

Peer Review File

Manuscript Title: Rhythmic IL-17 production by $\gamma\delta$ T cells maintains adipose de novo lipogenesis

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Douglas et al. presents a set of experiments designed to explore how circadian expression of gd T cell-derived IL-17 associates with adipose tissue lipogenesis and body temperature. Notably, the authors present data indicating that expression of IL-17 by gd T cells, as well as their intrinsic mitochondrial metabolism, follows oscillatory patterns. gdT cell-derived IL-17 associated with adipose lipogenesis, while germline Il17 mutants displayed altered lipogenesis.

The manuscript includes a significant set of descriptive data and provides incremental insight on the role of IL -17 in lipid metabolism. Despite addressing an interesting hypothesis, the conceptual framework of this work is not entirely novel, and the manuscript contains conclusions that are not supported by the experimental data and approaches. For example, there is a general lack of formal mechanistic genetic links to sustain the main manuscript claims. Finally, the main parts of the text are fragmented and loosely connected, which further contributes to a manuscript that reads as a compilation of incomplete independent studies.

1. Fig.1 and 2 are mostly descriptive focusing on splenic and lymph node gd T cells. It is well established that tissue-resident lymphocyte signatures are significantly shaped by their local environment. Thus, descriptive analysis of diurnal expression patterns must be performed on gd T cell purified from adipose tissues.

2. In most tissues and immune cells, Arntl peaks at the transition between the active and rest phase; Nr1d1 peaks around the beginning of the light phase; and Per1 peaks at the transition between the rest and active phase. This is in large contrast to the data shown Figs. 1E, F and Suppl Figure 1C, which seemingly display a phase shift of these clock genes. This discrepancy likely results from the in vitro expansion and serum shock approach taken by the authors, which fails to represent the complex diurnal regulation of adipose-resident gd T cells. This approach is misleading.

3. By performing in vitro time course analysis from gd T cells purified at one single time point from the animal, the authors are not taking into account direct or indirect cues (neuronal, endocrine, feeding-related, etc) that may act on these cells beyond that specific timepoint in vivo. Thus, the expansion/serum shock strategy is not adequate to explore physiologically relevant oscillations in

adipose-tissue resident gd T cells. The authors must define the diurnal expression of clock genes and gdT cell functions and/or genetic signatures from purified adipose tissue gd T cells, ie, mRNA analysis of FACS purified adipose gdT cell in 4-6 timepoints/24h. Cosinor or akin statistical analysis must be also provided in full.

4. Fig.1 in vitro experiments. The presence of the grey area in the plots, which is assumed to represent the dark phase, should not be used since the zeitgeber in this case, is the time point of the serum shock. While these experiments can elucidate cell-intrinsic maintenance of diurnal oscillations in vitro, this claim must be validated in relevant in vivo settings. Animals must be put in constant darkness followed by mRNA analysis of FACS purified adipose gdT cell in 4-6 timepoints/24h. Only then can the authors claim that adipose gd circadian rhythms are self-sustained.

5. Data analysis presents inconsistencies that are puzzling. For example, in Suppl Figure 1B-D, clock gene expression is normalized to Ppib, while Actb is used for normalization in Figure 1E-H. Why this inconsistency? This makes interpretation of the data uncertain.

6. In Sup Fig 1F, ZT03, ZT15 and ZT18 do not display error bars. What is the sample size? This is not specified in the figure legend.

7. Figure 1M, diurnal expression of IL-17A (MFIs) produced by ex vivo adipose-resident gd T cells must be shown. IL-17F data should also be provided (% and MFIs). Are the total numbers of gd T cells and IL-17 producing gd t cells in the adipose tissue oscillating in a diurnal manner?

8. How do putative adipose gd T cell rhythms compare to the daily pattern of IL-17A production by other immune cells and in other adipose depots (BAT, scWAT and epiWAT)?

