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Expression of the miR-17~92a cluster of microRNAs by regulatory T cells controls blood glucose homeostasis

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ABSTRACT

Regulatory T cells (Tregs) are a specialised immune cell type that plays important roles in regulating immune responses. However, those found in adipose tissue, particularly visceral adipose tissue (VAT), have also been shown to exert metabolic regulatory functions. In this study, we investigate the requirement of the miR-17~92a cluster of microRNAs in VAT Tregs and the impact on blood glucose. This cluster of microRNAs is one that we previously showed to be important for the fitness of Tregs found in secondary lymphoid organs. We found that male mice with Treg-specific miR-17~92a deficiency are resistant to impaired glucose tolerance induced by a high-fat diet. However, high-fat feeding still impaired glucose tolerance in female mice with Treg-specific miR-17~92a deficiency. There was an increase in KLRG1$^-$ naïve Tregs and a loss of KLRG1$^+$ terminally differentiated Tregs in the VAT of Treg-specific miR-17~92a deficient male mice but not female mice. The protection of male mice from high-fat feeding was also associated with increased IL-10 and reduced IFNγ expression by conventional CD4$^+$ T cells and reduced IL-2 expression by both CD4$^+$ and CD8$^+$ T cells in the VAT. Together this suggests that expression of miR-17~92a by VAT Tregs regulates the effector phenotype of conventional T cells and in turn the metabolic function of adipose tissue and blood glucose homeostasis.

KEYWORDS

MicroRNA, regulatory T cells, adipose tissue, blood glucose

INTRODUCTION

Adipose tissue is the main energy store of the body and is important for the regulation of metabolic homeostasis. It consists of adipocytes, which stores fat, surrounded by a matrix of dispersed immature adipocytes, immune cells and other cell types [1]. The immune compartment is particularly diverse, which includes macrophages, dendritic cells and T cells, and under normal conditions have roles in the elimination of apoptotic cell debris and the regulation of adipose tissue function [2].

Regulatory T cells (Tregs) are a specialised lineage of T cells that is critical for immune homeostasis by regulating the activation and function of other immune cells. They are characterised by the expression of the lineage-defining transcription factor FOXP3 [3]. Mutations in the *Foxp3* gene are associated with Treg deficiency and severe systemic autoimmune disorders [4]. There are two sources of Tregs in the body. Those that develop within the thymus from $CD4^+CD8^+$ double positive thymocytes are known as thymic or natural Tregs [5]. In addition, Tregs can differentiation from naïve conventional $CD4^+$ T cells in peripheral tissues following appropriate antigenic stimulation and these are known as induced Tregs [5].

The Tregs found in adipose tissue and particularly visceral adipose tissue (VAT), have critical roles in regulating metabolic homeostasis [3]. Specific depletion of these cells in mice induces local inflammation, insulin resistance and impaired glucose tolerance [6]. Gain-of-function experiments in which mice are treated with the cytokine IL-10, which is normally secreted by adipose Tregs, or with IL-2 plus anti-IL-2 antibody to expand Treg numbers, both lead to improved insulin sensitivity and glucose tolerance following a high-fat diet (HFD) [6, 7].

The frequency and function of adipose-resident Tregs appear to be influenced by a range of factors. Metabolic status is one. The number of Tregs found in the VAT of male mice is reduced the ob/ob and Ay/a mouse models of obesity [6, 8]. VAT Tregs are also lost in wildtype male mice fed a HFD. These findings point to another factor, sexual dimorphism. VAT Tregs from male and female mice display distinct transcriptional profiles and these are influenced by sex hormones, which ultimately translates to different responses to high caloric intake and adipose inflammation [9, 10]. Age is another factor. The number of Tregs found in adipose tissue increases with age but, counterintuitively, this increase appears to contribute to the development of age-associated insulin resistance [11]. Selective depletion of adipose-resident Tregs attenuates many of the hallmarks of age-associated metabolic dysregulation, such as insulin resistance and impaired glucose tolerance [11].

Adipose-resident Tregs display a transcriptional profile that distinguishes them those found in lymphoid organs [12]. Moreover, they are also distinguishable from Tregs found in other non-lymphoid tissues. The resident Tregs found in different tissues have been shown to express unique cell surface markers, transcription factors, effector molecules and distinct repertoires of T cell receptors [12]. VAT Tregs distinctly express the transcription factor PPAR-γ and a unique pattern of chemokine receptors[6]. PPAR-γ is required for the localization and function of adipose-resident Tregs [13]. PPAR-γ is also essential for the differentiation and function of adipocytes [14]. This suggest that the identity of VAT Tregs may be determined by the local environment in which they are found. It has been shown that this is at least partly dependent on the adipose-resident macrophages [15]. Id2, IRF4 and BATF are other transcription factors that are highly expressed in adipose-resident Tregs and have been shown to be important for their accumulation and function within adipose tissue [16-18]. These appear to regulate the expression of the IL-33R, and IL-33 is a cytokine that is important for differentiation and maintenance of adipose Tregs [18].

While recent studies have revealed much about the transcriptional networks in adipose-resident Tregs, there is still much that we do not understand about the molecular pathways that that govern the identity and function of these cells. MicroRNAs (miRNAs) may have a role, which are short ~22 nucleotide non-coding RNAs that inhibit the translation and stability of protein-coding messenger (m)RNAs [19]. The miRNA pathway is critical for the development and function of Tregs, at least those found in lymphoid organs. Treg-specific deletion of the genes encoding DROSHA or DICER, the two RNase III enzymes that are essential for the biogenesis of microRNAs, results in uncontrolled lethal inflammation in mice [20-22], a phenotype that is identical to mice with FOXP3 deficiency. Moreover, FOXP3 drives the transcription of specific miRNA genes in Tregs, suggesting that FOXP3 exerts at least some of its functions via miRNAs [23]. Tregs highly express a number of miRNA species. These include those from the well-characterized miR-17~92a miRNA gene cluster [24]. We and others previously showed that this cluster of miRNAs is critical for the homeostasis of lymphoid Tregs by regulating the proliferation, apoptosis and effector function of these cells [25-27].

While it is clear that the miRNA pathway is critical for the development and function of lymphoid Tregs, it remains unknown whether this pathway is also important in the adipose-resident or other tissue-resident Tregs. In this study, we investigate the requirement of the miR-17~92a cluster in adipose-resident Tregs and the influence on blood glucose homeostasis.

