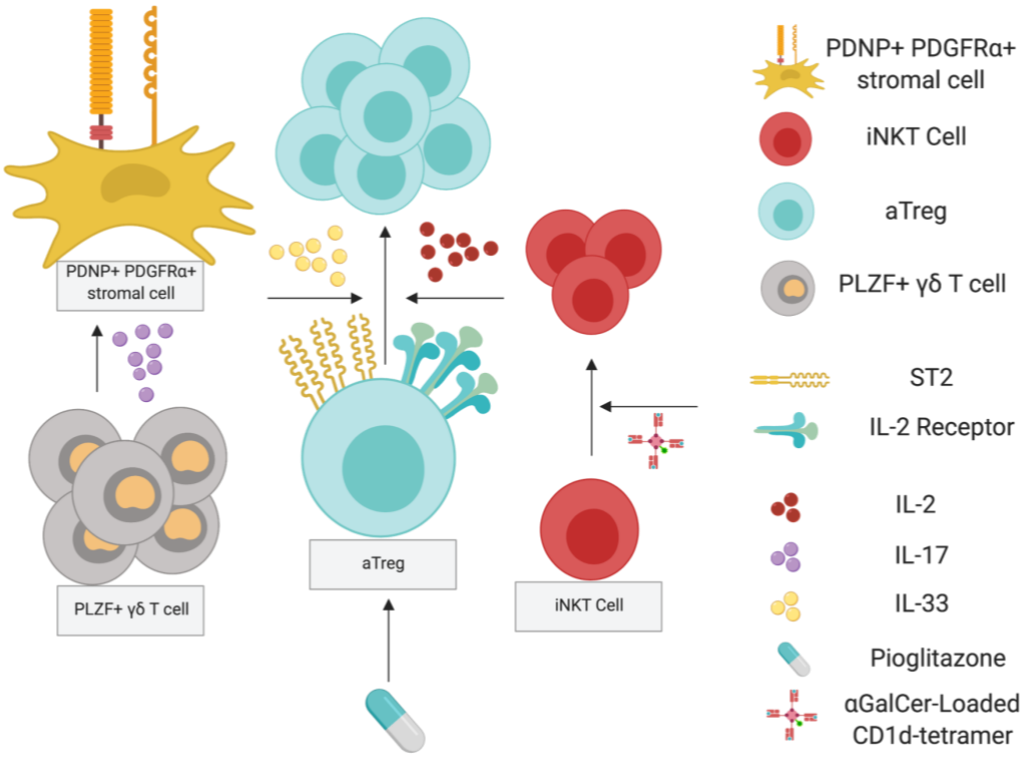

Figure 5. Adipose-resident Treg expansion signals

Adipose-resident Tregs are dependent on various cells and signals. PLZF+ γδ T cells produce IL-17 that stimulates PDNP+ PDGRα+ stromal cells to produce IL-33. IL-33 signals through the ST2 receptor on aTregs. IL-2 signals through CD25 on aTregs. Stimulating iNKT cells with α-GalCer can induce additional IL-2 that further expands aTregs. Additionally, aTregs are receptive to expansion by Pioglitazone.

2.0 Effects of loss of microRNA-155 on T cell development and function with loss of microRNA-155

2.1 Foreword

This chapter is adapted from a previously published manuscript in The Journal of Immunology. Adolfo B. Frias Jr., Eric J. Hyzny, Heather M. Buechel, Lisa Y. Beppu, Bingxian Xie, Michael J. Jurczak and Louise M. D'Cruz. 2019. The Transcriptional Regulator Id2 Is Critical for Adipose-Resident Regulatory T Cell Differentiation, Survival, and Function. J. Immunol. Vol: 203 no. 3 658-664. Copyright © 2019. The American Association of Immunologists, Inc. DOI: https://doi.org/10.4049/jimmunol.1900358

2.2 Introduction

Invariant Natural Killer T (iNKT) cells are a versatile T cell population, expressing a semi-invariant TCR (Vα14-Jα18 in mice) and with the ability to rapidly respond to their cognate glycolipid to produce an array of cytokines and rapidly mobilize an immune response[44-88]. Although the unique miRNA profile associated with iNKT cells during activation is currently unclear, there is precedence for studying the role of miRNAs in iNKT cell development, lineage differentiation and function. Conditional loss of Dicer (the RNAase II enzyme responsible for miRNA processing) led to significant reduction in iNKT

cells during development and in peripheral tissue, strongly indicating that miRNAs play a role in iNKT cell development[89-91]. Substantial evidence also exists for a function for individual miRNAs in development of iNKT cells. Mir-150 is required for iNKT cell maturation and its loss results in increased IFN-γ production[92], miR-181 is essential for early development of iNKT cells[93] and Let-7 microRNAs specifically target PLZF expression during iNKT cell development in the thymus[94]. Recently, it was shown that the miRNA cluster miR17~92 regulates iNKT cell development via regulation of TGF-β[95].

MiR-155 was one of the first miRNAs discovered in mammals and is expressed by multiple cells of the immune system. In B cells, overexpression of miR-155 led to transformation and development of lymphoma, suggesting a role for miR-155 in regulating cell proliferation[96]. In T cells, miR-155 appears to play multiple unique functions. Initially, T cells from mice with germline miR-155 deficiency were shown to skew toward the Th2 lineage, possibly due to loss of miR-155 negative regulation of the transcription factor c-Maf (a positive regulator of IL-4)[97-98]. MiR-155 has been shown to regulate both the $CD8^+$ effector T cell response and the NK cell response to viral infection, through effects on proliferation of these cells, although miR-155 appears to target different mRNAs during proliferation depending on cell type[99-102]. In one study, survival of $CD8^+$ effector T cells was also impaired in the absence of miR-155[99]. MiR-155 has been reported to regulate cell lineage decisions, with central memory $CD8^+$ T cells expressing higher levels of miR-155 than effector memory subsets[103-104]. A role for miR-155 in the survival of regulatory T cells through its action on SOCS1 has also been observed[105-106]. Aside from a function in proliferation and survival, miR-155 can also influence cytokine production by immune cells. Indeed, miR-155 can positively regulate IFN-γ production by CD4 and CD8 T cells

through its repression of the phosphatase SHIP1, a negative regulator of IFN-γ expression[107-108].

Here we examined a role for miR-155 in development and function of iNKT cells. Using germline deficient miR-155 mice, we showed that loss of miR-155 subtly affected the frequency of iNKT cells in the thymus but not in peripheral tissues. Loss of miR-155 also resulted in marginally more $CD44^{+}NK1.1^{+}$ stage 3 cells in the thymus. While miR-155 functions to regulate conventional T cell proliferation and cytokine production in peripheral tissue, these parameters were unaffected by loss of miR-155 in peripheral iNKT cells. We thus concluded that IFN-γ production by iNKT cells is regulated by a mechanism independent of miR-155.

2.3 Materials and Methods

2.3.1 Mice

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). MiR-155 KO mice were purchase from Jackson Laboratories and bred to C57BL/6 mice to generate WT littermate controls. Mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of Pittsburgh. α-GalCer (2μg, Avanti polar lipids, Alabaster, AL) was administered intraperitoneally.

2.3.2 Flow Cytometry

Single-cell suspensions were prepared from thymus and spleen. Bone marrow (BM) was prepared by isolating lymphocytes from the femurs of mice and preparing a single cell suspension. Liver single cell suspensions were prepared as previously described[109]. Adipose single cell suspensions were prepared as previously described[110]. Lung single cell suspensions were prepared by mincing lung, incubating minced tissue with collagenase IV at 37C before filtration. The following antibodies were used: anti-TCRβ FITC (clone H57-597), anti-CD4 FITC (L3T4), anti-CD24 (M1/69), CD1dTetramer Alexa 488 (NIH Tetramer Core Facility (mCD1d/PBS57)), CD1d Tetramer PE (NIH Tetramer Core Facility (mCD1d/PBS57)), anti-TCRβ PE (H57-597), anti-PLZF PE (Mags.21F7), anti-NK1.1 PerCP Cy5.5(PK136), anti-NK1.1 APC (PK136), anti-TCRβ APC (H57-597), anti-TBET APC (4B10), CD1d Tetramer Alexa 647 (NIH Tetramer Core Facility (mCD1d/PBS57)), Ki-67 Pacific Blue (SolA15) and Annexin V PE (BD Pharmingen). All antibodies were purchased from eBioscience unless otherwise specified. Samples were collected on a FACS LSRII, FACS Fortessa or FACS Aria (BD Biosciences) and were analyzed with FlowJo software (TreeStar). TCRβ$_+$CD1d-tetramer$_+$ iNKT were sorted with a FACS Aria to at least 98% purity.

2.3.3 qPCR

For miR-155, samples were reversed transcribed using TaqMan™ MicroRNA Reverse Transcription Kit ((P/N 4366597, Applied Biosystems). Mouse MiR155 TaqMan™ MicroRNA assays were purchased from applied Biosystems (P/N 4427975). Sno202 was used to normalize.

2.3.4 Statistical analysis

Two-group comparisons were assessed with an unpaired Student's t-test. Graph-Pad Prism software (version 5) was used for statistical analyses. P values of less than 0.05 were considered significant.

2.4 Results

2.4.1 MiR-155 deficiency results in increased iNKT cell frequency in the thymus

Using miR-155 germline deficient (miR-155 KO) mice, we first characterized conventional T cell development in the thymus of these animals. We observed that total thymocyte frequency and cell number were not significantly different between wildtype (WT) and miR-155 KO mice (Figure 6A). To characterize the functional importance of miR-155 in the development of iNKT cells, we determined the frequency and absolute number of iNKT cells in the thymus of WT and miR-155 KO mice. Although the frequency of iNKT cells was significantly increased in the absence of miR-155, we observed no substantial difference in absolute iNKT cell number in the thymus between WT and miR-155 KO mice (Figure 6B).

Similar to conventional T cells, iNKT cells go through sequential stages of development in the thymus. The first, stage 0, is characterized by high CD24 expression. Gating on CD1d-tetramer^{+} CD24^{+} iNKT cells, we noted no difference in the frequency or absolute number of iNKT cells expressing CD24 in the thymus. We concluded that progenitor stage 0 iNKT cells were unaffected by the absence of miR-155 (Figure 6C). After stage 0, iNKT cells downregulate CD24 becoming CD44^{-} NK1.1^{-} stage 1 iNKT cells

before upregulation of CD44 (stage 2) and NK1.1 (stage 3). Closer analysis of iNKT cell stage development in the thymus revealed that loss of miR-155 resulted in marginally decreased stage 2 ($CD44^+$ $NK1.1^-$) and increased stage 3 ($CD44^+$ $NK1.1^+$) iNKT cells (Figure 6D). Stage 1 ($CD44^-$ $NK.1^-$) cells were unaffected by loss of miR-155 expression (Figure 6D). We conclude that miR-155 deficiency results in increased frequency of stage 3 $CD44^+$ $NK1.1^+$ iNKT cells in the thymus.

Recent reports have indicated that transcription factor expression can define iNKT cell subsets[111]. Gating on iNKT cells and examining TBET, PLZF and RORγt expression, we found no substantial difference in expression of these transcription factors by WT and miR-155 KO cells (Figure 6E). Additionally, cell surface proteins expressed by iNKT cells including CD122, PD1 and CD69, were not differentially expressed by miR-155 KO cells (Figure 6E). Gating on thymic iNKT cells and examination of NKT1, NKT2 and NKT17 cells by transcription factor expression, we noted a slight decrease in NKT2 cells and increase in NKT1 cells (Figure 6E) consistent with the subtle decrease we observed in stage 2 and increase in stage 3 cells (Figure 6D). NKT17 cell frequency remained unchanged in the absence of miR-155 expression (Figure 6E).

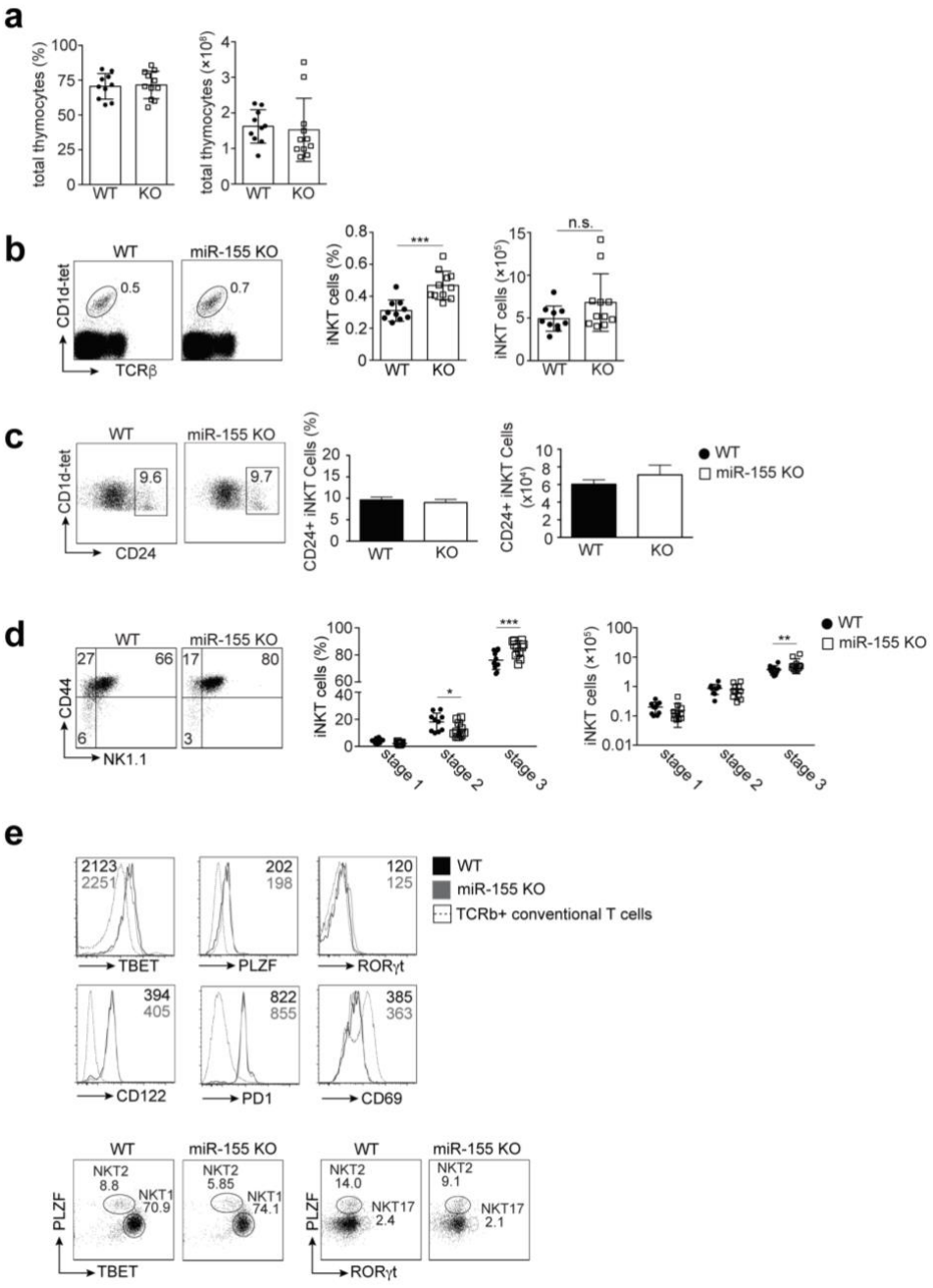

Figure 6. Loss of miR-155 promotes maturation of thymic iNKT cells

T cells from the thymus of wild type (WT) and miR-155 germline deficient (miR-155 KO) mice were characterized. (a) Graphs indicating frequency and total thymocytes from WT and miR-155 KO (KO) mice. (b) Flow cytometric analysis of iNKT cell frequency in WT and miR-155 KO gated lymphocytes from the thymus. Numbers indicate percentage of cells within the drawn gate. Graphs indicate the average frequency and absolute number (+/- SEM) of CD1d-tet$_+$ TCR-β$_+$ gated iNKT cells from the indicated genotype. (c)