9. The authors extensively correlate NFIL3 expression levels in gd T cells with IL-17 expression, indicating that, similarly to Th cells, NFIL3 may regulate gd IL-17 in a circadian manner, presumably in a cell autonomous manner via Rorc. This discussion is not supported by the data and the literature is controversial in this regard. While germline NFIL3 mutants display altered Th IL-17 (Yu et al 2013), this is unlikely due to the T cell intrinsic clock machinery, as demonstrated by the analysis of the master circadian Arntl mutants indicating that Th17 differentiation is independent of the cell-intrinsic clock activity in Th cells (Hemmers et al 2015). Such claims by the authors require additional experimental evidence.

10. A vast majority of body cells display circadian oscillations. Thus, it is not novel to see circadian oscillation of IL17, as demonstrated by previous studies. The authors correlate circadian gene and IL17 circadian oscillation, but fail to demonstrate that IL-17 production by adipose gd T cells is regulated by the molecular clock. Understanding whether rhythms in IL-17 expression and production are dependent on a functional cell-intrinsic molecular clock or on cell-extrinsic circadian signals is critical. In vivo conditional deletion of a master circadian regulator, such as Arntl, is required to claim that "Circadian expression of IL-17A by adipose gd T cells" impacts lipogenesis as referred in the title or in the text, eg,

Lines 175-176: “While IL-17 production by lymph node $\gamma\delta$ T cells is regulated by the molecular clock...”. The authors do not present clear and unequivocal demonstration that IL17 production by adipose gd T cells is controlled by their circadian machinery.

11. Data shown in Fig 2 (metabolic phenotype of gd T cells) does not connect to the main message of the manuscript and is fully dispensable to main story. Still, it would be important to have a full diurnal characterization (4-6 timepoints) of the basal, max and spare respiration.

12. Figure 2B does not show that lipid uptake follows a diurnal oscillation, this is shown in Figure 2C. Please correct (line 151). What is the cause of gd T cells displaying increased mitochondrial metabolism during the light phase?

13. Fig 3K. The authors must perform a circadian characterization, analyzing 4-6 timepoints, in order to determine complete inversion of the circadian profile. This should be demonstrated by circadian statistical analysis of the parameter from the Cosinor model or using another suitable method (eg.: CircaCompare).

14. Inversion of light-dark cycles results in the inversion of whole-body behavior and physiology, including mouse feeding behavior. Since the adipose tissue can respond to many physiological signals, including feeding/fasting cycles, it is not clear whether IL-17 production by adipose tissue gd T cells is entrained by light, by feeding or by other cue (microbial, temperature...). Together with cell-intrinsic clock disruptions (see point 10), definition of extrinsic circadian factors contributing to gd circadian regulation is critical to sustain the authors' claims.

15. In Fig.4K the authors must demonstrate full inversion of IL-17A rhythms expression rhythms and provide circadian analysis, including values and statistics for the acrophase, etc.

16. Data in Figure 4I are representative of one independent experiment, n=2. These data need to be replicated, the n must be increased and statistical analysis must be provided. This raises important uncertainty about data analysis.

17. The novelty and excitement in the finding that gd T cell-derived IL-17 shapes lipid metabolism, is taken away by previous studies, from this group and others, showing a role for IL17a and IL17f in adipose tissue physiology. The use of double IL17a and IL17f mutants is incremental.

18. What is the outcome of IL17a/f $-/-$ mice upon cold exposure and/or high fat diet?

19. Do Tcrd $-/-$ mice display impaired de novo lipogenesis, altered respiratory exchange ratio (RER), blunted circadian RER rhythms, decreased body temperature / dampened circadian fluctuation of body temperature?

20. The authors do not demonstrate which IL-17-producing immune(s) effector(s) are mediating de novo

lipogenesis in the BAT. Whether this is mediated by gd T cells or by other(s) innate or adaptive IL-17-producing immune cells remains to be addressed. Cell-autonomous in vivo strategies altering gd IL-17 expression must be employed to support the author's claims.

21. Do mice with conditional ablation of the molecular clock in gd T cells recapitulate the de novo lipogenesis and whole-body metabolic phenotype of *Il17a/f -/-* mice?