RESULTS

Treg-specific miR-17~92a deficiency protects male mice from impaired glucose tolerance induced by high-fat feeding

To investigate whether miR-17~92a is required for the function of adipose-resident Tregs, we analysed conditional *Mir17hg*^{lox} mice harbouring a *Foxp3*$^{IRES\text{-}Cre}$ knockin (hereinafter referred to as Mir17hg Foxp3-cre mice), which results in deletion of the *Mir17hg* gene only in FOXP3-expressing cells. This deletion occurs in all Tregs, including those found in adipose tissue and secondary lymphoid organs [26]. The *Foxp3* gene is located on the X-chromosome. To account for random X-chromosome inactivation, female mice were maintained as *Mir17hg*$^{lox/lox}$ *Foxp3*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (homozygous for both alleles), while male mice were maintained as *Mir17hg*$^{lox/lox}$ *Foxp3*$^{IRES\text{-}Cre/Y}$ (hemizygous for the Cre knockin). *Mir17hg*$^{lox/lox}$ *Foxp3*$^{IRES\text{-}Cre/Y}$ male mice were mated with wildtype female mice to produce control *Mir17hg*$^{lox/+}$ *Foxp3*$^{+/Y}$ male and *Mir17hg*$^{lox/+}$ *Foxp3*$^{IRES\text{-}Cre/+}$ female mice. We previously showed that while miR-17~92a is important for the homeostasis of lymphoid Tregs, the lymphoid organs of Mir17hg Foxp3-cre mice only display minor perturbations. Importantly they do not display any obvious signs of autoimmunity [26] and thus, the metabolic status of young animals can be assessed.

Mir17hg Foxp3-cre and control mice were fed on normal chow or HFD for eight weeks from weaning then subjected to glucose tolerance testing. On a normal chow diet, Mir17hg Foxp3-cre male and female mice were indistinguishable from control mice (Figure 1a–c). Chow-fed Mir17hg Foxp3-cre mice cleared glucose as the same rate as control mice. However, while high-fat feeding impaired the response of control male mice, this impairment was not observed in Mir17hg Foxp3-cre male mice (Figure 1a–c). HFD-fed Mir17hg Foxp3-cre male mice reached a lower maximal blood glucose than HFD-fed control male mice and cleared the glucose over the 2 h challenge. In fact, HFD-fed Mir17hg Foxp3-cre male mice cleared glucose with the same efficiency as chow-fed mice. HFD-fed control male mice did not return to baseline blood glucose levels within the course of the challenge.

Interestingly, this protection from high-fat feeding was not observed in Mir17hg Foxp3-cre female mice (Figure 1a, c). Their response was equally impaired as control female mice. Thus, expression of the miR-17~92a cluster of miRNAs plays an important modulatory function in Tregs and the regulation of blood glucose homeostasis but only in males.

Mir17hg Foxp3-cre male mice fed on a HFD were found to weigh the same as those fed on normal chow diet, whereas control male and female mice fed a HFD were statistically heavier than those

fed on a normal chow diet (Supplementary figure 1). There was also a trend for HFD-fed Mir17hg Foxp3-cre female mice to be heavier than those fed a normal chow diet.

Treg-specific miR-17~92a deficiency results in a loss of peripheral-induced Tregs but not thymic-derived Tregs in the VAT of male mice

It is the Tregs found in VAT that play a critical role in metabolic homeostasis and increasing their numbers or function improves glucose tolerance following HFD-feeding [6, 7]. We thus analysed the VAT of Mir17hg Foxp3-cre mice (on a normal chow diet) for $FOXP3^+$ Tregs (Supplementary figure 2). As previously reported [10], Tregs are more frequent in the VAT male mice than female mice. However, there was no difference in either the frequency (Figure 2a) or absolute numbers (Supplementary figure 3a) of VAT Tregs between Mir17hg Foxp3-cre and control mice of both sexes.

Thymic-derived Tregs are distinguishable from peripheral induced Tregs by their expression of neuropilin-1, also known as CD304 [28]. No difference in the frequency of $CD304^+FOXP3^+$ thymic Tregs was observed between Mir17hg Foxp3-cre and control mice of both sexes, which accounted for approximately two-thirds of the Tregs found in VAT (Figure 2b). However, there was a significant reduction in the frequency of $CD304^-FOXP3^+$ peripheral induced Tregs in Mir17hg Foxp3-cre male mice compared to controls (Figure 2c), while no difference was observed in female mice.

Treg-specific miR-17~92a deficiency alters the balance between naïve and terminally differentiated Tregs in the VAT of male mice

We next analysed the maturation status of the VAT Tregs. Expression of KLRG1 marks terminally differentiated Tregs, while naïve Tregs lack expression of this marker [29]. There was a reduction in the frequency of $KLRG1^+$ cells in both the thymic-derived (Figure 2d) and peripheral-induced (Figure 2e) Treg compartments in Mir17hg Foxp3-cre male mice compared to control male mice, and thus an increase in $KLRG1^-$ naïve cells. This was somewhat unexpected because an increase in terminally differentiated Tregs might have been predicted to correlate with the resistance to impaired glucose homeostasis. The reduction in $KLRG1^+$ cells in both thymic-derived and peripheral-induced compartments means that miR-17~92a deficiency must be acting on at least two distinct levels of VAT Treg maturation, the differentiation of induced Tregs and the terminal differentiation of all Tregs, whether induced or thymic-derived. No difference in either Tregs subpopulation were observed between Mir17hg Foxp3-cre and control female mice.

Perturbations in Treg subpopulations in Treg-specific miR-17~92a deficient male mice are maintained with HFD feeding

We next analysed the impact of a HFD on VAT Treg populations in these mice. As expected [30], HFD-feeding resulted in a reduction in the total frequency of Tregs in the VAT mice, regardless of miR-17~92a genotype (Figure 3a & Supplementary figure 3a). The altered proportions of Treg subpopulations previously observed in chow-fed mice were maintained in these HFD-fed mice, with Mir17hg Foxp3-cre male mice exhibiting a reduction in $CD304^{-}FOXP3^{+}$ peripheral induced Tregs but not $CD304^{+}FOXP3^{+}$ thymic Tregs, as well as a reduction in $KLRG1^{+}$ terminally cells in both the thymic-derived and induced Treg compartments compared to control male mice (Figure 3b–e). Again, there was no statistical differences in Treg subpopulations between Mir17hg Foxp3-cre and control female mice (Figure 3b–e).