Flow cytometry plots indicating CD24 expression on gated iNKT cells in the thymus. Graphs indicate the average frequency and absolute number (+/- SEM) of $CD24_+$ gated iNKT cells from the indicated genotype. (d) Flow cytometric analysis of $CD44_+$ and $NK1.1_+$ expression by gated iNKT lymphocytes from the indicated genotype. Graphs indicate average frequency and absolute number (+/- S.D.) of maturation stages as defined by CD44 and NK1.1 expression on CD1d-tet_+ TCR-β_+ gated iNKT cells from the indicated genotype. (e) Histograms showing TBET, PLZF, RORγt, CD122, PD1 and CD69 expression by gated iNKT cells from WT and miR-155 KO mice. Numbers indicate the median fluorescence intensity (MFI) from the indicated genotype with WT shown in black and miR-155 KO shown in grey. The dotted line indicates expression by conventional T cells for comparison. Data are representative of 3 independent experiments with 2-4 mice per group per experiment. Statistical significance was evaluated with unpaired Students t-test, where n.s.=not significant, *P<0.05, ***P*<0.01 and ****P*<0.001.

2.4.2 Loss of miR-155 does not affect peripheral iNKT cells

We next determined whether the modest increase in the frequency of stage 3 iNKT cells that we observed in miR-155 KO thymocytes was maintained in peripheral iNKT cells. We compared the frequency and absolute number of iNKT cells in the spleens of WT and miR-155 KO mice. Here we observed no difference in the frequency and absolute number of iNKT cells in the spleen between WT mice and those with germline loss of miR-155 expression (Figure 7A). When we examined iNKT cell stages, we also noted that there was no significant difference between stage 1 ($CD44_{lo}$, $NK1.1_{lo}$), 2 ($CD44_{hi}$, $NK1.1_{lo}$) and 3 ($CD44_{hi}$, $NK1.1_{hi}$) cells in the periphery of WT and miR-155 KO mice (Figure 7B). To investigate cell death, we performed Annexin V and 7-AAD staining of WT and miR-155 KO iNKT cells and noted no difference in cell death (Figure 7C). As in the thymus, expression of the transcription factors TBET, PLZF and RORγ was unaffected by loss of miR-155 (Figure 7D). Finally, in other peripheral lymphoid tissues such as the liver, bone marrow, lung and adipose tissue, we did not observe a significant

difference in the frequency of iNKT cells (Figure 7E). Thus we concluded that the increased frequency of iNKT cells, and specifically stage 3 iNKT cells, that we observed in the thymus was lost as the cells migrated from the thymus and matured in peripheral tissue.

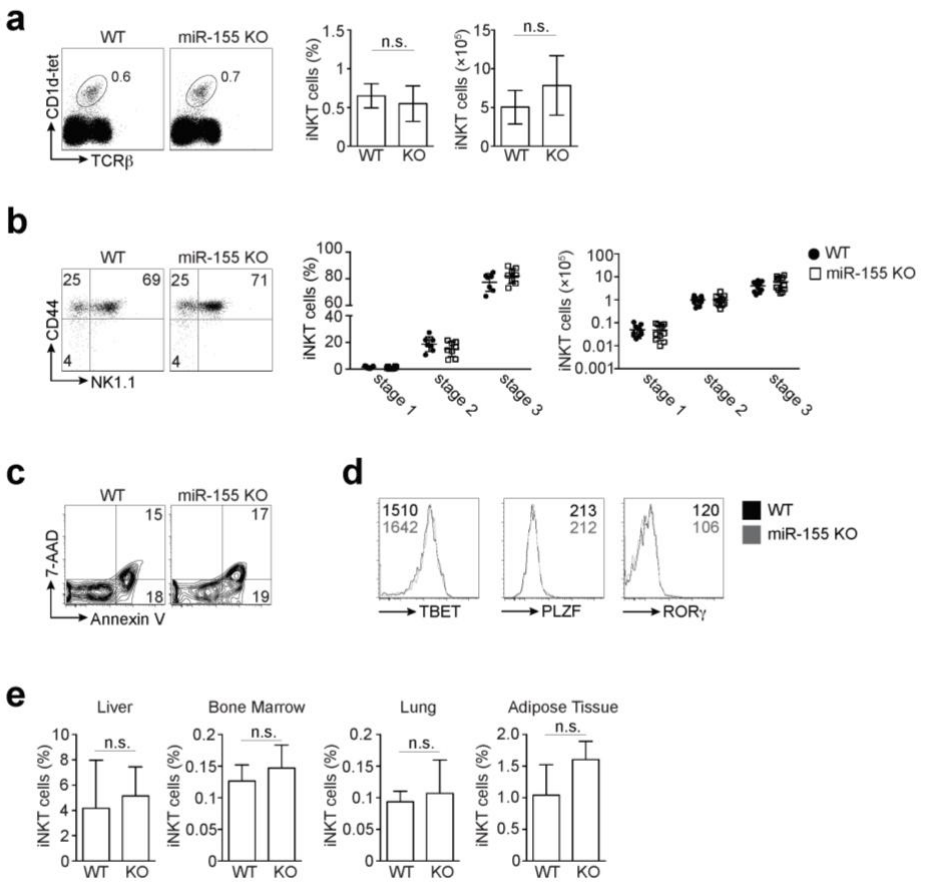

Figure 7. MiR-155 expression is dispensable for iNKT cell homeostasis in peripheral tissue

Wild type (WT) and miR-155 germline deficient (miR-155 KO) mice were characterized. (a) Flow cytometric analysis of iNKT cell frequency in WT and miR-155 KO lymphocytes from the spleen. Numbers indicate percentage of cells within the drawn gate. Graphs indicate the average frequency and absolute number (+/- SEM) of CD1d-tet$_+$ TCR-β$_+$ gated iNKT cells from the indicated genotype. (b) Flow cytometric analysis of CD44$_+$ and NK1.1$_+$ expression by gated iNKT lymphocytes from the indicated genotype. Graphs indicate average frequency and absolute number (+/- SEM) of maturation stages as defined CD44 and NK1.1 expression on CD1d-tet$_+$ TCR-β$_+$ gated iNKT cells from the indicated genotype. Graphs are the average of 10 mice from 3 independent experiments. (c) Flow cytometry plots indicating Annexin V and 7-AAD expression on gated iNKT lymphocytes from the indicated genotype. (d) Histograms showing TBET, PLZF and RORγt expression by gated iNKT cells from WT and miR-155 KO mice. Numbers indicate the median fluorescence intensity (MFI) from the indicated genotype. (e) Graphs indicating the frequency of

iNKT cells from WT and miR-155 KO (KO) mice from the indicated lymphoid organ. Statistical significance was evaluated with unpaired Students t-test, where n.s.=not significant.

2.4.3 Loss of miR-155 does not affect iNKT cell cytokine production

Previous data have indicated that miR-155 can indirectly positively regulate IFN-γ expression in conventional T cells, through targeting of the src homology 2 domain-containing inositol phosphatase 1 (SHIP1) for degradation. Therefore, we determined whether miR-155 could affect cytokine production by peripheral iNKT cells. IFN-γ and IL-4 production can be detected by iNKT cells 1-3 hours after activation with their cognate ligand α-GalactosylCeramide (α-GalCer). First, we analyzed expression of miR-155 in WT iNKT cells after α-GalCer treatment. Sorting iNKT cells from WT mice after in vivo activation, we observed upregulation of miR-155 ~2-fold after three hours and ~30-fold after three days of activation relative to naïve iNKT cells (Figure 8A). Activating WT and miR-155 KO mice with α-GalCer for three hours, we could detect no difference in frequency or absolute number of splenic iNKT cells (Figure 8B). When we examined IFN-γ and IL-4 production by intracellular staining WT and miR-155 KO iNKT cells at three hours after activation *in vivo*, we observed no difference in production of these cytokines between the WT and miR-155 KO iNKT cells (Figure 8C). Thus, we conclude that mir-155, despite its increased expression within three hours after iNKT cell activation, is dispensable for production of canonical cytokines by iNKT cells.

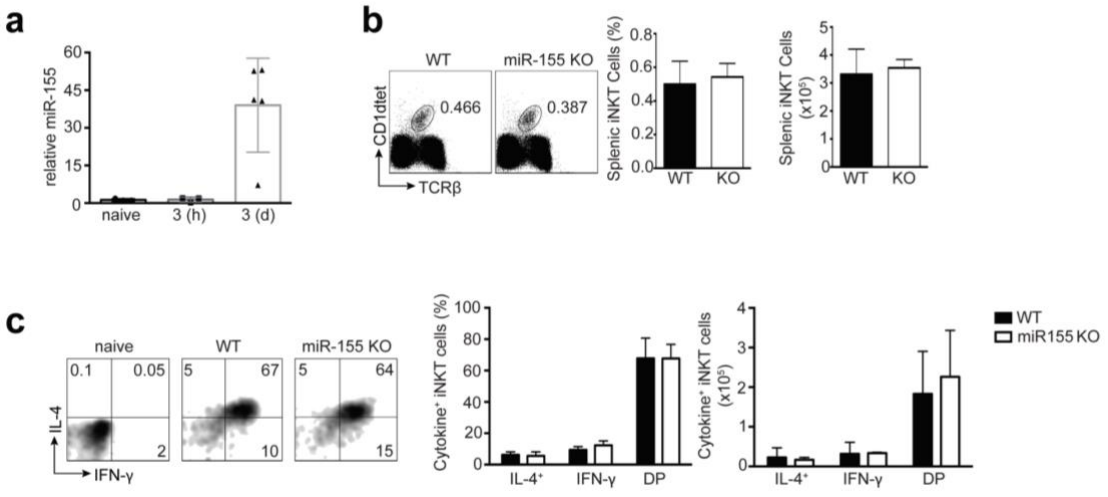

Figure 8. MiR-155 is upregulated in activated iNKT cells

(a) QPCR indicating relative expression of miR-155 by sorted CD1d-tet+ TCR-β+ iNKT cells from naïve mice or mice injected with 2 µg α-GalCer for 3 hours or 3 days. (b) Flow cytometric analysis of iNKT cell frequency in the spleen after i.p. injection with 2 µg α-GalCer for 3 hours. Graphs indicate the average frequency and absolute number (+/- SEM) of gated iNKT cells from the indicated genotype. (c) Flow cytometry and bar graphs indicate frequency and cell number of IFN-γ and IL-4 expression by gated splenic iNKT lymphocytes from the indicated genotype. DP=double positive for IFN-γ and IL-4. Graphs are representative of two experiments with 2 mice per group.

2.4.4 Mir-155 is dispensable for the iNKT cell response at day 3 after activation

MiR-155 was previously reported to affect proliferation of conventional CD8+ effector T cells[99-101]. Furthermore, we observed ~30-fold increase in miR-155 expression in iNKT cells upon activation with α-GalCer at three days after activation (Figure 8A). To determine a role for miR-155 in iNKT cells three days after activation, we compared WT and miR-155 KO iNKT cells at this time point. We examined iNKT cells in the spleen, liver and bone marrow (BM) and all trended towards reduced frequency of iNKT cells in the absence of miR-155 (Figure 9A). However, the absolute number of iNKT cells in these tissues was unaffected by loss of miR-155 expression (Figure 9A). Moreover, we could

determine no differences in NK1.1 and CD44 expression in iNKT cells from miR155 KO mice relative to WT, although as previously reported NK1.1 downregulation after activation with α-GalCer was observed in both WT and miR-155 KO iNKT cells (Figure 9B). Previous reports have indicated that PD1 is upregulated on activated iNKT cells[112]. Therefore, we examined PD1 expression on WT and miR155 KO iNKT cells three days after activation. As with overall iNKT cell development, we observed no differences in PD1 expression on activated iNKT cells in the absence of miR-155 (Figure 9C). To determine proliferation, we examined Ki-67 expression in WT and miR-155 KO iNKT cells three days after activation with α-GalCer. In all tissues examined, a subset of iNKT cells were dividing ($Ki\text{-}67^{+}$) (Figure 9D). Importantly, loss of miR-155 in iNKT cells did not affect cell division by these cells in peripheral tissue after activation (Figure 9D). Finally, we examined transcription factor expression and could detect no difference in expression of TBET, PLZF or RORγ in WT or miR-155 KO iNKT cells after activation (Figure 9E). Thus, we have established that despite high miR-155 expression in iNKT cells at day three after activation, miR-155 is dispensable for the proliferation of iNKT cells in peripheral tissues.