Referee #2 (Remarks to the Author):

In the paper, Douglas and colleagues describe circadian expression of clock genes in innate T cells in the lymph node and white adipose tissue. Specifically, IL-17 is rhythmically expressed in these cells. Lack of both IL-17A and IL-17F reduces the amplitude in core body temperature in affected animals, yet the circadian rhythmicity remains.

While the link between IL-17 and circadian lipogenesis and body temperature is novel, it has previously been shown that IL-17 is important for thermogenesis by the authors themselves (PMID: 29670241). Since thermogenesis also is circadian (e.g. PMID: 19717669), it is thus not that surprising that there is a link between the two phenomena. Furthermore, the authors show a known circadian phenomenon - mitochondrial fission and fusion - in innate T cells. Thus, overall novelty is hampered by a combination of known circadian rhythms in a cell type where this has not been shown before.

The manuscript discusses circadian rhythms at length, yet there is no single functional circadian experiment performed, for example in the form of genetic ablation of circadian rhythms, ideally in a lineage-specific manner. In general, the manuscript is very descriptive, with the first functional intervention in the form of loss of IL-17 shown in Figure 4. Other data are shown in the main figure that seem to replicate each other (lymph node data and WAT data for gamma delta T cells), which should be placed (for the LN) in the supplements. Thus, the manuscript suffers from an overall lack of mechanistic insights.

Major points:

line 72: It is stated that the authors show that 'molecular clock components.... mediates circadian expression of ROR γ t, IL-17A, IL-17F, and their cellular metabolism'. There is no data in the paper showing that components of the clock mediate this circadian expression.

Line 77: '...demonstrate a crucial role for IL-17 which is strongly regulated by the molecular clock'. Again, there is no data supporting this claim.

Figure 1C: Clock genes exhibit strong time of day differences, with different clock genes peaking at

different times. It is thus not clear how data can be merged when the time of analysis is not known and a molecular clock gene score on the expression is provided. E.g. example Bmal1 expression is high when Per1 expression is low. It is not clear how these can then be merged. Clearly, in both cases clock genes are expressed and rhythmic expression of clock genes is occurring. How were these individual genes weighted? Given that these molecules are rhythmically expressed, the amplitude in the rhythm in each subset would be much more functionally relevant than mere expression. The legend would also need to be adapted as not all clock genes were analyzed, e.g. the clock genes Nr1d2, Cry2, Per2/3 are missing.

Figure 1E-H: light/dark conditions are indicated in grey shades; however, these experiments were performed in vitro without light input. Arntl should be high at the respective onset of the day not the night. These should be circadian time points and not Zeitgeber time points. What is ZT0 referring to? 0 h after serum shock? This is normally too early a time point after serum shock. The Y-axes should start at 0 or in case the Y-axis is cut, this should at least be indicated to not misrepresent relatively small oscillations (also for other Figures, e.g. 1K, 1M, 2K, 2L).

Figure 1I: These should be circadian time points as no Zeitgeber is present, i.e. time after serum shock. The general circadian terminologies should be applied to facilitate understanding. In the text, it should be stated how long cells were stimulated for. Line 124: How do the authors link the in vitro time points with the in vivo time points? The 'ZT18' should I presume be time after serum shock? Given then Arntl peaks in a phase-shifted manner in the previous data set, the authors need to verify which in vitro time point matches to which in vivo time point. In addition, the peak time point appears to be between 'ZT18' and 'ZT0'. The authors should include a 24h time point. In general, it should also always be stated whether samples were harvested from tissues and directly analyzed or whether they were kept in culture; this is not always clear (e.g. Figure 3B).

Figure 1L-N: The experimental setup is not clear - in general the paper would benefit from diagrams explaining the in vitro setups: Are T cells harvested and some are left for hours in vitro before being stimulated? Surely, this has an effect on cell phenotype and survival before the stimulation is performed?

Figure 2H: The images are not clear. There should be co-stainings and higher power analyses to be able to make any kind of claim.