Conventional $CD8^{+}$ T cells are increased in the VAT of Treg-specific miR-17~92a deficient male mice

The interplay between Tregs and other immune cells, particularly conventional $CD4^{+}$ and $CD8^{+}$ T cells, within VAT is thought to be important for the regulation of adipose tissue function. Depletion of Tregs results in an increase in $CD4^{+}$ and $CD8^{+}$ T cells numbers within VAT, while IFNγ from Th1 $CD4^{+}$ T cells inhibits Treg differentiation [31]. We therefore investigated whether conventional T cell populations in the VAT are affected by Treg-specific miR-17~92a deficiency in these mice. The frequency (Figure 4a) or number (Supplementary figure 3b) of conventional ($FOXP3^{-}TCR\beta^{+}$) $CD4^{+}$ T cells in the VAT of Mir17hg Foxp3-cre mice was no different to control mice in both males and females, and whether fed on normal chow or HFD. However, there was an increase in conventional $CD8^{+}$ T cells in Mir17hg Foxp3-cre male mice whether fed on normal chow or HFD (Figure 4b & Supplementary figure 3c). There was also a trend for an increase in $CD8^{+}$ T cells in Mir17hg Foxp3-cre female mice (Figure 4b & Supplementary figure 3c). Because miR-17~92a deficiency is restricted only to Tregs, this increase in $CD8^{+}$ T cells must be a downstream consequent of altered Treg function.

Increased IL-10 expression and decreased IFNγ expression by conventional $CD4^{+}$ T cells in the VAT of Treg-specific miR-17~92a deficient male mice

We next investigated the phenotype of the conventional T cells in these mice. The VAT $CD4^{+}$ T cells of Mir17hg Foxp3-cre male mice were found to express higher levels of IL-10 and lower levels of IFNγ than those of control mice, following high-fat feeding (Figure 5a, c). These cells also expressed lower levels of IL-2. Interestingly, the VAT $CD8^{+}$ T cells of Mir17hg Foxp3-cre male mice expressed similar levels of IFNγ compared to control mice but lower levels of IL-2. In fact,

there was a substantial increase in the frequency of IFNγ^{+}IL-2^{-} CD8^{+} T cells in Mir17hg Foxp3-cre male mice.

The VAT CD4^{+} T cells of Mir17hg Foxp3-cre female mice displayed similar expression of IL-10, IL-2 and IFNγ compared to control mice (Figure 5b, c). However, like in their male counterparts, there was a reduction in IL-2 expressing CD8^{+} T cells in Mir17hg Foxp3-cre female mice. There was similarly a substantial increase in the frequency of IFNγ^{+}IL-2^{-} CD8^{+} T cells.

Finally, we found an increase in the frequency of KLRG1^{+} effector CD8^{+} T cells in the VAT of both male and female Mir17hg Foxp3-cre mice compared to control mice (Figure 5d).

Together, these data suggest that a shift in the conventional CD4^{+} T cell compartment towards an anti-inflammatory IL-10-expressing phenotype may potentially underlie the protection of Mir17hg Foxp3-cre male mice from high-fat feeding. However, the phenotypic changes in conventional CD8^{+} T cells are less likely to explain the resistance because the same changes were evident in Mir17hg Foxp3-cre female mice.

Ablation of the microRNA pathway in all T cells does not affect glucose tolerance

Given that miR-17~92a deficiency in Tregs protected male mice from high-fat feeding, we wondered if deficiency of the entire miRNA pathway might result in an even greater tolerance to high fat. To investigate this, we analysed conditional *Drosha^{lox}* knockout mice harbouring a *CD4-cre* transgene (hereinafter referred to as Drosha CD4-cre mice), which deletes the *Drosha* gene at the CD4^{+}CD8^{+} stage in T cell development. This results in DROSHA deficiency in all αβ T cells, include FOXP3^{+} Tregs [20]. These mice also harbour a *FOXP3$^{IRES-GFP}$* knockin [32] that allows the identification of Tregs by GFP expression. Unlike *Drosha$^{lox/lox}$ FOXP3$^{IRES-Cre/IRES-Cre}$* mice, in which deletion is restricted only to Tregs, these mice with DROSHA deficiency in all T cells survive to adulthood and do not develop obvious disease until much later in life, typically 6 to 9 months of age [20]. Young healthy mice could therefore be analysed for glucose tolerance. Moreover, macrophages and adipocytes, the other targets of VAT Tregs, remain microRNA sufficient in these mice. *Drosha$^{lox/lox}$* lacking the CD4-cre transgene were analysed as controls. We previously showed that *Drosha$^{lox/lox}$* mice are indistinguishable from wildtype mice or mice only harbouring the CD4-cre transgene [20].

Drosha CD4-cre and control mice were fed on a normal chow or HFD for eight weeks from weaning then subjected to glucose tolerance testing. Somewhat unexpectedly, there was no

difference between the response of Drosha CD4-cre and control mice, both in males and females, and whether fed on normal chow or a HFD, but high-fat feeding impaired glucose responses in all mice (Figure 6).

T cell specific DROSHA deficiency results in a loss of both VAT Treg and conventional T cells

We next analysed the Treg and conventional T cell compartments in the VAT of Drosha CD4-cre mice. We previously showed that there is a 50% reduction in both Treg and conventional T cell populations in the lymphoid organs of these mice [20]. Consistent with this, total VAT Tregs were reduced by approximately half in Drosha CD4-cre mice, in both males and females, and whether fed on normal chow or a HFD (FIG 7A). Both thymic-derived and peripherally induced VAT Tregs were equally affected by DROSHA deficiency (Supplementary figure 3). However, while naïve Tregs were lost, the frequency of terminally differentiated Tregs appeared largely unaffected (Supplementary figure 3).

Compared to lymphoid organs [20], the loss of conventional T cell populations was even more severe in the VAT of Drosha CD4-cre mice (Figure 7b, c), with an almost total absence of $CD8^+$ T cells, in both male and female mice, and whether fed on normal chow or HFD. Thus, DROSHA deficiency from the $CD4^+CD8^+$ stage in T cell development dramatically affects both Treg and conventional T cell populations in the VAT but this does not alter responses to high-fat feeding. This suggests that any impacts of microRNA deficiency on the metabolic function of VAT Tregs must be via the interaction with conventional T cells rather than via direct interactions with macrophages or adipocytes.

DISCUSSION

We have shown that expression of the miR-17~92a cluster of miRNAs by Tregs has a critical role in metabolic control, with Treg-specific miR-17~92a deficiency rendering male mice resistant to impaired blood glucose homeostasis caused by high-fat feeding. The metabolic functions of adipose-resident Tregs is tought to involve complex interactions with other immune cells as well as directly with adipocytes. In Treg-specific miR-17~92a deficient male mice, protection from high feeding is likely to involve increased IL-10 and decreased IFNγ expression by conventional $CD4^+$ T cells.