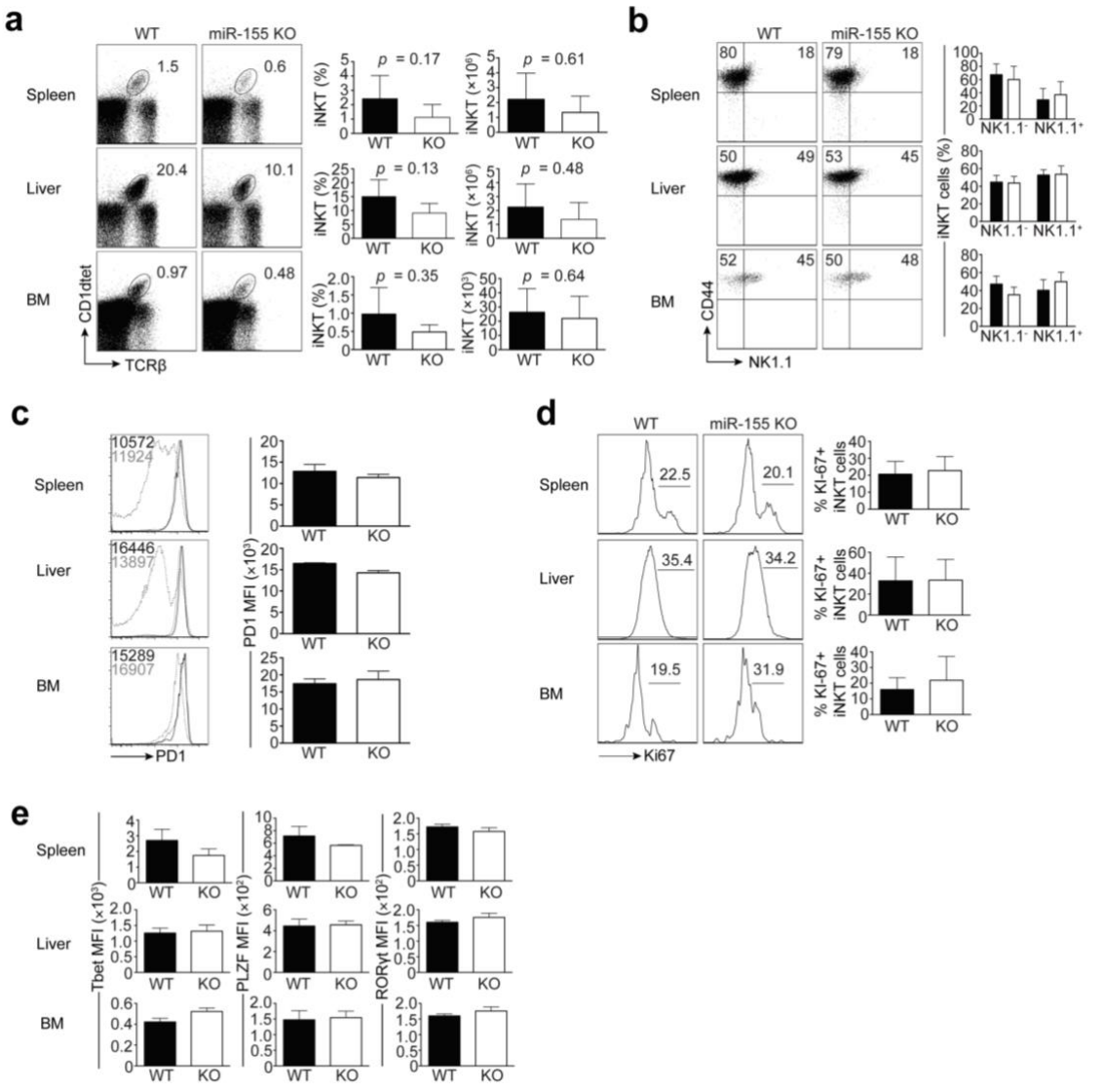

Figure 9. Loss of miR-155 does not affect iNKT cells at 3 days after activation

(a) Flow cytometric analysis of iNKT cell frequency in the spleen, liver and bone marrow (BM) at 3 days post i.p. injection with 2 μg α-GalCer. Bar graphs indicate iNKT cell frequency and absolute number (+/- SEM) in the indicated tissue. (b) Flow cytometric analysis of $CD44_+$ and $NK1.1_+$ expression by gated iNKT lymphocytes from the indicated genotype and tissue. Graphs indicate average frequency (+/- SEM) of $NK1.1_-$ $CD44_+$ or $NK1.1_+$ $CD44_+$ CD1d-tet_+ TCR-$β_+$ gated iNKT cells from the indicated genotype and tissue. (c) Flow cytometric analysis and MFI of PD1 expression by gated iNKT lymphocytes from the indicated genotype and tissue. Black line indicates WT, grey line indicates miR-155KO and dotted line indicates conventional T cell control for comparison. (d) Histograms indicating Ki-67 expression in iNKT cells from

the indicated genotype and tissue at day 3 after activation. Graphs indicate average frequency (+/- SEM) of Ki-67$_+$ CD1d-tet$_+$ TCR-β$_+$ gated iNKT cells from the indicated genotype and tissue. (e) Graphs showing TBET, PLZF and RORγt MFI by gated iNKT cells from WT and miR-155 KO mice after 3 days activation with α-GalCer. Graphs are representative of 4-6 mice from two independent experiments.

2.5 Discussion

Here we show that miR-155 has a minor role in the development of iNKT cells in the thymus. Loss of miR-155 resulted in a small but reproducible increased in stage 3 cells, with a corresponding decrease in stage 2 cells. Although thymic development of iNKT cells was slightly perturbed in the absence of miR-155, this defect was not apparent in peripheral tissues, and activation, and cytokine production by iNK cells in the periphery were unaffected by loss of miR-155 expression. Moreover, miR-155 expression, although high in iNKT cells at day 3 after activation, was dispensable for their proliferation at this timepoint.

Our lab also performed a Nanostring microRNA analysis on naïve, 3hr α-GalCer post stimulated, and 30-day α-GalCer post-stimulated splenic iNKT cells and compared the expression of microRNAs at two stimulated time-points relative to naïve cells (Figure 22). Among the most differentially expressed microRNAs, miR-21 and miR-146a were found to be upregulated at all time points post α-GalCer stimulation.

A few interesting targets were downregulated at 3-days post-α-GalCer stimulation during iNKT cell's proliferative burst, but then upregulated at 30-days post-α-GalCer stimulation when iNKT cells undergo hyporesponsiveness. From our top-hit list, these included miR-467f, miR-339-5p, miR-1894-5p, miR-690, miR-29a, and miR-15b. From

this same list, a few miRNAs went from being upregulated at 3 days post-α-GalCer stimulation, and then downregulated during hyporesponsiveness: miR-475c-5p, miR-18a, miR-467a.

Importantly, our data are largely consistent with a previously published report in which miR-155 was overexpressed using an Lck-miR-155 transgene[113]. Here it was shown that overexpression of miR-155 resulted in an increase in stage 2 cells[113]. In this miR-155 overexpression study, peripheral iNKT cell frequency and cell number were perturbed[113], in contrast to our findings here in which peripheral iNKT cells lacking expression of miR-155 were unaffected. It is possible that loss of miR-155 expression is compensated for by other microRNAs or even by other proteins in peripheral iNKT cells, which would account for normal iNKT cell homeostasis and activation observed in the periphery in the absence of miR-155.

Previous work has also indicated that miR-155 can indirectly regulate IFN-γ expression, mediated in part through its repression of SHIP1, in conventional $CD4^+$ and $CD8^+$ T cells[107]. iNKT cells can produce IFN-γ within a matter of hours after activation, a process that was unaffected by loss of miR-155, indicating that activation-induced IFN-γ production is independent of miR-155 in these cells upon activation. Overall, we find that miR-155 has a moderate effect on the development of iNKT cells in the thymus but that loss of miR-155 does not affect peripheral homeostasis, cytokine production or activation of iNKT cells in peripheral tissues.

3.0 The Transcriptional regulator id2 is critical for adipose-resident regulatory T cell differentiation, survival and function

3.1 Foreword

This chapter is adapted from a previously published manuscript in Immunology. Frias, A. B., Buechel, H. M., Neupane, A. and D'Cruz, L. M. (2018), Invariant natural killer T-cell development and function with loss of microRNA-155. Immunology, 153: 238-245. doi:10.1111/imm.12836

3.2 Introduction

Visceral adipose tissue (VAT) is enriched for a population of $CD4^+$ $Foxp3^+$ $CD25^+$ Tregs, relative to Treg populations in lymphoid tissue, and these cells are reduced in frequency and cell number in different models of insulin resistance, including HFD-induced obesity [57]. Adipose Tregs (aTregs) are thymically derived [78], maturing in the spleen before seeding the visceral adipose tissue at approximately 10 weeks of age, reaching a peak of ~40% total $CD4^+$ T cells in adipose tissue by ~25 weeks of age [57-80]. aTreg peripheral development is complex and a recent report indicated a two-step process by which differentiation of these cells occurs[80]. aTreg precursor cells can be identified in the spleen by low expression of PPARγ and downregulated expression of the transcriptional regulator Id3[80-114]. Their TCR repertoire is more restricted than

conventional Tregs, suggesting VAT antigen-specific activation followed by clonal expansion [78]. A number of surface markers and transcription factors are characteristic of aTregs including ST2, KLRG1, CCR2 and GATA3, and a key feature of these cells is their ~10-fold increased production of IL-10 relative to conventional Tregs [57-82-86].

Detailed gene expression analysis of these cells revealed the transcription factor PPARγ to be highly expressed by aTregs [79-86]. Treg-specific loss of PPARγ did not affect conventional lymphoid-resident Tregs but resulted in a substantial reduction in the frequency and absolute number of aTregs. Moreover, PPARγ-deficient aTregs displayed a gene expression profile more similar to conventional lymphoid Tregs, including reduced Foxp3 expression [86]. The transcriptional factors BATF and IRF4 to regulate expression of the IL-33 receptor ST2 and PPARγ in aTregs. IL-33, signaling through ST2, is required to both recruit and maintain the aTreg population in adipose tissue [78-82-83]. Importantly, aTregs are critical for suppressing chronic systemic inflammation observed under conditions of obesity [57-83]. Under HFD conditions aTregs are reduced but can be restored through administration of IL-33 or the thiazolidinedione drug pioglitazone, an approved T2DM drug treatment [78-83-86]. Thus, it is becoming increasingly apparent that aTregs are critical for regulating and maintaining metabolic homeostasis.

The E and Id families of transcriptional regulators have been implicated in many aspects of lymphocyte differentiation [115-118]. E proteins (e.g. E2A, HEB) are basic helix-loop-helix (HLH) transcriptional activators/repressors that bind to DNA at E-box sites, controlling expression of genes crucial to lineage commitment, targeting antigen-receptor gene rearrangement, and enforcing key developmental checkpoints [115-118]. Id proteins (Id1-Id4) heterodimerize with the E proteins, preventing DNA binding at their target E-box

site. Thus, Id proteins function as natural dominant-negative regulators of E protein activity (Figure 10).

To investigate the function of E proteins in the thymic development of conventional Tregs, E2A and HEB conditional mice were crossed to a tamoxifen-inducible Rosa26-ER-Cre line [75]. Administration of tamoxifen caused deletion of the E proteins E2A and HEB and resulted in a dramatic increase in Foxp3+ Tregs in the thymus[75]. Moreover, conditional loss of Id2 and Id3 using CD4-Cre led to a decrease in thymic Foxp3+ Tregs [75]. These data strongly suggest a role for the E and Id proteins in the regulation of lymphoid Tregs. However, the use of non-Treg specific Cre lines and a focus on analysis of the thymically-derived Treg populations, precluded significant advancement in understanding the function of these proteins in aTregs. More recently, E2A and HEB were shown to be critical for effector Tregs with Foxp3-Cre mediated loss of E proteins resulting in substantial increases in Tregs in peripheral tissues such as the small intestine, lung and liver, although the Treg population residing in adipose tissue was not examined [76]. The function of Id2 and Id3 in conventional Tregs has also been examined by crossing Id2 and Id3 floxed animals to a Foxp3-Cre line [119]. Treg-specific loss of both Id2 and Id3 resulted in a decrease in Tregs in lymphoid tissues and a corresponding increase in spontaneous inflammation and autoimmunity in these animals [119]. Another study analyzed the function of Id3 in germline deficient mice, concluding loss of Id3 resulted in the generation of fewer Tregs in the thymus and spleen [120]. However, the function of the Id proteins in aTregs was not examined in either of these studies. Using Id3-GFP reporter mice, it was recently shown that Id3 expression is gradually reduced in effector regulatory T cells as they seed peripheral tissue [114]. Therefore, although it is clear that E and Id

proteins play reciprocal roles in thymic Treg development and homeostasis, the function of these proteins in aTregs has not been determined.

Here we examined the role of Id2 in the differentiation, survival and function of aTregs. We show that Id2 is highly upregulated in aTregs, while Id3 is expressed predominantly in lymphoid-resident Tregs. Id2 was required for the survival of aTregs and its loss resulted in substantially decreased aTreg population. Moreover, RNA-seq analysis revealed increased Fas expression in Id2-deficient aTregs, providing a possible mechanistic explanation for the increased cell death we observed in the absence of Id2. Loss of Id2 in Tregs resulted in increased systemic inflammation, inflammatory macrophage and $CD8_+$ effector T cell infiltration and glucose intolerance under standard diet conditions, indicating that Id2 is critical for the survival and function of aTregs in order to maintain adipose-tissue homeostasis.

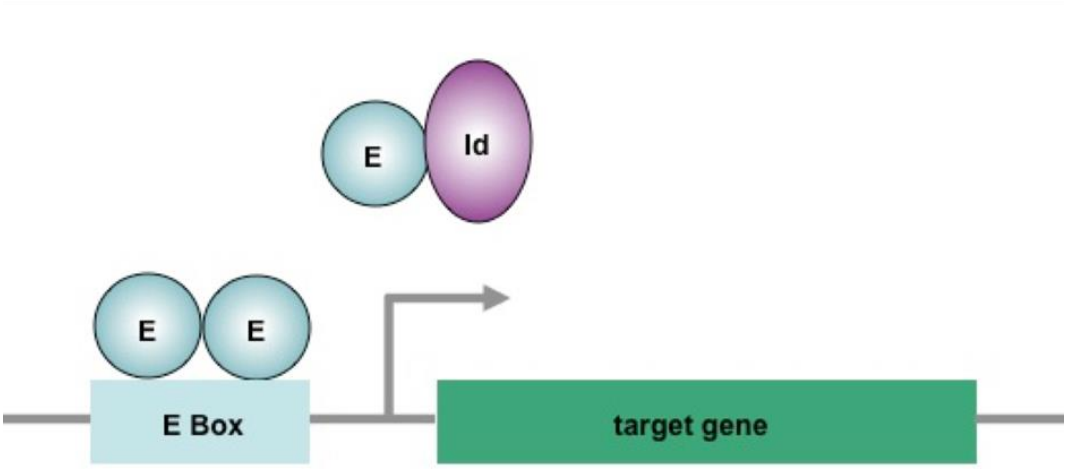

Figure 10. E and Id Proteins

E proteins are basic helix-loop-helix proteins that can homodimerize with each other or heterodimerize with other E proteins or Id (Inhibitor of DNA binding) proteins. E protein dimerization allows them to bind to DNA box sites whose canonical binding site is CANNTG. E protein activity can be both positive and negative regulation. Id proteins dimerize with E proteins and negatively regulate their activity as Id proteins do not contain the DNA binding domain.