IFNγ has pleiotropic impacts on adipose tissue function. It can directly act on adipocytes to inhibit insulin signalling and to reduced lipid storage [33, 34]. Moreover, it inhibits the differentiation of pre-adipocytes [33]. IFNγ also promotes the polarization of macrophages towards a proinflammatory M1 phenotype that secrete other proinflammatory cytokines like TNFα to further inhibit insulin signalling [35]. Furthermore, the IFNγ in adipose tissues has been shown to inhibit Treg differentiation and function [31], which ultimately contributes to a feedback loop between conventional T cell and Tregs that promotes adipose inflammation. Thus, the reduced IFNγ expression in the VAT of miR-17~92a deficient male mice is likely to contribute to the protection from a high-fat diet.

IL-10 is anti-inflammatory cytokine that is thought to suppresses adipose tissue inflammation and insulin resistance [36]. This may be occurring via its action on various immune cell types, such as by suppressing conventional $CD4^+$ and $CD8^+$ T cell function [37]. IL-10 has also been shown to inhibit the expression of TNFα-induced pro-inflammatory genes in adipocytes, including chemokines like CCL5 that would otherwise recruit other pro-inflammatory cells to the tissue [6]. Furthermore, IL-10 prevents TNFα from inhibiting insulin signalling in adipocytes [6]. Paradoxically, however, IL-10 also has been shown to repress the expression of thermogenic genes in adipocytes to promote lipid storage [38]. Tregs, M2 macrophages and conventional T cells have all been shown to be sources of IL-10 in adipose tissue [35]. The IL-10 produced by Tregs appears to contribute to insulin resistance by directly acting on adipocytes to suppress thermogenesis [39]. Thus, it could be that the consequences of IL-10 in adipose tissue depends on both source and target. Clearly, the roles that IL-10 plays in metabolic homeostasis are complex and remain to be clarified.

Regardless of these complex cytokine-cell interactions, high levels of IL-10 do appear to have a therapeutic benefit. Administration of IL-10 improves insulin sensitivity and glucose tolerance following high-fat feeding [6, 7]. Thus, the elevated IL-10 expression by conventional $CD4^+$ T cells in

the VAT of miR-17~92a deficient male mice is also likely to contribute to the protection of these mice from a high-fat diet.

The increased IL-10 and decreased IFNγ expression by VAT $CD4^+$ T cells resulting from Treg-specific miR-17~92a deficiency occurred in male but not female mice. Sexual dimorphism in VAT Tregs has been reported previously [10]. The greater numbers of Tregs found in the VAT of male mice than in that of female mice appears to be dependent on the androgen-regulated differentiation of adipose stromal cells that produce IL-33 [10]. The impact of miR-17~92a deficiency in the Tregs of male mice could simply be due to males having a larger number of these cells. However, it may also be related to the direct actions of the sex hormones on the Tregs themselves. Both androgen and estrogen can directly signal Tregs [40, 41]. The interaction of sex hormones with adipose tissue cells is almost certainly involved in the male-specific impact miR-17~92a deficiency on glucose responses because there was no difference in the impact of miR-17~92a deficiency on lymphoid Tregs between male and female mice [26]. Thus, miR-17~92a deficiency may be interacting with one or both hormones, which then manifests as a differential resistance to a high-fat diet.

EOS is a zinc finger transcription factor that is an essential coregulator of FOXP3-mediated gene transcription and the mRNA encoding this factor has been shown to be a target of miR-17 [27]. This could potentially be a contributing mechanism in VAT Tregs but miR-17 along with the other miRNAs in this cluster regulates numerous targets. In conventional $CD4^+$ T cells, miR-17 has been shown to regulate the *Tgfbr2* and *Creb1* mRNAs, while miR-19b regulates *Pten* [42]. Whether these are also targets in Tregs remains to be determined. Together, these suggest it is quite possible that the miR-17~92a cluster operates as an inhibitor of the suppressor functions of VAT-resident Tregs.

Deletion of the *Mir17hg* gene in Tregs has also been shown to impair their function, that is, miR-17~92a is required for the suppressor function of Tregs. We showed that the fitness of lymphoid Tregs is impaired by miR-17~92a deficiency [26]. Another study showed that mice with Treg-specific miR-17~92a deficiency develop more severe disease in the experimental autoimmune encephalomyelitis model of multiple sclerosis [25]. This was associated with impaired differentiation of naïve Tregs into IL-10-expressing effector Tregs. Thus, the role of miR-17~92a in Tregs appears to be dependent on physiologic context. To understand the molecular mechanism underlying the protection of Treg-specific miR-17~92a deficient male mice from high-fat feeding, it will be important in future studies to determine the targets of miR-17~92a miRNAs in specifically within VAT Tregs.

METHODS

Mice

Treg specific miR-17~92a deficient mice have been described previously [26], which harbour a conditional *Mir17hg*^{lox} allele and *Foxp3*$^{IRES\text{-}Cre}$ knockin. Control animals were generated by crossing Treg specific miR-17~92a male mice with wildtype female mice to produce offspring with the genotype *Mir17hg*$^{lox/+}$ *Foxp3*$^{IRES\text{-}Cre/+}$ or *Mir17hg*$^{lox/+}$ *Foxp3*$^{Y/+}$. *Drosha*$^{lox/lox}$ *CD4-cre* mice have been previously described [20]. They were crossed to *Foxp3*$^{IRES\text{-}GFP}$ knockin mice [32], which allowed for identification of Tregs by expression of GFP.

Mice were fed with standard chow pellets or modified high fat pellets (diet SF01-025, Specialty Feeds, Glen Forrest) *ad libitum* from weaning for eight weeks before analysis. The total fat content of the high-fat diet was 22.3%, compared to 4.8% of the normal chow diet. The calculated energy of the high-fat diet was 14.3 MJ Kg^{-1} compared to 18.3 MJ Kg^{-1} of the normal chow diet.

The mice were housed at St Vincent's Hospital Melbourne's BioResources Centre. Experiments were approved by the Animal Ethics Committee of St Vincent's Hospital Melbourne and performed under the Australian code for the care and use of animals for scientific purposes.

Glucose tolerance testing

A 30% w/v glucose (Sigma-Aldrich, St Louis) solution in sterile water was prepared fresh for each experiment. The mice were fasted for 6 h, then a blood glucose reading was taken by nicking the end of the tail with a scalpel blade to obtain a drop of blood. Mice were subsequently weighed and injected intraperitoneally with a 2g kg^{-1} bolus of glucose. Blood glucose readings were then taken at 15, 30, 45, 60, 75, 90 and 120 min after the glucose injection by warming the tail nick under a heat lamp.

VAT preparations

To obtain leukocytes from visceral adipose tissue, the tissue was cut into small pieces with scissors and digested with collagenase B (Sigma-Aldrich, St Louis) in RPMI (Sigma-Aldrich, St Louis) at 37°C for 20 min in a shaking incubator (at 200rpm). The digest was then filtered through a 70µm strainer and washed twice with cold PBS (Sigma-Aldrich, St Louis). If required, red blood cells were removed by treating the pellet with RBC lysis buffer (Thermo Fisher Scientific, Waltham) between the two washes. The cells were then resuspended in RPMI + 10% FCS (GE Healthcare Life Sciences, Chicago), filtered again through a 70µm strainer and allowed to recover at 37°C for 1h before antibody staining. For intracellular cytokine analyses, the cells were allowed to recover in

RPMI + 10% FCS supplemented with 50ng mL^{-1} phorbol 12-myristate 13-acetate (Thermo Fisher Scientific, Waltham), 1μg mL^{-1} ionomycin (Thermo Fisher Scientific, Waltham) and Protein Transport Inhibitor Cocktail (Thermo Fisher Scientific, Waltham) for 4h prior to antibody staining.

Flow cytometry

For the analysis of leukocytes from Mir17hg Foxp3-cre mice, the cells were first stained with antibodies against cell surface antigens, before fixing and staining with an anti-FOXP3 antibody using the FOXP3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham). For the analysis of intracellular cytokine expression, the cells were first stained antibodies against cell surface antigens, before fixing and staining with antibodies against cytokines using the Intracellular Fixation and Permeabilization Buffer Set (Thermo Fisher Scientific, Waltham). The cells were then resuspended in PBS 0.5% BSA 2mM EDTA for analysis. For the analysis of leukocytes from Drosha CD4-Cre mice, the cells were simply stained with antibodies against cell surface antigens in PBS 0.5% BSA 2mM EDTA (Sigma-Aldrich, St Louis). All antibodies used were purchased from (Thermo Fisher Scientific, Waltham) and are listed in Supplementary table 1. Data acquisition was performed on an LSRFortessa (BD Biosciences, Franklin Lakes) then analysed on FlowJo ver10.7 (Ashland).

Statistical analyses

All statistical analyses were performed on Prism ver9 (GraphPad, San Diego). Comparisons between multiple groups (male versus female and genotype) were assessed by ANOVA with Tukey's *post hoc* test. Two-way comparisons were analysed by a *t*-test.

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FIGURE CAPTIONS

Figure 1: Treg-specific miR-17~92a deficiency protects male mice from high fat-impaired glucose tolerance. Mir17hg Foxp3-cre and control mice were fed on normal chow or HFD for 8 weeks from weaning, then subjected to glucose tolerance testing. **(a)** Blood glucose (B.G.) measurements (mean ± S.D. of n = 5 or 6 mice per group analysed over 3 experiments) were taken at the indicated times following injection of glucose. Area under curve (AUC, mean ± S.D. shown) of blood glucose levels comparing chow versus HFD-fed **(b)** male and **(c)** female mice (C = control, KO = Mir17hg Foxp3-cre). Blood glucose curves were assessed by ANOVA and AUC were assessed by a *t*-test (* $P < 0.05$, ** $P < 0.005$ compared to littermate controls).

Figure 2: miR-17~92a deficiency perturbs Treg subpopulations in the VAT of male but not female mice. Mir17hg Foxp3-cre and control mice were fed on normal chow for 8 weeks from weaning, then the VAT was analysed for Treg populations by flow cytometry. Tregs were identified by intracellular staining for FOXP3 protein. Shown are the frequencies of **(a)** total $FOXP3^+$ Tregs, **(b)** thymic-derived $CD304^+$ Tregs and **(c)** peripheral induced $CD304^-$ Tregs as a percentage of total $CD45^+$ leukocytes and the frequency of $KLRG1^+$ terminally differentiated cells within the **(d)** $CD304^+$ thymic-derived subset and **(e)** $CD304^-$ peripheral-induced subset. Each dot is an individual animal (n = 5–10 mice per group analysed over 5 experiments), with means indicated by the lines. Comparisons were assessed by ANOVA (*** $P < 0.0005$, only control versus knockout comparisons are indicated).

Figure 3: High-fat feeding depletes VAT Tregs regardless of miR-17~92a expression, but the perturbations in Treg subpopulations caused by miR-17~92a deficiency are maintained. Mir17hg Foxp3-cre and control mice were fed on a HFD for 8 weeks from weaning, then the VAT was analysed for Treg populations flow cytometry. Tregs were identified by intracellular staining for FOXP3 protein. **(a)** The frequency of total $FOXP3^+$ Tregs as a percentage of total $CD45^+$ leukocytes comparing male to female, knockout to control and chow-fed to HFD-fed mice. The data for chow-fed mice is derived from Figure 2. Each dot is an individual animal (n = 4–10 mice per group), with means indicated by the lines. Shown are the frequencies of **(b)** thymic-derived $CD304^+$ Tregs and **(c)** peripheral induced $CD304^-$ Tregs as a percentage of total $CD45^+$ leukocytes and the frequency of $KLRG1^+$ terminally differentiated cells within the **(d)** $CD304^+$ thymic-derived subset and **(e)** $CD304^-$ peripheral-induced subset in HFD-fed mice. Each dot is an individual animal (n = 4–6 mice per group, analysed over 4 experiments), with means indicated by the lines. Comparisons were assessed by ANOVA (** $P < 0.005$, *** $P < 0.0005$, only control versus knockout comparisons are indicated).