3.3 Materials and Methods

3.3.1 Mice

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The Foxp3YFPiCre mice were obtained from Jackson Laboratories [121]. The Id2 floxed mice were a gift from Anna Lasorella (Columbia University), the Id3 floxed mice were a gift from Yuan Zhuang (Duke University), Id2-YFP mice from Ananda Goldrath (UC San Diego), and Id3-GFP mice from Cornelis Murre (UC San Diego). Mice were bred and housed in specific pathogen-free conditions in

accordance with the Institutional Animal Care and Use Guidelines of the University of Pittsburgh.

Mixed bone marrow chimeras were generated by transferring either 5×10^6 B220$^-$CD3$^-$NK1.1$^-$ bone marrow cells from a CD45.2 Foxp3YFPiCre$^+$ donor WT donor or 5×10^6 B220$^-$CD3$^-$NK1.1$^-$ bone marrow cells from a CD45.2 Id2$^{f/f}$ Foxp3YFPiCre$^+$ donor into lethally irradiated (1000 rads) CD45.1 recipient mice. All chimeras were rested at least 8 weeks to allow reconstitution of the host.

For the HFD studies, mice were fed a rodent diet of 60% kcal fat from Research Diets (D12451) for 12 weeks.

3.3.2 Flow cytometry and cytokine stimulation assay

Single-cell suspensions were prepared from thymus and spleen. Bone marrow was prepared by isolating lymphocytes from the femurs of mice and preparing a single-cell suspension. Gonadal adipose single-cell suspensions were prepared as previously described [110]. All antibodies were purchased from BD Biosciences and eBioscience (San Diego, CA, USA) unless otherwise specified. Samples were collected on a Cytek Aurora (Cytek Biosciences), FACS LSRII, FACS Fortessa or FACS Aria (BD Biosciences, San Jose, CA, USA) and were analyzed with Flowjo software (TreeStar, Ashland, OR, USA).

For intracellular cytokine staining we stimulated the cells with PMA (50ng/ml) (Sigma) and ionomycin (1nM) (Sigma) for 4 hours. We added protein transport inhibitor (eBiosciences) to the culture at the recommended concentration. Cells were surfaced stained, before fixing, permeabilizing and intracellular staining according to the manufacturer's instructions (eBioscience Foxp3 staining kit).

3.3.3 Quantitative PCR

Total RNA was isolated from spleen or adipose tissue single cell suspensions using the RNeasy Mini Purification Kit (Qiagen) and cDNA was obtained using Superscript First Strand cDNA synthesis kit (GeneCopoeia). qPCR was performed using Taqman probes for the indicated gene (Applied Biosystems). Target gene expression was normalized to housekeeping gene *HPRT*.

3.3.4 RNA sequencing and analysis

RNA was isolated from sorted Tregs ($CD4^+$, $CD25^+$, $CD127^-$) from the spleen or adipose tissue. Libraries were prepared using Ultra DNA library preparation kits. RNA sequencing analysis was performed on Illumina NextSeq500 using 500bp paired-end reads by Health Science Sequencing core facility at University of Pittsburgh. Raw sequence reads were trimmed of adapter sequences using CLC Genomics Suite. The trimmed reads were mapped onto the mouse genome and gene expression values (TPM; transcripts per million kilobase) were calculated using CLC Genomics Suite Workbench 11. Differential gene expression was analyzed using Partek Genomics Suite and graphs generated using Graphpad Prism with values normalized as follows: (TPM value for gene X in condition A)/ (mean TPM of gene X in all conditions for that sample). These data can be accessed through the Gen Omnibus Expression database (GEO). Accession number GSE131591. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131591

3.3.5 Determination of fasting blood glucose, insulin and glucose tolerance testing.

Mice were fasted overnight before fasting blood and insulin levels were measured. Blood glucose was measured using a handheld glucometer (Contour NEXT EZ, Bayer) and plasma insulin was measured by ELISA (Alpco). For the glucose tolerance tests (GTTs), we administered glucose (2g/kg of body weight) by intraperitoneal injection after an overnight fast. We measured changes in plasma insulin at 15, 30 and 120 minutes and blood glucose at 15, 30, 45, 60 and 120 minutes after glucose injection.

3.3.6 Statistical analysis

All graphs were created using GraphPad Prism 7 and statistical significance was determined with the two-tailed unpaired Student's t-test or using one-way ANOVA adjusted for multiple comparisons where appropriate.

3.4 Results

3.4.1 Id2 and Id3 are differentially expressed in aTregs.

We first determined the expression of Id2 and Id3 mRNA in aTregs by sorting $CD4^+$ $CD25^+$ Tregs from the spleen and visceral adipose tissue (VAT) of 25-week-old male C57BL/6 mice. We found that Id2 mRNA was expressed at ~4-fold higher in aTregs relative to splenic Tregs (Figure 11A). In contrast Id3 mRNA expression appeared approximately equal in splenic and aTregs (Figure 11A). To confirm these findings, we used Id2-YFP and Id3-GFP reporter mice. In agreement with our qPCR data, we

observed high Id2-YFP expression in aTregs compared to their splenic counterparts (Figure 11B). We also found low Id3-GFP expression in aTregs and high expression in splenic Tregs (Figure 11B). These data are consistent with recently published data showing that Id3 expression is high in splenic Tregs and reduced in tissue Tregs [114]. Additionally, gating on Id2-YFP^{+} and Id2-YFP^{-} Tregs in the spleen, we noted higher KLRG1 expression in the Id2-YFP^{+} Tregs and slightly elevated ST2 expression, while the converse was true of Id3-GFP^{+} versus Id3-GFP^{-} Tregs (Figure 11C). KLRG1 is a marker associated with effector Tregs, while ST2, the IL-33 receptor, is upregulated after Tregs migrate to peripheral tissue [80]. Therefore, these data support a model whereby loss of Id3 and concurrent upregulation of Id2 marks effector-like Tregs that seed peripheral tissue, including the VAT [80-114]. We concluded from these data that Id2 is preferentially expressed by aTregs, while Id3 is expressed by lymphoid tissue resident Tregs.

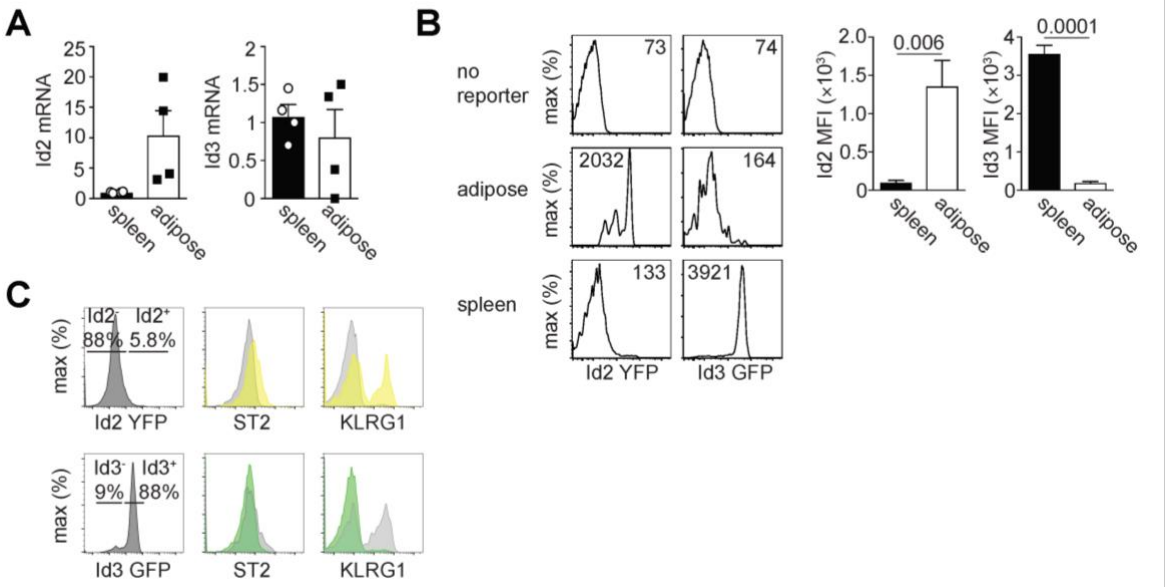

Figure 11. Id2 and Id3 expression in aTregs

(A) qPCR showing Id2 and Id3 mRNA expression in sorted CD4+ CD25+ Tregs isolated from the spleen or visceral adipose tissue. Id2 or Id3 expression was normalized to HPRT. Each dot indicates cells isolated from one animal. (B) Flow cytometry histograms showing Id2-YFP or ID3-GFP expression in gated CD4+ CD25+ Tregs isolated from the spleen or visceral adipose tissue. B6 mice were used as a 'no reporter' control. Numbers in histograms indicate the median fluorescence intensity (MFI). Bar graphs indicate the average MFI in Tregs isolated from the indicated tissue. (C) Histograms indicating the frequency of Id2+ or Id3+ Tregs identified in the spleen (left) and their expression by flow cytometry of ST2 and KLRG1 (right). Yellow peaks indicate Id2-YFP+ cells and green peaks indicate Id3-GFP+ cells. Grey peaks indicate Id2-YFP- or Id3-GFP- cells, respectively. Data are representative of two independent experiments with 1-3 mice per group. P values were calculated using the student's t-test.

3.4.2 Id2 is critical for maintenance of aTregs.

To determine the specific function of Id2 in Tregs, we crossed Id2 floxed mice to the Foxp3-YFP-iCre line [121] to generate mice in which Id2 was conditionally deleted in Tregs. We called these mice Id2 CKO (conditional knock out) and compared them to Foxp3-YFP-iCre only littermate controls (Ctrl, WT). We first examined the frequency of

Tregs in adipose tissue, lymph nodes (LNs) and spleen in the absence of Id2 expression. Gating on total $CD4_+$ T cells, we observed that Treg-specific loss of Id2 expression resulted in a significant reduction of $Foxp3_+$ $CD25_+$ aTregs but not Tregs in the LNs and spleen (Figure 12A). The frequency of Tregs in the thymus was not affected by the absence of Id2 (data not shown).

We next assessed the phenotype of the aTreg, focusing on the markers CCR2, ST2 and KLRG1, comparing the aTreg in WT and Id2-deficient mice. Expression of CCR2, ST2 and KLRG1 were all significantly reduced in the Id2-deficient aTregs relative to the WT (Figure 12B). We also determined expression of GATA3, a transcription factor associated with tissue-resident Tregs. Notably, GATA3 expression was reduced in Id2-deficient aTregs relative to WT Tregs (Figure 12C). We also examined expression of Foxp3 in these cells and observed no difference in Foxp3 expression in the absence of Id2 in aTregs (Figure 23). Thus, these data show that Id2 expression is required for the maintenance of the aTregs population and for differentiation of the aTreg phenotype.

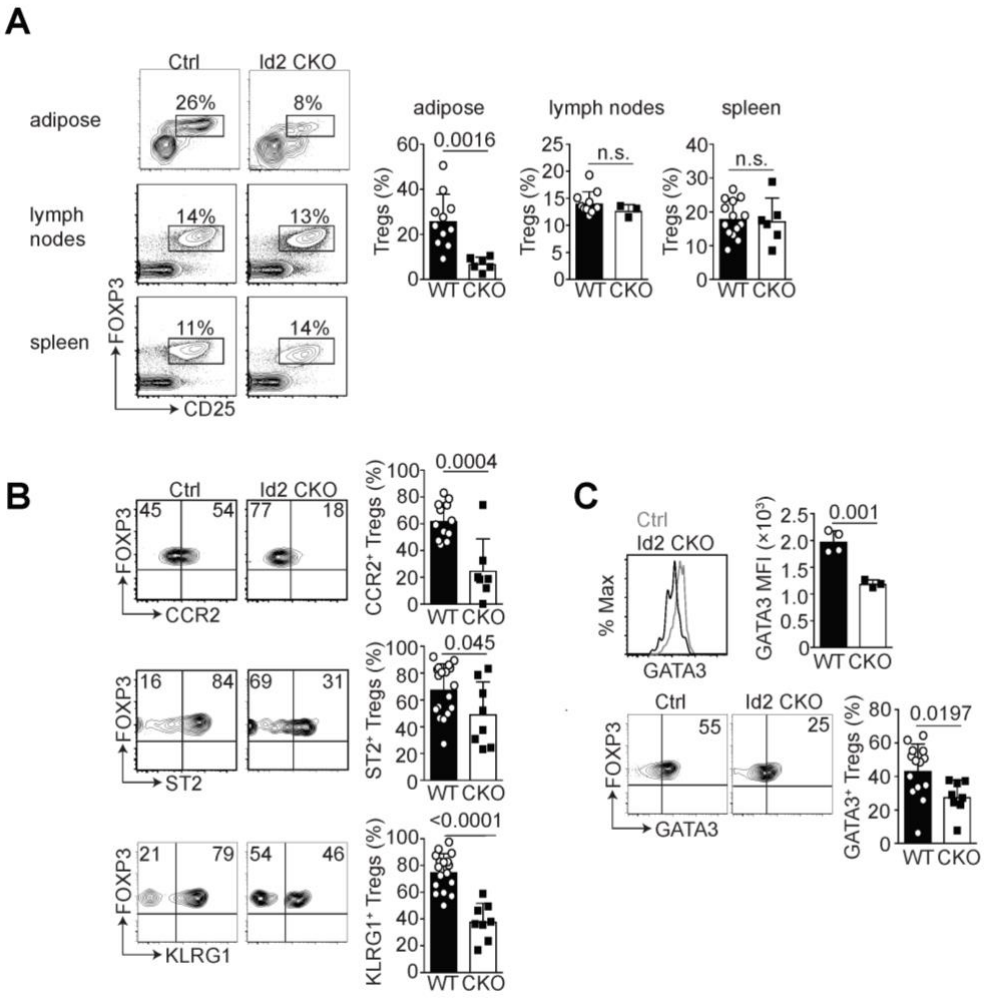

Figure 12. Id2-deficiency results in reduced frequency of aTregs

(A) Flow cytometry plots showing the frequency of Foxp3+ CD25+ gated CD4+ T cells from the indicated tissue in Control (Ctrl) and Id2-deficient (Id2 CKO) mice. Bar graphs indicate the frequency of Foxp3+ CD25+ Tregs from the adipose, lymph nodes or spleen. Each dot indicates one animal. (B) Flow cytometry plots showing CCR2, ST2 and KLRG1 expression on gated Foxp3+ CD25+ CD4+ T cells in the adipose tissue in Ctrl or Id2 CKO mice. Bar graphs indicate the frequency of CCR2+, ST2+ and KLRG1+ aTregs from wildtype (WT) and Id2 CKO (CKO) mice. (C) Histogram, flow cytometry plots and bar graphs indicating the median fluorescence intensity (MFI) and frequency of GATA3 expression in gated aTregs from WT and Id2 CKO mice. Data are representative of at least four independent experiments with 1-5 mice per group. P values were calculated using the student's t-test.