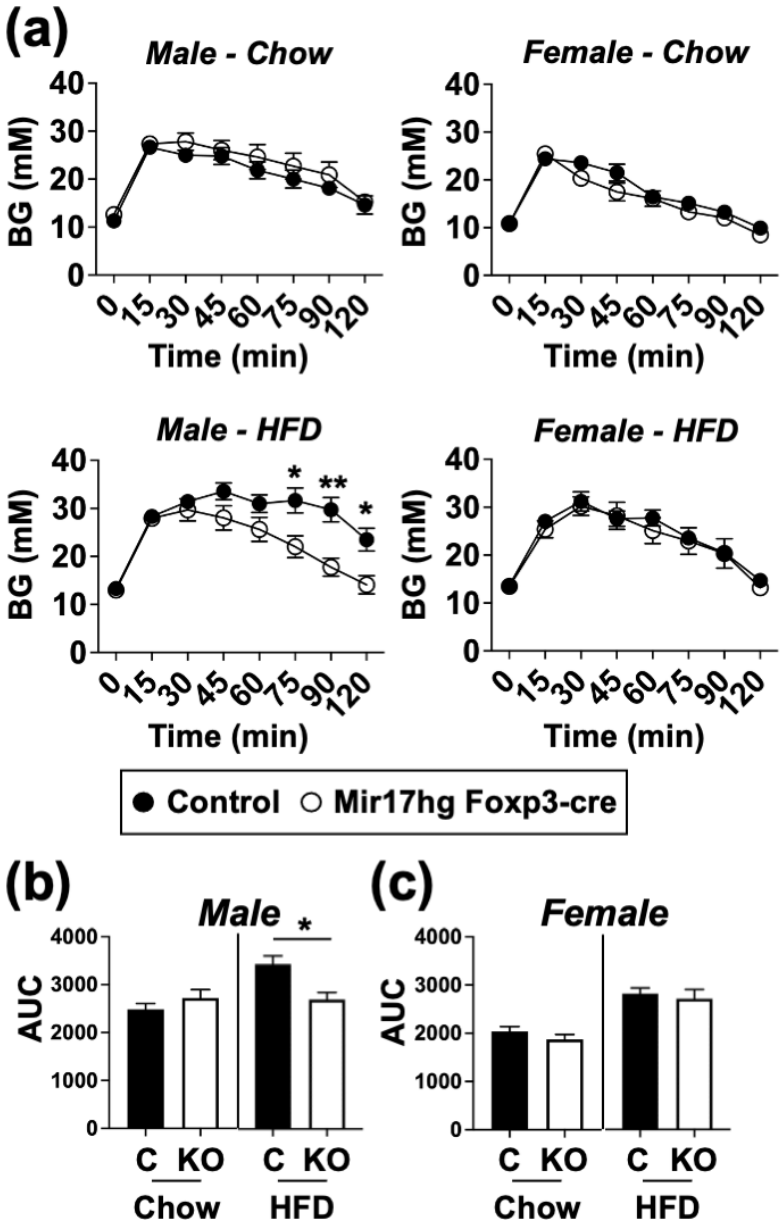
Figure 4: Treg-specific miR-17~92a deficiency results in an increase of conventional CD8$^+$ T cells in VAT. Mir17hg Foxp3-cre and control mice were fed on normal chow or HFD for 8 weeks from weaning, then the VAT was analysed for conventional T cell (TCRβ$^+$FOXP3$^-$) populations. By flow cytometry. Shown are the frequencies of **(a)** CD4$^+$ and **(b)** CD8$^+$ T cells as a percentage of total CD45$^+$ leukocytes comparing male to female, knockout to control and chow-fed to HFD-fed mice. Each dot is an individual animal (n = 4–10 mice per group, analysed over 5 experiments), with means indicated by the lines. Comparisons were assessed by ANOVA (* $P < 0.05$, ** $P < 0.005$, only control versus knockout comparisons are indicated).

Figure 5: Increased IL-10 expression by conventional CD4+ T cells and other cytokine perturbations in the VAT of Treg-specific miR-17~92a deficient male mice. Mir17hg Foxp3-cre and control mice were fed on a HFD for 8 weeks from weaning, then the conventional T cell (TCRβ$^+$ FOXP3$^-$) populations in the VAT were analysed for cytokine expression by flow cytometry. Shown is a representative set of flow cytometric plots (out of 3 sets of mice analysed over 3 experiments) for IL-10 versus IFNγ or IL-2 versus IFNγ expression by CD4$^+$ and CD8$^+$ T cells in **(a)** male and **(b)** female mice (KO = Mir17hg Foxp3-cre). **(c)** The pooled data from the 3 sets of mice analysed. Each dot is an individual animal, with means indicated by the lines. Comparisons were assessed by ANOVA (*p <0.05, **p<0.005, ***p<0.0005, only control versus knockout comparisons are indicated). **(d)** The frequency of KLRG1$^+$ effector CD8$^+$ T cells as a percentage of total CD45$^+$ leukocytes in the VAT of mice. Each dot is an individual animal (n = 5–10 mice per group), with means indicated by the lines. Comparisons were assessed by a *t*-test (* P <0.05, ** P <0.005 compared to littermate controls).

Figure 6: Mice with DROSHA deficiency in all αβ T cells exhibit normal responses to glucose challenge. Drosha CD4-cre and control mice were fed on normal chow or HFD for 8 weeks from weaning, then subjected to glucose tolerance testing. **(a)** Blood glucose (B.G.) measurements (mean ± S.D. of n = 4–8 mice per group analysed over 4 experiments) were taken at the indicated times following injection of glucose. Area under curve (AUC, mean ± S.D. shown) of blood glucose levels comparing chow versus HFD-fed **(b)** male and **(c)** female mice (C = control, KO = Drosha CD4-cre). Blood glucose curves were assessed by ANOVA and AUC were assessed by a *t*-test (no statistical difference found).

Figure 7: Severe depletion of both Tregs and conventional T cells in the VAT of mice with DROSHA deficiency in all αβ T cells. Drosha CD4-cre and control mice were fed on a normal

chow or HFD for 8 weeks from weaning, then the VAT was analysed for T cell populations by flow cytometry. Tregs were identified by expression of a FOXP3-IRES-GFP reporter. Shown are the frequencies of **(a)** total GFP(FOXP3)$^+$ Tregs, **(b)** conventional CD4$^+$(GFP$^-$) and **(c)** CD8$^+$(GFP$^-$) T cell populations as a percentage of total CD45$^+$ leukocytes comparing male to female, knockout to control and chow-fed to HFD-fed mice. Each dot is an individual animal (n = 4–7 mice per group analysed over 4 experiments), with means indicated by the lines. Comparisons were assessed by ANOVA (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, only control versus knockout comparisons are indicated).



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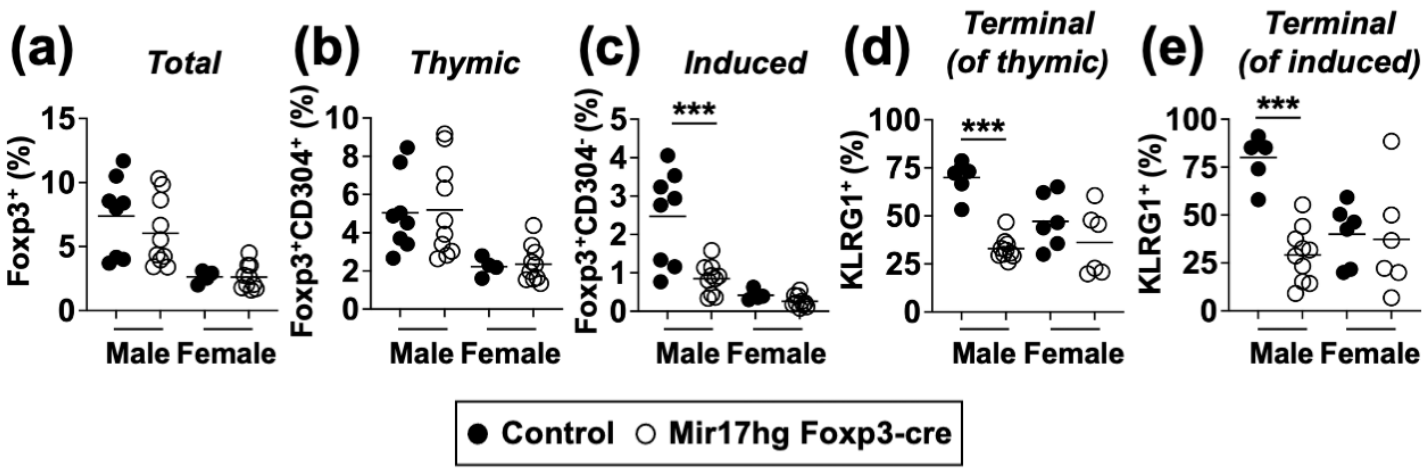

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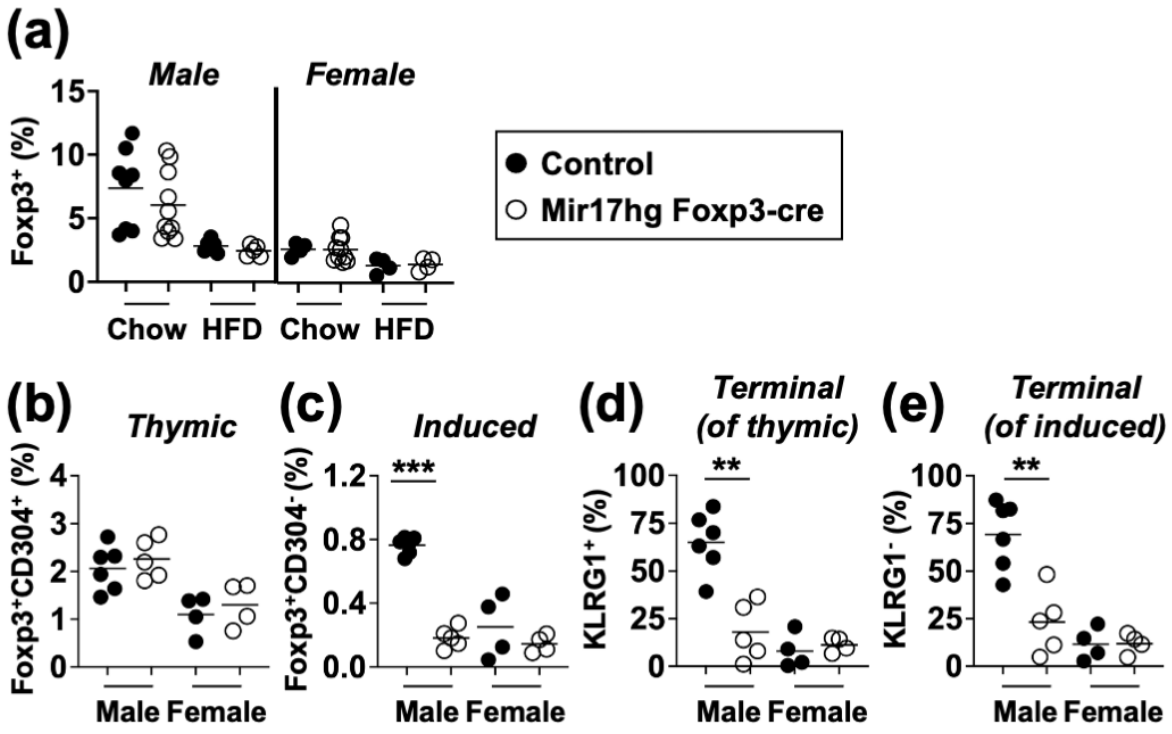

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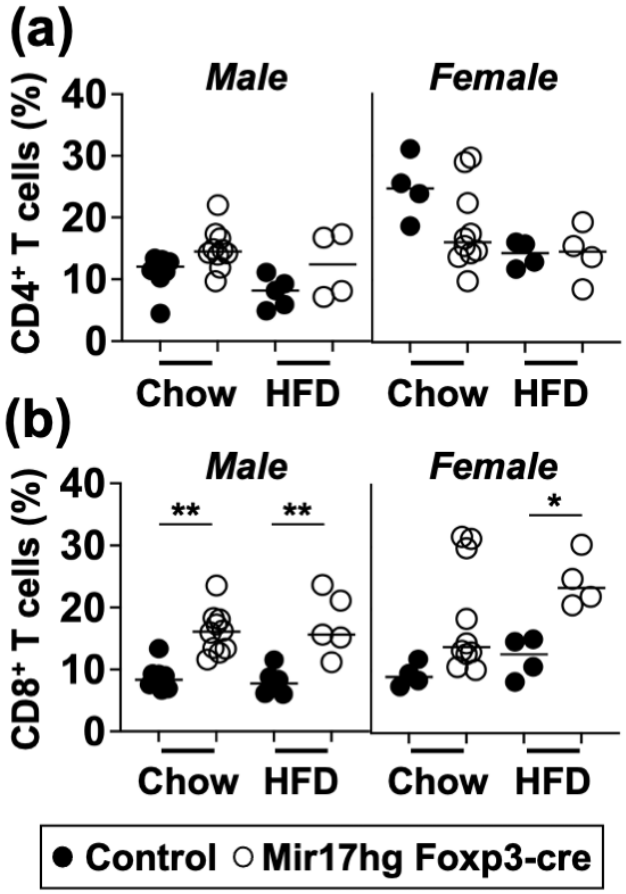

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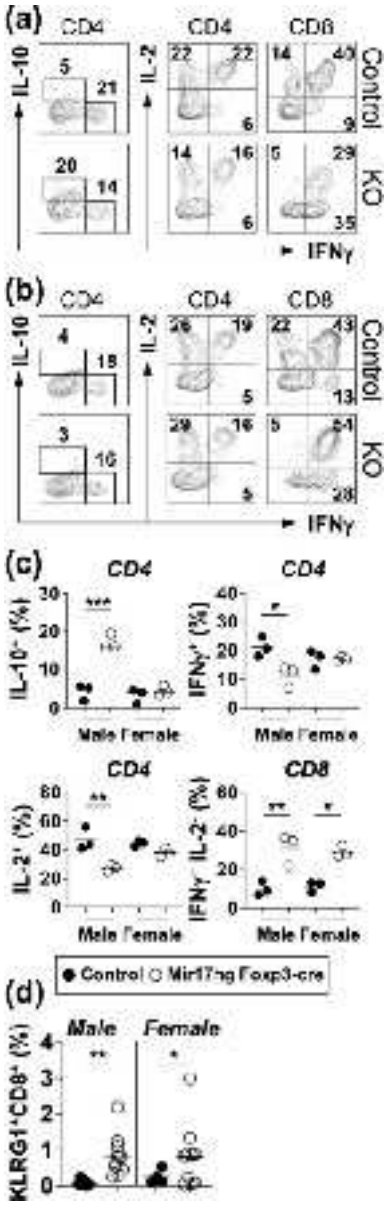