3.4.3 Id2 expression is required for aTreg survival.

As we observed a substantial reduction in the frequency of the aTreg population in the absence of Id2 (Figure 12A), we next wanted to determine the cause of this decrease in aTregs. To address this, we performed transcriptomic analysis of Tregs sorted from the spleen or visceral adipose tissue of >15-week old WT or Id2 CKO male mice. Genes previously reported to be associated with aTregs were substantially upregulated in our sorted aTreg dataset compared to splenic Treg, indicating our gene expression analysis was robust (data not shown). Closer inspection revealed that a number of genes associated with cell death and survival, transcription and metabolism were up- or downregulated in the Id2 CKO aTregs, relative to splenic Tregs (Figure 13A). In particular, we were intrigued by the upregulation of Fas in the Id2-deficient aTregs as these data suggested that Id2-deficient aTregs may be more prone to cell death.

To confirm that Id2 was required for the survival of a Tregs, we used a fixable viability ‘zombie’ dye and noted an increase in dead aTregs in the absence of Id2 relative to WT aTregs (Figure 13B). Moreover, a greater frequency of Id2-deficient aTregs stained positive for AnnexinV and 7AAD compared to their WT counterparts (Figure 13C). When we tested aTregs for expression of Fas, we observed Id2-deficient aTregs express more Fas relative to their WT counterparts (Figure 13D), which was consistent with our gene expression analysis. We thus concluded that one mechanism by which Id2 regulates aTregs is by promoting their survival in VAT.

Our RNA-seq data revealed that Id3 expression was increased in aTregs in the absence of Id2 (Figure 12A). To test whether Id3 plays a functional role in aTregs, we isolated aTregs from WT and Treg-specific Id3-deficient mice. We observed a slight

increase in the frequency of aTregs in the absence of Id3 (Figure 24A) but this increase did not affect the phenotype or function of the aTregs (Figure 24B, 25C). We thus concluded that Id2 expression by aTregs was critical for their survival, an effect that could not be compensated for by Id3 expression.

From our gene expression analysis, we had also noted the decreased expression of *Hif1*α in the Id2-deficient aTregs (Figure 13A). Hif1α (Hypoxia Inducible Factor 1α) is critical for cell survival under low oxygen conditions. Relative to splenic Tregs, we observed that more aTregs express Hif1α (>50%) (Figure 25). However, in agreement with our gene expression data, Id2-deficiency resulted in reduced Hif1α protein expression on aTregs. Taken together, our data show that Id2 is critical for expression of proteins required for the survival of aTregs.

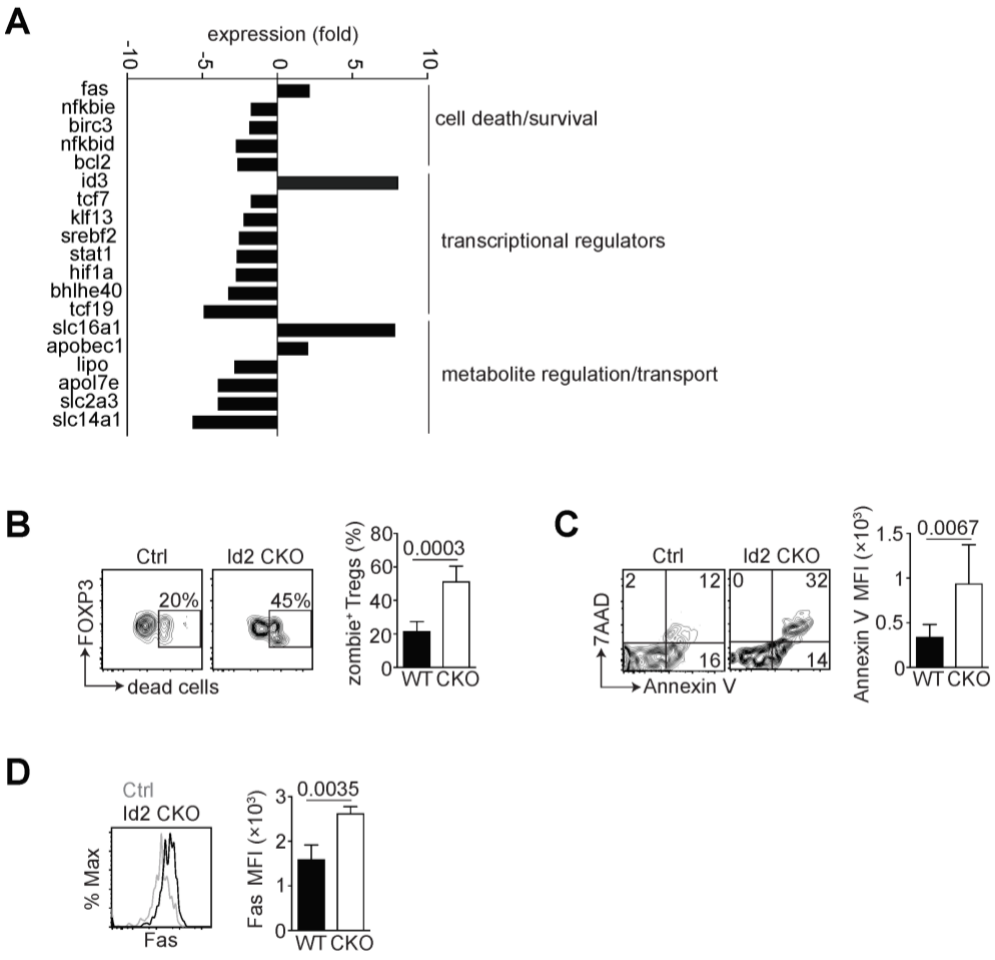

Figure 13. Loss of Id2 decreases survival of aTregs

WT and Id2 CKO aTregs were sorted from the spleen or adipose tissue of 15-20-week-old male mice for RNA-sequencing. (A) Bar graphs indicating expression of transcripts involved in various pathways (indicated) in Id2 CKO aTregs relative to WT aTregs. (B) Flow cytometry plots and bar graph from Ctrl and Id2 CKO mice showing the frequency of viability dye (zombie)+ gated CD4+ Foxp3+ aTregs. (C) Flow cytometry plots and bar graph indicating the Annexin V frequency and MFI on WT vs CKO aTregs. (D) Histogram and bar graph indicating Fas MFI in WT vs CKO aTregs. Data are representative of two independent experiments with 1-3 mice per group. P values were calculate using the student's t test.

3.4.4 Id2 expression is required for aTreg cytokine production.

Previous data have shown that aTregs are potent IL-10 producers [57-82] and it is likely this IL-10 contributes to an anti-inflammatory environment in non-obese adipose tissue. Additionally, Il-13 production by Tregs was recently shown to support macrophage IL-10 secretion and efferocytosis to promote resolution of inflammation [122]. Stimulation of aTregs *in vitro* revealed reduced production of IL-10 and IL-13 by the Id2-deficient aTregs (Figure 14A and 14B). Previous reports indicate that Tregs can produce little to no IFN-γ [123] and we could not detect a difference in the limited IFN-γ production by aTregs from WT versus Id2 CKO mice (Figure 14C). Overall, our data suggest that Id2 is required for optimal reduced anti-inflammatory cytokine production by aTregs.

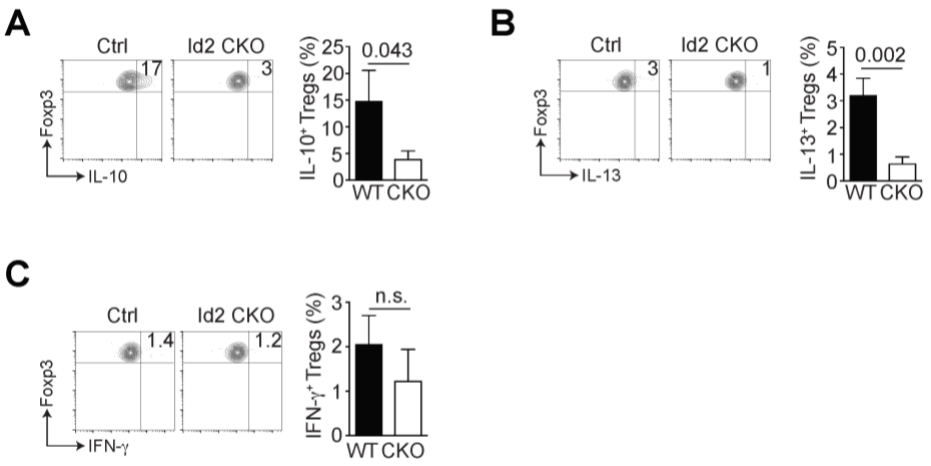

Figure 14. Cytokine production by Id2-deficient aTregs

The total stromal vascular fraction (SVF) isolated from the adipose tissue of WT or Id2 CKO mice was cultured with PMA and ionomycin for 4 hours and cytokine production by gated $CD4_+$ $Foxp3_+$ aTregs analyzed by flow cytometry. (A) Flow cytometry plots and bar graph indicating IL-10 expression by gated $CD4_+$ $Foxp3_+$ WT vs CKO aTregs. (B) Flow cytometry plots and bar graph indicating IL-13 expression by gated $CD4_+$ $Foxp3_+$ WT vs CKO aTregs. (C) Flow cytometry plots and bar graph indicating IFN-γ expression by gated $CD4_+$ $Foxp3_+$ WT vs CKO aTregs. Data are representative of two independent experiments with 2-3 mice per group. P values were calculate using the student's t test.

3.4.5 Loss of Id2 in aTregs results in reduced aTreg frequency under standard or high fat diet conditions.

It has previously been reported that under high fat diet (HFD) conditions, there is a loss of Tregs from the VAT [57-82-86]. To test this, and whether the requirement for Id2 in Tregs was cell-intrinsic, we generated bone marrow chimeras (BMC) in which we transferred congenically marked WT or Id2 CKO bone marrow into sublethally irradiated CD45.1 hosts. At four weeks post-reconstitution, we placed these chimeric mice on high fat diet (HFD) for 12 weeks, leaving the control groups on control diet (CD). As reported previously, there was a significant reduction in the frequency of aTregs in WT mice on

HFD relative to their CD counterparts (Figure 15A). Importantly, we observed that the reduction in aTregs in mice with loss of Id2 expression on CD was more severe than in WT mice on HFD (Figure 15A) [57]. Additionally, ST2, CCR2, KLRG1 and GATA3 were all reduced in the Id2-deficient aTregs, irrespective of diet (Figure 15A). We concluded that Treg-specific deficiency in Id2 resulted in loss of aTregs that was comparable with the frequency of aTregs in WT mice on HFD.

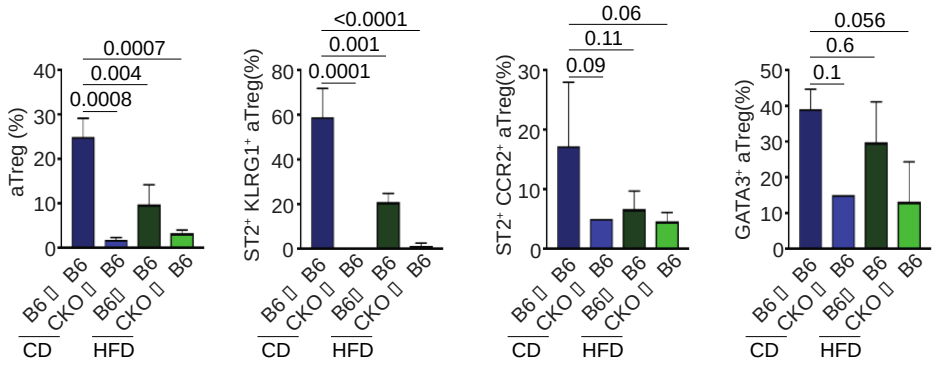

Figure 15. Id2-deficient aTregs under high-fat-diet

CD45.1 mice were irradiated and injected with bone marrow cells from WT or Id2 CKO mice. Four weeks after reconstitution, mice were placed on CD or HFD for 12 weeks. Bar graphs indicating the frequency of CD4+ Foxp3+ aTregs, ST2+ KLRG1+, ST2+ CCR2+ and GATA3+ gated aTregs in the indicated BMC under CD or HFD conditions. Data are representative of two independent experiments with 2-3 mice per group. P values were calculate using the student's t test or one-way ANOVA.

3.4.6 Id2-deficiency in aTregs results in perturbed systemic inflammation and metabolism.

Loss of aTregs due to genetic perturbations or HFD can also affect other adipose-resident immune cells [57-82-86]. We tested how loss of Id2 in aTregs affected other adipose-resident cells and found a significant increase in F4/80+ macrophages in the adipose tissue of Id2 CKO mice (Figure 16A). Moreover, the frequency of CD11c+ F4/80+ M1 macrophages was substantially increased in the absence of Id2, although the CD206+ M2 macrophages remained unchanged (Figure 16A). When we cultured the total stromal vascular fraction (SVF) from the adipose tissue with PMA and ionomycin, we noted that IL-10 production by the F4/80+ macrophages was substantially reduced in the Id2 CKO cohort (Figure 16A). Additionally, we observed an increased frequency of CD8+ effector T cells in the absence of Treg-specific Id2 (Figure 16A). Taken together, these data show

that there is an increase in pro-inflammatory macrophages and CD8+ effector T cells in the absence of Id2+ aTregs.

To determine how Id2-mediated aTreg loss affected glucose metabolism and insulin action in these animals, we fasted CD WT and Id2 CKO mice overnight and measured their fasting blood glucose and plasma insulin levels. Although weight, fasting glucose and insulin levels were unaffected by Treg-specific loss of Id2, these mice displayed impaired glucose tolerance during the GTT (Figure 16B). Plasma insulin levels were also slightly elevated in the glucose-challenged Id2 CKO mice over time, relative to the WT mice (Fig. 16B). Additionally, mRNA analysis revealed that inflammatory mediators such as *Tnf*α, *Il6* and *A20* (*Tnfaip*3) were all increased in total adipose tissue relative to spleen in the Id2-deficient animals (Fig. 16C). Together our data show that loss of Id2 in Tregs in mice on CD results in increased inflammation, increased inflammatory immune cells and metabolic perturbation comparable with WT mice on HFD.

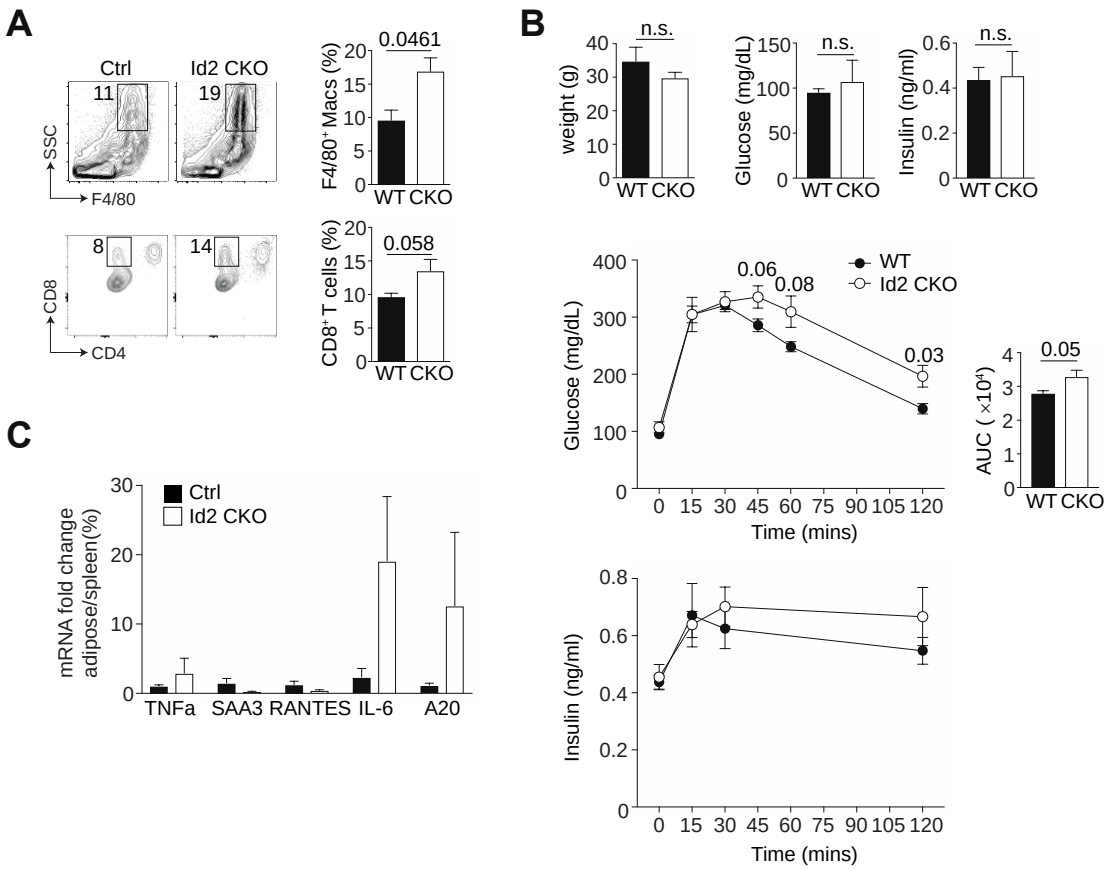

Figure 16. Physiological function of Id2+ aTregs

(A) Flow cytometry and bar graphs indicating the frequency of total macrophages (F4/80+), M1 (CD11c+ F4/80+), M2 (CD206+ F4/80+), IL-10+ F4/80+ macrophages and CD8+ T cells isolated from the adipose tissue of WT or Id2 CKO mice on CD. (B) Graphs indicating weight, fasting glucose, fasting insulin and blood glucose and insulin over time following GTT. Calculated area under the curve (AUC) from all mice tested by GTT. (C) Graph indicating the expression of TNFα, SAA3, RANTES, IL-6 and A20 mRNA fold change in total adipose tissue SVF versus spleen. Data are representative of two independent experiments with 2-3 mice per group (A and C) and one experiment with 6 mice per group (B). P values were calculate using the student's t test or one-way ANOVA.

3.5 Discussion

The role of the immune system in influencing systemic chronic inflammation, insulin sensitivity and glucose tolerance associated with obesity and adipose tissue has recently been elucidated. We now know that increasing adiposity resulting from increased caloric intake results in reduction in immune cells associated with anti-inflammation such as Th2 cells, M2 macrophages, iNKT cells, ILC2s and aTregs and a corresponding increase in inflammatory cells such as M1 macrophages, Th1 cells and cytotoxic $CD8_+$ T cells [124]. In our work here, we explore the transcriptional requirements of aTregs and show the transcriptional regulator Id2 is critical for their survival and function.

In other immune cell contexts, it was previously shown that Id2 is critical for cell survival. In the $CD8_+$ T cell immune response to infection, Id2 is substantially upregulated during the effector response and loss of Id2 resulted in loss of responding effector T cells due to unrestricted E protein activity [125-126]. Similarly, in invariant Natural Killer T (iNKT) cells, Id2 was critical for survival of hepatic iNKT cells, as loss of Id2 resulted in loss of Bcl-2 expression and increased cell death of these cells [127]. In peripheral Tregs, recent gene expression analysis revealed that loss of E proteins resulted in upregulation of proteins associated with proliferation and survival such as Bcl-2, Ki-67 and Bcl-11b [76]. Here we showed that Id2 is a critical factor for the survival of aTregs and that loss of Id2 results in upregulation of the pro-apoptotic protein Fas. Notably, we did not see upregulation of Bim expression in the Id2-deficient aTregs (data not shown). However, we did observe that Id3 was substantially upregulated in the absence of Id2, suggesting that Id3 upregulation can compensate for some Id2 loss and negatively regulate E protein transcriptional activity at certain genomic sites.

One interesting chemokine receptor associated with aTregs is CCR2 (the receptor for CCL2) which is predominantly expressed on macrophages and monocytes, but is also found on activated T cells [128]. With loss of Id2 in aTregs, we observed reduced CCR2 expression, leading to concerns that migration of aTregs from the spleen to the adipose tissue affected their accumulation in the adipose tissue. However, loss of CCR2 on T cells resulted in increased accumulation and function of Tregs in the spleen and colon [128], suggesting that reduced CCR2 expression in the absence of Id2 should not affect migration of aTregs.

Regarding the function of Id2-deficient aTregs, we observed increased localized adipose tissue inflammation, increased inflammatory macrophage and $CD8^+$ T cell infiltration and impaired glucose tolerance after fasting in the Id2 Treg-deficient animals relative to WT controls. Additionally, we observed reduced IL-10 and IL-13 production by the Id2-deficient aTregs upon stimulation *in vitro*. IL-10 has been shown to be positively regulated by E proteins in conventional T cells [129]. However, in peripheral tissue Tregs it was recently reported that IL-10 production by tissue Tregs is likely negatively regulated by E2A and HEB [76], suggesting that E protein activity in T cells is context-dependent. With loss of Id2 and unchecked E protein activity, we see reduced IL-10 production, suggesting that 1) IL-10 is negatively regulated by E2A and HEB in aTregs and 2) loss of Id2 in aTregs reduces their ability to suppress local and systemic inflammation, resulting in perturbed glucose tolerance.

We noted that Hif1α, responsible for survival under hypoxic conditions, is highly expressed in aTregs and reduced in the absence of Id2. Hif1α has been reported to be as upregulated in adipocytes under HFD conditions [130-131], presumably to influence

survival under increasing hypoxic conditions which occurs when the increased adipose tissue isn't as vascularized as before. Its expression is also associated with induction of Foxp3, increasing abundance and function of Tregs [132]. It is possible that loss of Hif1α in the absence of Id2 in aTregs helps to contribute to their increased cell death and loss of functionality in adipose tissue, although Treg-specific loss of Hif1α would help to definitively show this.

As Id2 expression is low in Tregs in the spleen and LNs, it is interesting to speculate on the factors that control Id2 upregulation. Previous studies in Tregs and effector CD8+ T cells have shown that TCR stimulation and common γ chain cytokines such as IL-2 can upregulate Id2 expression *in vitro* [119-133]. Additionally, aTreg differentiation and proliferation is thought to be driven by IL-33 [82-83]. We acknowledge that a more thorough investigation of the factors regulating Id2 expression in Tregs is warranted but speculate that it is likely driven by TCR stimulation and adipose tissue specific cytokines.

In summary, here we provide evidence of a new role for the transcriptional regulator Id2 in the homeostasis, differentiation, survival and function of aTregs. Treg-specific loss of Id2 did not impact Tregs in the secondary lymphoid tissues but led to dramatic loss of these cells in adipose tissue. We showed that Id2 expression was critical for the survival of aTregs and that loss of these cells resulted in increased local and systemic inflammation, increased immune infiltration into adipose tissue and impaired glucose tolerance.

4.0 Summary and Future Directions

4.1 MicroRNA-155 deficiency skews iNKT cells towards the NKT1 subtype

In our studies on mice lacking miR-155, iNKT cell development was skewed significantly towards an NKT1 subtype in the thymus while showing little change in the periphery. We also examined iNKT cells at steady-state and under α-GalCer stimulation. Importantly, we found that miR-155 KO did not impact cytokine expression of iNKT cells. Thus, it may be worth testing α-GalCer treatment combined with therapies that target miR-155 to reduce inflammatory cytokines from cells dependent on miR-155, while maintaining iNKT cell function.

Moving forward, it may be necessary to use disease models to test the capabilities of miR-155 deficient iNKT cells, since without any additional stress the iNKT cell subsets in the periphery reached an equilibrium similar to WT controls. Future studies should focus on examining models of disease dependent on NKT2 or NKT17 subsets, such as helminth infection or mouse experimental arthritis model, respectively, to assess whether this increase of NKT1 cells in thymus impacts performance of iNKT cells in the periphery. Due to the minor phenotype we observed, we did not continue our studies with more specific tools such as cre-expressing mice under the iNKT cell master transcription factor PLZF crossed to miR-155 floxed mice. Even this intervention may not be enough to witness a change in the iNKT phenotype since the miR-155 global knockout did not show much change.

Studies show that multiple miRNAs can have redundant regulatory effects on the translation of transcripts, while individual miRNAs only mediate a fraction of the regulatory effect [31]. In a similar scenario, it may be that miR-155 works in collaboration with other regulatory signals, including other miRNAs, to apply greater regulation over target transcripts.

4.2 Upregulation of Id2 in Tregs

Our work has found that expression of Id2 is necessary for the differentiation, survival of aTregs, and overall homeostasis in the VAT (Figures 17 and 18). While Id2 is highly expressed in aTregs, it still remains a mystery as to precisely when Id2 is upregulated. We showed that Id2 is expressed in a small population of splenic Tregs, indicating that this population of Tregs expressing Id2 may eventually seed the VAT. In our RNAseq gene analysis, there weren't any significant differences in expression of PPARγ in Id2 CKO aTregs compared to WT controls, providing evidence that PPARγ regulation is independent of Id2. Currently, we are attempting to stain for PPARγ by flow cytometry to determine whether the Id2+ splenic Treg population is the same population of Tregs described to express low PPARγ and seed the VAT.

4.3 Id2 regulation of gene expression in Tregs

We are also interested in determining whether Id2 primes peripheral Tregs for entry into the VAT. Recently, we performed ATAC-seq on Id2-deficient splenic Tregs to obtain clues on what genes regions are open prior to entering the VAT, although thus far we have only validated WT and Id2 CKO samples and will need to repeat the experiment (Figure 19). From this ATAC-sec gene analysis, we expect to obtain clues about genes down- or up-regulated in splenic Tregs important for entry into the VAT. We expect Id2-deficient splenic Tregs to have increased chromatin accessibility around E box sites where targets of Id2, the E proteins, bind DNA.

Through qPCR and flow cytometry we have shown that Id2 is highly expressed in aTregs. This brings up two questions, 1) is Id2 expression downregulated in aTregs under HFD conditions, and 2) is Id2 expression required to maintain the aTreg population? In our Id2 CKO mice, aTregs express a similar phenotype to their WT HFD counterparts, which have a reduction in the frequency of aTregs along with other canonical markers. From our RNA-seq data, we know that Id3 expression is upregulated in our Id2 CKO mice, but it is unknown if the differential expression of E proteins is important in HFD. The second question arises because it seems that Id2 deficiency results in the impaired differentiation of Tregs that can survive in the VAT. Another questions that arises is: Once the aTregs differentiate, is Id2 necessary to maintain the aTreg unique transcription profile?

4.4 MCT1 is upregulated in Id2 deficient Adipose-resident Tregs

In our RNA-seq gene expression analysis, Id3 was upregulated in Id2 CKO aTregs, as reported previously. We also observed the gene *Slc16a1* was upregulated (Figure 13A). This gene encodes the protein monocarboxylate transporter 1 (MCT1) which is a proton-coupled transporter of monocarboxylates, with a higher affinity for pyruvate import.

We are currently crossing MCT1 floxed mice to the Id2 CKO mouse line in order to obtain compound mutants. Although we see an increase in cell death in aTregs from Id2 CKO mice, the sharp increase in MCT1 expression we observed in our RNA-seq gene analysis suggests that increased import or export of monocarboxylates, such as lactate or pyruvate, could be playing a detrimental role in aTregs. A pilot experiment using MCT1 CKO mice revealed increased frequency of Tregs in the VAT compared to WT controls, but no changes in Treg frequency in the spleen (Figure 20). In addition, when we examined the MCT1 CKO aTregs, they expressed elevated levels of aTregs markers (Figure 21). Upon generation of the MCT1/Id2 compound mutant mice, we will examine whether the upregulation of MCT1 plays a role in the phenotype we see in Id2 CKO aTregs.

4.5 Concluding remarks on Id2 expression in aTregs

Taken together, our studies reveal an important role for Id2 expression in adipose-resident Tregs. Ultimately, we want to know what signals leads to the upregulation of Id2 in aTregs whether it is programmed into Tregs destined for the VAT, dependent on a

Treg's TCR, determined later on in the periphery, or even a combination. Upon entry into the VAT, the expression of Id2 in aTregs is elevated. Thus, there may be a signal of VAT-origin that first primes Tregs in the periphery to induce low expression of Id2, which is further increased upon Treg entry into the VAT.

In line with previous studies, the remaining aTregs in our Id2 CKO mice displayed elevated Id3 in our RNAseq gene analysis. What would happen then if Id3 expression was precluded as well? Miyazaki et al. showed that Id2/Id3 double CKO Tregs had a reduction in accumulation in the lungs [119], but how would this apply to other tissues such as the VAT? Currently, we are working to generate mouse models where we can control the expression of Id3 to tease out its role in Tregs.

In future studies, it will be important to figure out what genes are regulated by Id2 to determine the genes that restrain the aTreg gene expression profile. In our studies, the frequency of aTregs is severely diminished, but not completely ablated, while the remaining Tregs display decreased expression of many known adipose-resident Treg markers. Thus, Id2 must provide critical signaling supporting the aTreg gene expression profile, including genes important for cell survival. However, microarray from data from Kolodin et al. show that Id2 transcript in aTregs is impacted by HFD [78], and thus responsive to changes in metabolic cues whether directly or indirectly.