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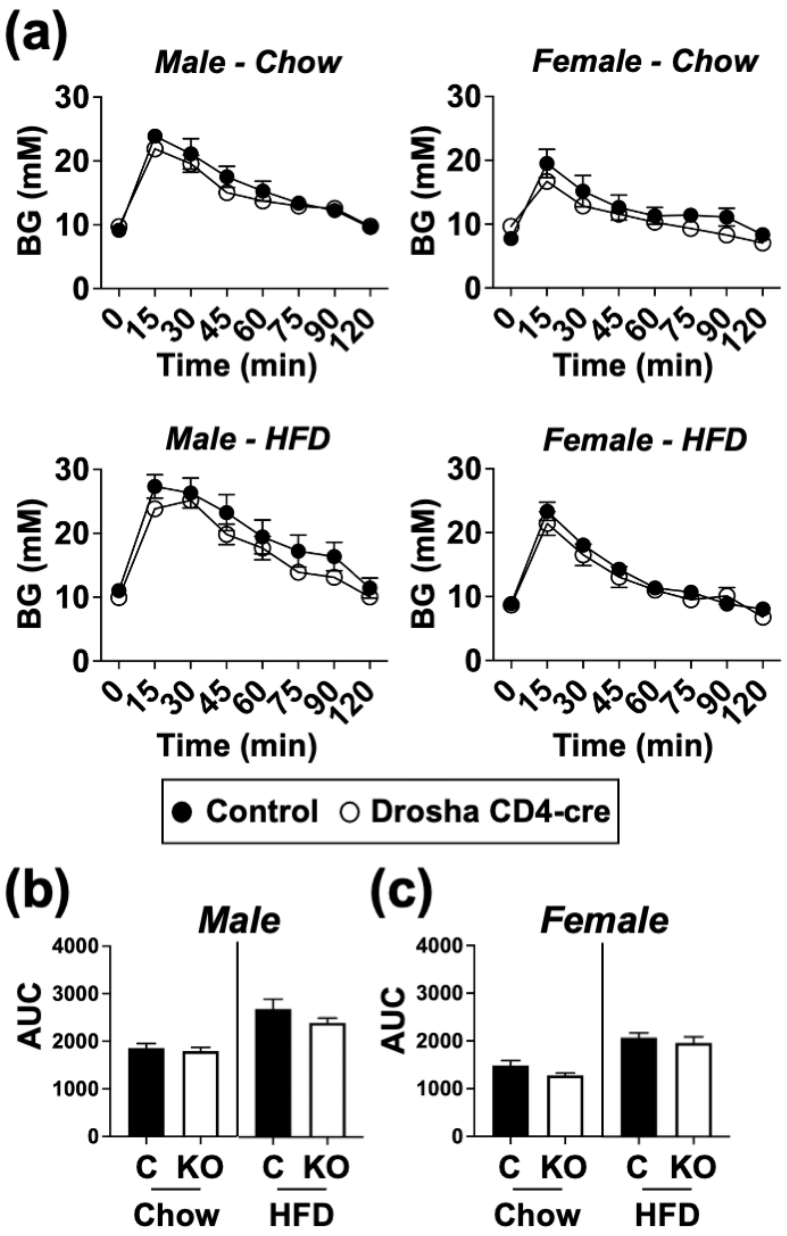

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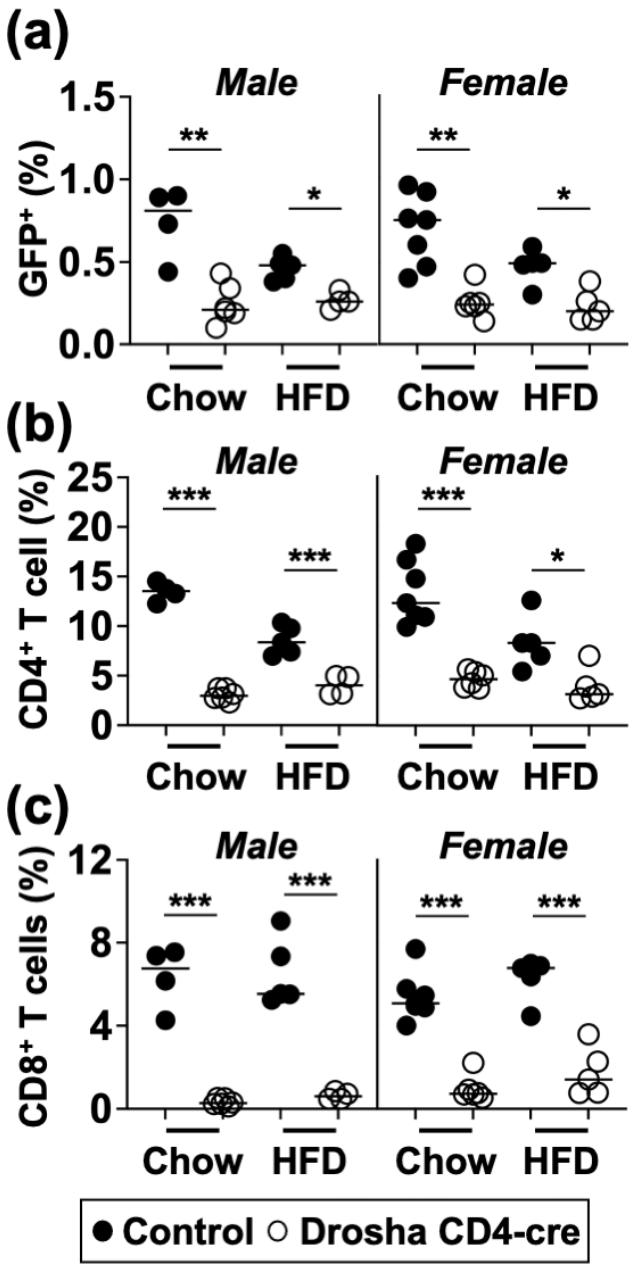

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