It is important to point out is that Id2 is not specific to aTregs or adipose tissue. A recent Treg study included Id2 as part of a nonlymphoid identity of markers[134], where an increase in the expression Id2, is associated with tissue residence of a Treg. This raises other questions such as whether Id2 performs a similar or different role in other tissue

Tregs? Understanding the regulation of Id2 expression and its role in tissue Tregs will enhance our ability to modulate Tregs for therapeutics.

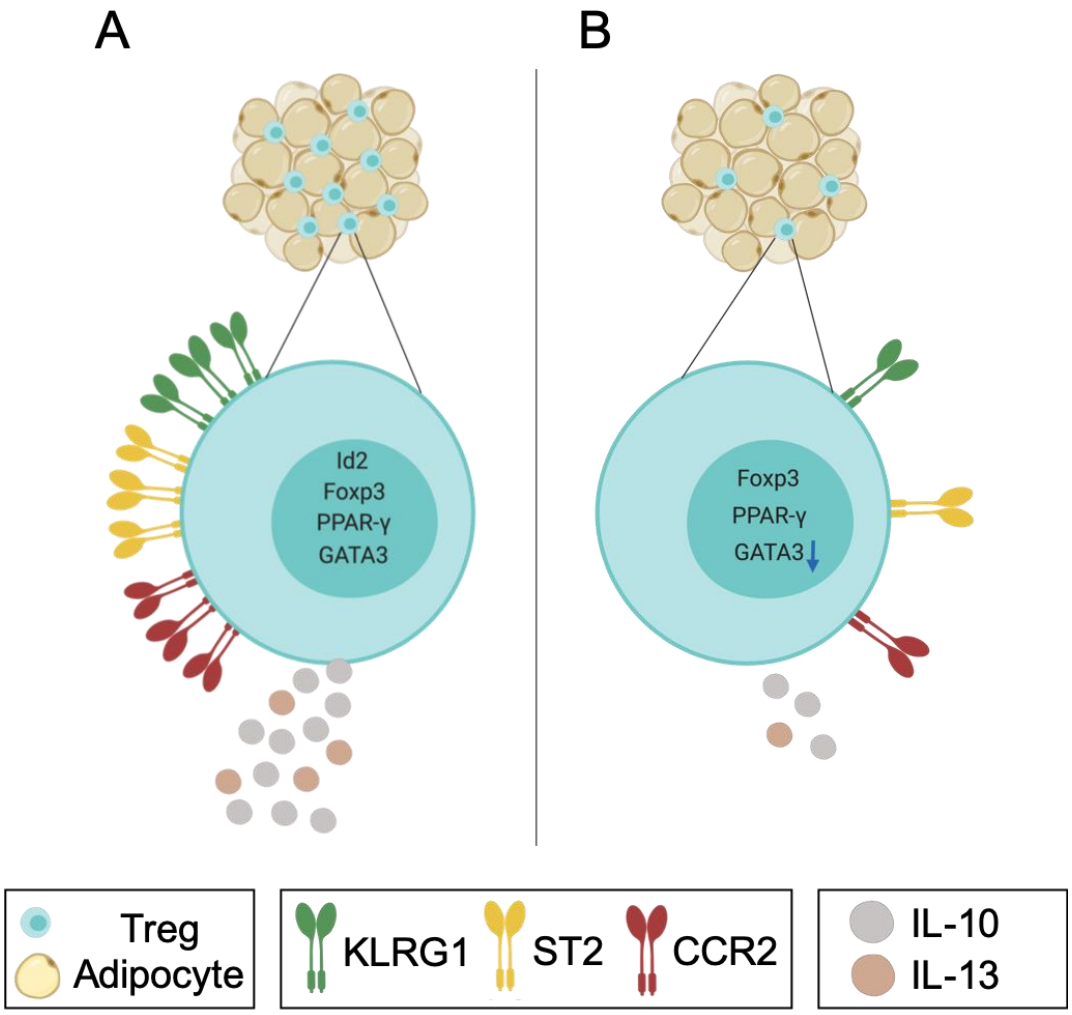

Figure 17. Summary of adipose resident Tregs in WT versus Id2 conditional knockout mice

(A) Lean visceral adipose tissue (VAT) is an immune rich region where Tregs make up a large portion of the CD4+ population. These adipose-resident Tregs (aTregs) are not only dependent on the canonical Treg transcription factor Foxp3 but also on the adipose transcription factor PPAR-γ. These aTregs also express KLRG1 (green) - a marker of antigen experience, ST2 (yellow) -the receptor for the alarmin IL-33 , and CCR2 (red) - the chemokine receptor for CXCL2. When stimulated *in vitro* with PMA/ION, these aTregs express the anti-inflammatory cytokine IL-10 as well as the type-2 immunity cytokine IL-13. (B) In mice deficient for Id2 in cells expressing Foxp3 (Id2CKO), there is a reduction in the frequency of aTregs. In these same Tregs there is a reduction in the expression of KLRG1 (green), ST2 (yellow), and CCR2 (red). Upon stimulation with PMA/ION, these Id2CKO aTregs have a reduction in the expression of IL-10 and IL-13.

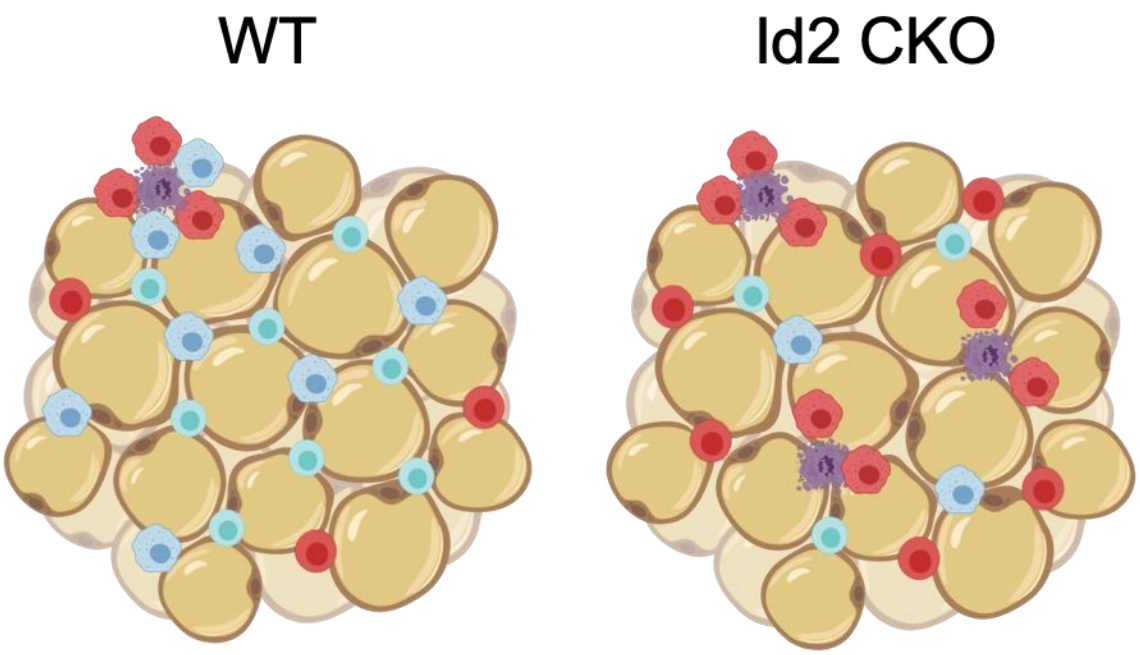

Figure 18. Schematic of adipose tissue in WT and Id2 CKO mice

The lean adipose tissue environment is made up of a majority of anti-inflammatory cell populations including alternatively activated macrophages and regulatory T cells (Tregs). In Id2 CKO mice, there is a reduction in the frequency of aTregs and increase infiltration of CD8+ T cells and macrophages similarly to what is seen in high-fat diet.

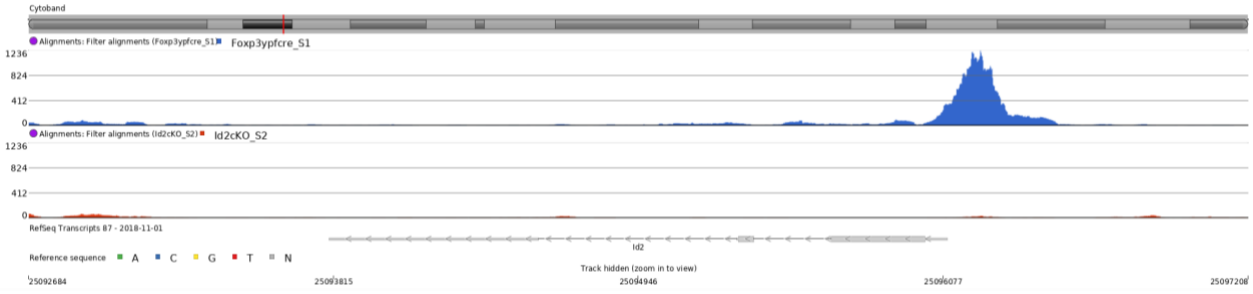

Figure 19. Analysis of Id2 from ATAC-seq data in WT and Id2 CKO splenic Tregs

WT and Id2 CKO spleens were enriched for CD4+ cells. Tregs were then sorted into TC (T cell) Media by gating on single-live CD45+ DumpGate- CD4+ and CD25+. Cells were also assessed for Foxp3 expressing through the Foxp3-YFPicre reporter.

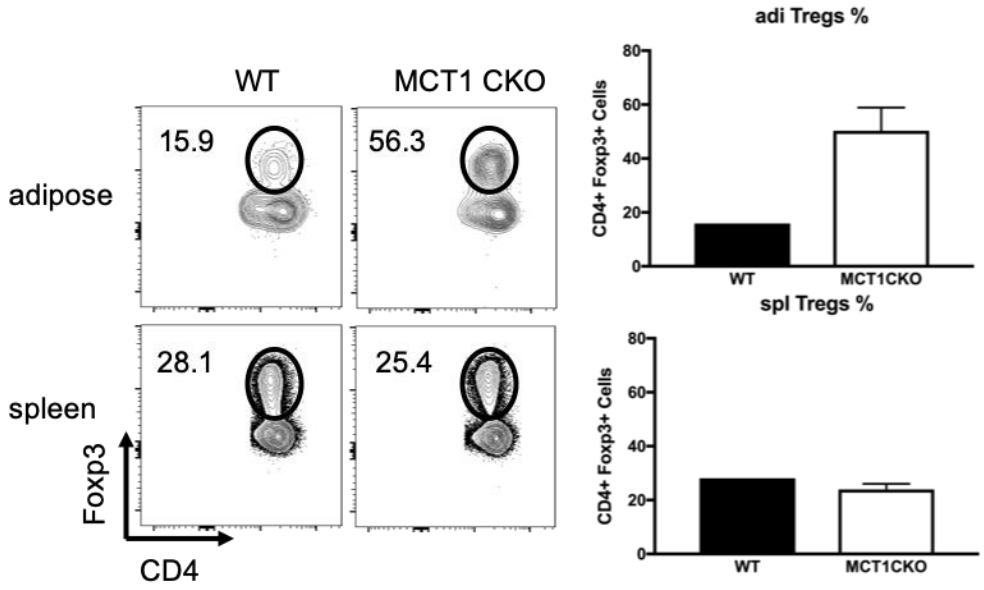

Figure 20. MCT1 deficiency in Tregs

30+ week old MCT1 CKO mice and one control were assessed for Tregs in the spleen and VAT. Cells are gated on single, live CD45+ CD4+ Foxp3+ cells.

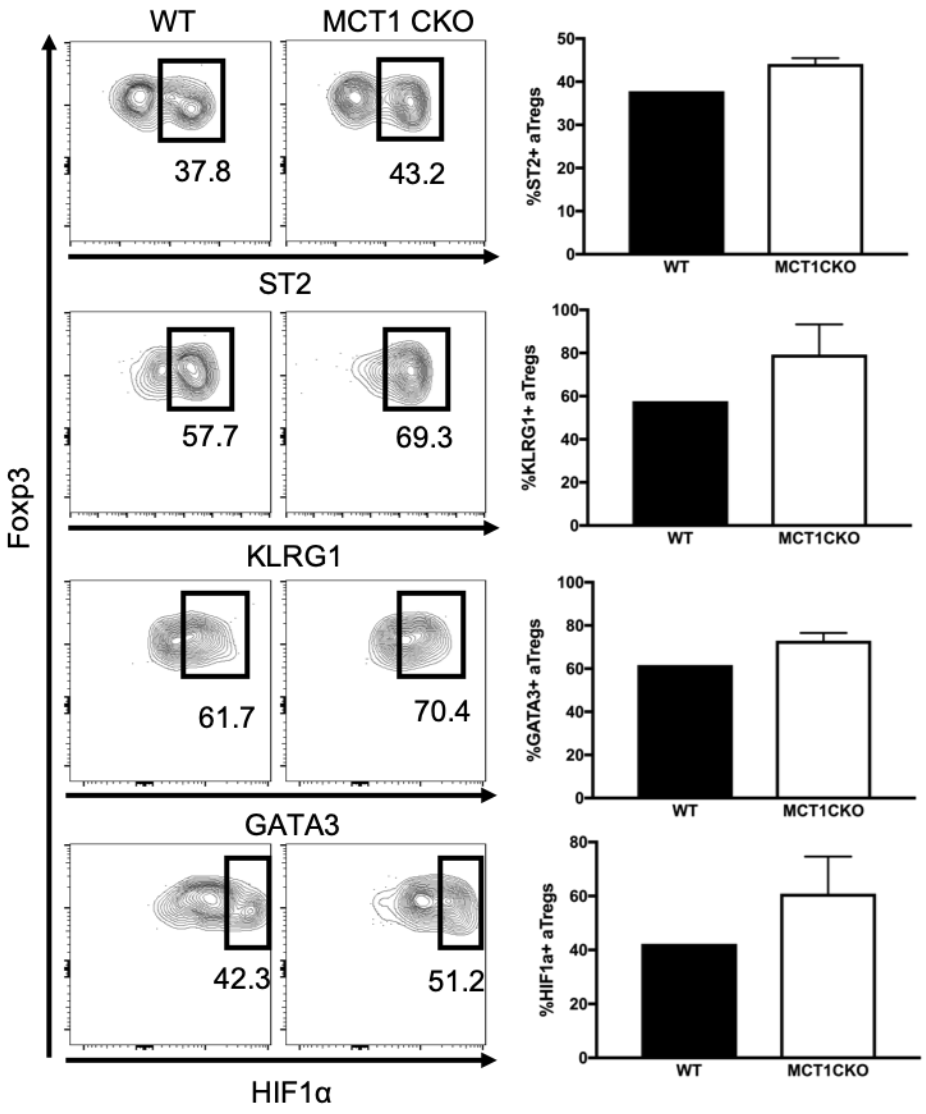

Figure 21. MCT1 deficiency in adipose-resident Tregs

CD4+ CD25+ aTregs in MCT1 CKO mice were asses for canonical markers: ST2, KLRG1, GATA3, and HIF1α.

Appendix A

Supplemental Figures

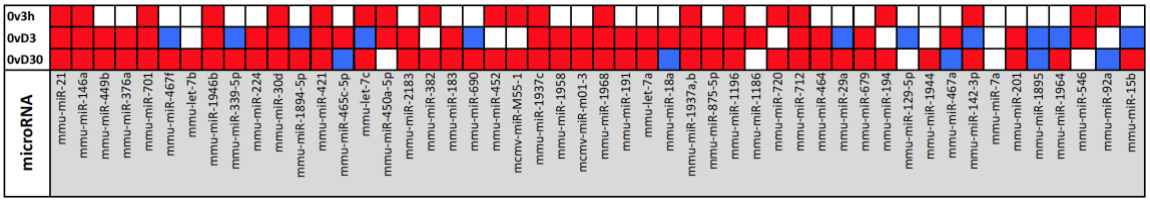

Figure 22. NanoString microRNA expression analysis

NanoString microRNA expression panel from splenic iNKT cells activated with α-GalCer at 3-hours, 3-days, and 30-days post-stimulation compared to naïve controls. Several miRNAs were significantly up- (red) or down-regulated (blue) in iNKT cells post α-GalCer stimulation.

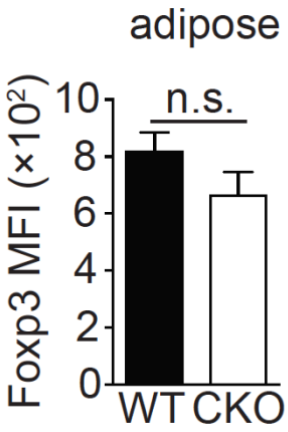

Figure 23. Foxp3 MFI is unchanged in Id2-deficient aTregs.

Bar graph indicating the median fluorescent intensity (MFI) of Foxp3 in WT or Id2 CKO Tregs isolated from the adipose tissue. Data are representative of three independent experiments.

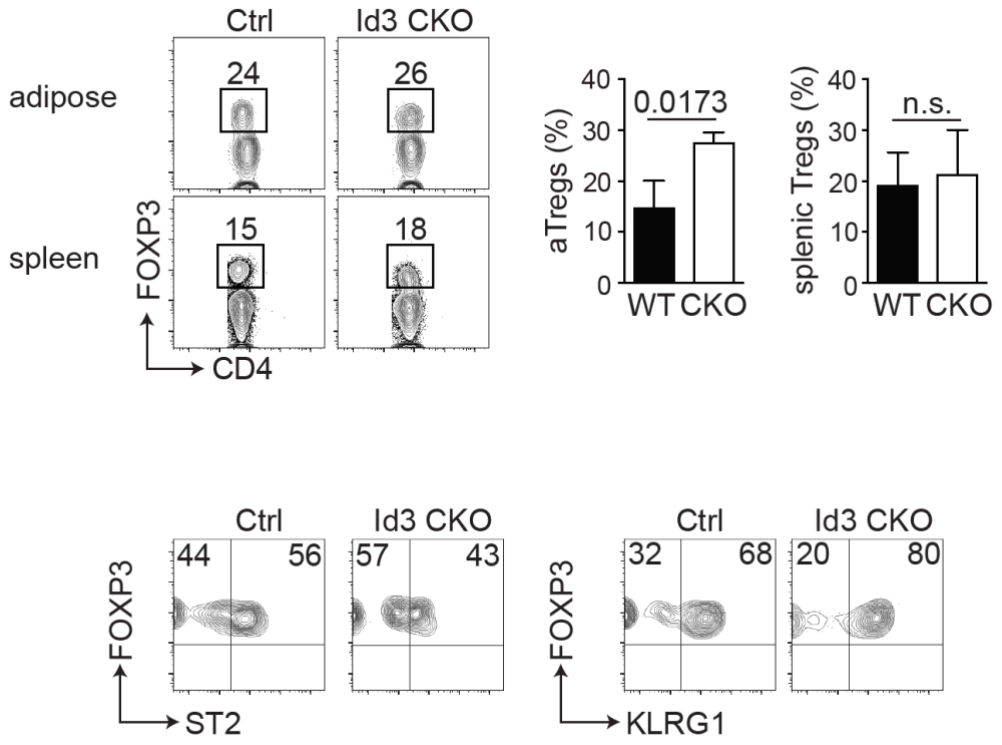

Figure 24. Id3-deficiency in aTregs.

Flow cytometry plots showing the frequency of Foxp3+ CD4+ T cells from the indicated tissue in Control (Ctrl) and Id3-deficient (Id3 CKO) mice. Bar graphs indicate the frequency of Foxp3+ Tregs from the adipose and spleen. Flow cytometry plots showing ST2 and KLRG1 expression on gated Foxp3+ CD4+ T cells in the adipose tissue in Ctrl or Id3 CKO mice. Data are representative of two independent experiments with 1-5 mice per group. P values were calculate using the student's t test or one-way ANOVA.

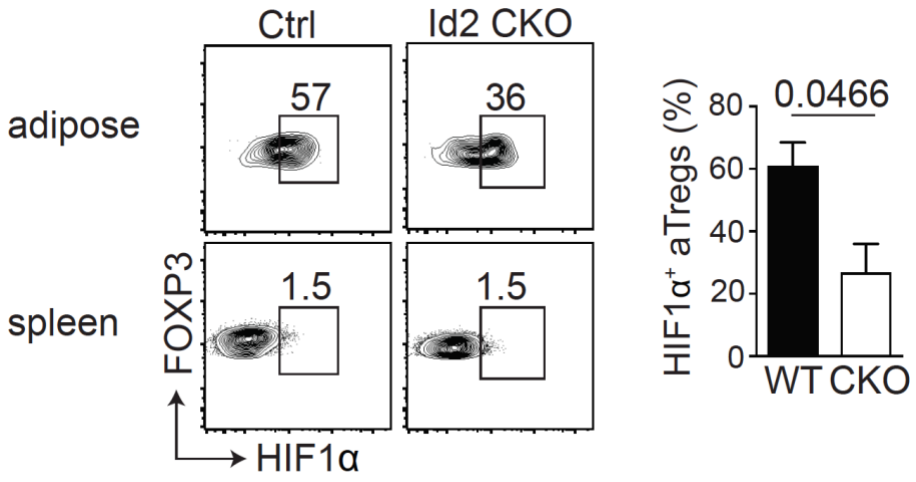

Figure 25. Hif1α expression in Id2-deficient aTregs.

Flow cytometry plots and bar graph indicating the frequency of Hif1α + Foxp3+ aTregs from WT and Id2 Treg-specific deficient male mice. Data are representative of two experiments with 2 mice per group. P values were calculate using the student's t test.

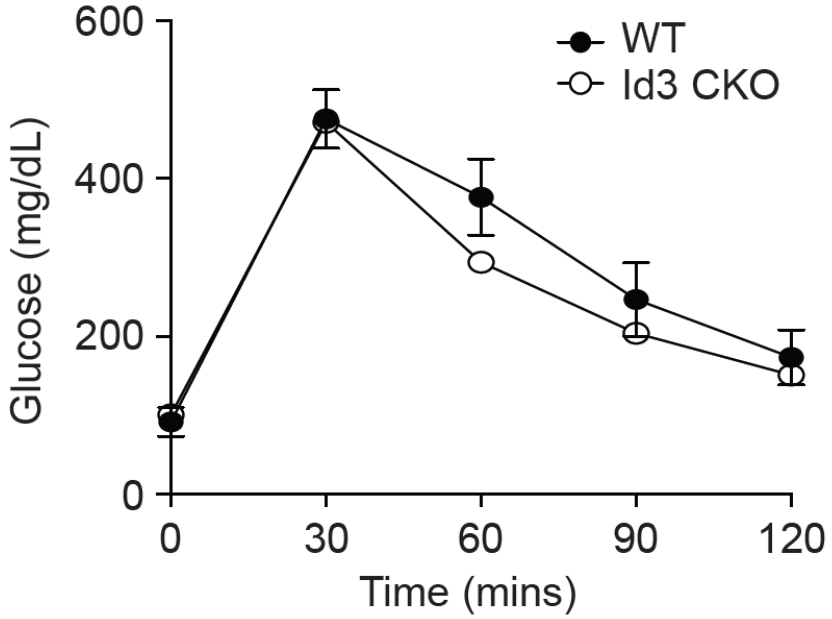

Figure 26. Glucose tolerance in Id3 CKO aTregs

Graph indicating the blood glucose over time following GTT. Data are representative of one experiment with 1-2 mice per group (C). P values were calculate using the student's t test or one-way ANOVA.

Appendix B

B.1 Extended Methods

B.1.1 C57BL/6Mice

For visceral adipose tissue experiments, male C57BL/6 were aged to a minimum 15 weeks for adequate frequency of aTregs and up to 25-30 weeks for maximum frequency of aTregs.

B.1.2 Isolation of lymphocytes from spleen

Materials: Microscope slides with frost edges x2 / sample, Petri dishes, FACS buffer, 15ml conical tube, Nylon filters, RBC Lysis Buffer

Place spleen in petri dish with 1mL FACS buffer and gently grind between frosted sides of microscope slides. Wash each slide with 1mL FAC buffer. If necessary, grind spleen again and repeat wash. Pipette solution through nylon mesh into 15mL tube. (Optional) Clean petri dish with 1mL of FACS and pipette solution through nylon mesh. Spin down – large centrifuge 1800 RPMS for 3 minutes; small centrifuge 3000RPMS for 3 minutes. Suck up supernatant and add 500mL RBC lysis buffer for ~ 30 seconds to 1 minute (solution will go from cloudy to clear). Dilute by a factor of 10 (e.g. 4.5mL FACS

for 0.5mL RBC lysis buffer). Spin down as before (1800 RPMS for 3 minutes. Resuspend in 1mL of FACS. (Optional) Filter again to remove any clumps.

B.1.3 Annexin V and 7AAD staining

To stain for annexin V and 7AAD, eBioscience kit and protocol were used. Make 1x binding buffer by diluting 10x stock in DI water. Create master mix with annexin V (1:200) and 7AAD (1:200) in 1x binding buffer. Stain cells in 100uL master mix in the dark at room temperature for 10-15 minutes. Finish by adding 100uL 1x binding buffer straight onto samples and run flow. NOTE: Samples must be run within an hour after the last step.

B.1.4 Reconstitution of α-GalCer

Materials: Pyrogen-free water, sucrose, L-histidine, Tween-20, glass-vials with caps, syringe with filter, needles. Prepare in sterile environment.

To make a 20uL stock solution to reconstitute α-GalCer, start by adding 10mL of pyrogen-free water. Then add 1.14 grams of sucrose, 150mg of L-histidine, and 100uL Tween-20. Adjust the pH to 7.2. Once the pH has been adjusted, fill up to 20uL to complete the stock solution. In a glass tube, add 500uL of stock solution. Try to syringe out the α-GalCer out of the bottle to keep sterility. Add up to 1mL stock solution and heat to dissolve. In the glass vial, sonicate in 69º C water bath for 15-20 minutes at standard amplitude. If still cloudy, move to heat-block for 10-15 minutes at 80º C or until solution is clear. Filter sterilize and aliquot 10uL (1ug/100uL) or 20uL (2ug/100uL) into glass vials.

Store at -20ºC. When ready for use, add up to 1mL sterile PBS.

B.1.5 Visceral Adipose Tissue Preparation

Materials: Adipose Buffer (Can be kept for unto 2 weeks): Low glucose DMEM, 50mM HEPES, 1% fatty acid poor BSA. Digestion Buffer (Prepare fresh on day of experiment): Low glucose DMEM, 50mM HEPES, 1% fatty acid poor BSA, 0.2mg/ml Liberase TL, 0.25mg/ml DNase I

Careful excise the visceral adipose tissue (VAT) using forceps and scissors and place into 2mL of adipose buffer. In male mice, this is the fat, also known as the perigonadal fat, attached to the gonads. Make sure to avoid collecting the gonads which can appear white in coloration, as opposed to pink. Place VAT into a petri dish and add 1mL of digestion buffer. Mince finely with a razor blade and then transfer to 50mL conical tube. If working with multiple samples, place on ice until other samples are ready. Otherwise, continue by adding 9mL of additional digestion buffer. Keep on ice to prevent collagenase activity before all samples are ready. Place samples in incubator/shaker for 30 minutes at 37C. If VAT hasn't dissolved after 30 minutes, leave for another 10 minutes. Place samples on ice, and stop collagenase activity by adding ice-cold adipose buffer up to the 30mL mark. Filter samples through 70uM filter and spin down at 2000 RPM for 5 minutes. If necessary, take syringe plunger to push VAT on the filter. Aspirate the supernatant, including the fat at the top, leaving only the pellet at the bottom made up of red blood cells (RBCs) and lymphocytes. Lyse RBCs with 500uL of RBC lysis buffer for

<1min by resuspending the pellet gently (avoid bubbles). Spin down at 2000 for 5 minutes. Aspirate supernatant and resuspend pellet in desired media and volume.

B.1.6 Bone Marrow Chimera Generation

Preparation: Donor mice should be at least 6-8 weeks old. They should be congenically distinct from the recipient cells. The exception to this rule is in the case of mixed chimeras where the WT bone marrow cells can be congenically identical to the recipient mice. For every donor mouse, 4-5 recipient mice are required. They should be 6-8 weeks old, although older doesn't matter. Should be congenically distinct from donor cells.

Day -1: Irradiate the recipient mice. B6 mice should get 1000 rads. Balb/c should only get 800 rads. Place the mice in newly autoclaved cages with clean bedding, food and antibiotic water. Water should be changed every 2-3 days for the first two weeks after cell transfer. One capful of antiobitcs per water bottle.

Day 0: Sacrifice donor mice and harvest leg bones. Using a 27 gauge needle, push the bone marrow out of the leg bones into a petri dish with FACS buffer. Make a single cell suspension of the bone marrow (BM) cells. Deplete BM of B and T cells using MACS beads (anti-CD3-Biotin and anti-B220-biotin). After MACS depletion, count cells. For single transfers, transfer 5 x 10_6 cells per recipient. Transfer cells by injecting host mice I.V. with BM cells. Transferred cells should be resuspended in sterile PBS. Six-to-eight weeks later, cheek bleed BM chimeric mice. Isolate lymphocytes from blood and stain for CD45.1 and CD45.2 to confirm peripheral reconstitution.

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