Figure 3A: This is another representation of the same data shown in Figure 2D, which seems odd. Figure 2D should just be cited instead of showing Figure 3A.

Figure 3A-F: This is very similar data (just white adipose tissue) to lymph node data already shown in previous figures. The data should only be shown once in the main Figure with the LN data going into supplements.

Figure 3G-K: This data is not surprising. Light is the main entrainment factor of the body and mice (and humans) simply adjust to new time zones over several days. The authors should have tried to tease light

and food input apart, as the latter as the source of energy would be an important entrainment factor for adipose tissue, presumably more important than light.

line 226: The authors state that they investigate whether 'absence of circadian IL-17 signalling impacted the circadian rhythm of de novo lipogenesis'. They use IL17a/f double KO mice to assess this but this is not absence of circadian IL-17 signaling, this is general absence of IL-17 signaling. These assays are not specific enough.

lines 271: 'Here, we show that tissue resident innate T cells express a molecular clock, which is more prominent in type 17 cells compared to type 1 cells, and this is important for maintaining normal physiological responses which change between night and day.' This claim cannot be made as there is no functional assessment of the importance the circadian clock plays in this process.

Minor points:

line 70: It is stated that 'Adipose $\gamma\delta$ 17 T cells have the highest expression of molecular clock components across multiple organs'. Among which cells, all cells, leukocytes, innate lymphoid cells? This needs to be clarified as currently the statement is not correct.

line 42: It should be stated which organism is being discussed here, likely mice. In humans, peak body temperature occurs in the evening, just before going to bed.

It would be much easier to review when each figure is directly followed by the respective figure legend.

Line 130: "gdIFN γ T cells displayed a weak circadian rhythm for IFN γ production..." This figure (Fig S1F) shows a two star significance by the cosinor analysis. This is the same degree of significance that gd17 T cells showed for their diurnal IL-17 production in Figure 1M, however, the rhythm of IL-17 production was called "robust" (Line 128). The discrepancy in the adjectives does not reflect the similarity in the circadian nature of both IL-17 and IFN γ production by their respective cell types following PMAi stimulation.

Figure 3B-C: It is unclear if or how adipose gd17 T cells were stimulated. Please clarify in figure legend

Referee #3 (Remarks to the Author):

Douglas et al. provide evidence for the circadian control of adipose tissue de novo lipogenesis through oscillating IL-17A levels from resident $\gamma\delta$ T cells. While the impact of IL-17A and IL-17F on adipose tissue DNL is quite convincing, the link to circadian regulation of de novo lipogenesis is less clear. The following points should be considered to strengthen the manuscript.

Major points:

From a circadian clock standpoint, Figure 1 raises some major conceptual concerns/questions. One such consideration is whether the publicly available data used for the analysis, is from experiments/harvest performed at similar times of day, which would affect the interpretation.

Most significantly, however, in Figure 1D, all the clock genes are higher in the $\gamma\delta$ 17 T cells, including the repressor Nr1d1 and the activator Arntl. This is perplexing given that Nr1d1 and Arntl physiologically oscillate in opposition to one another because Nr1d1 represses Arntl (see CircaDB database of circadian transcriptomes of organs throughout the body by John Hogenesch). This means that when Nr1d1 peaks, Arntl is near its nadir. This is further evidenced in Figure 1E and 1F, where Arntl and Nr1d1 exhibit overlapping oscillations, which need to be explained.

As a general important comment, the graphs and text throughout the paper imply day/night in the oscillatory curves but to my understanding these are data almost exclusively from serum shocked cells which have been expanded 3 days in vitro? This gives a misleading impression and day/night or time-of-day should only be used when tissue or cell samples were isolated from animals at that particular time and analyzed. To that end, sorting and directly analyzing transcriptional rhythmicity in $\gamma\delta$ T cells from mice at four or more times of day would be the best way to assess physiologically relevant rhythmicity.

Given that post serum shock ZT5 and ZT17 time points don't appear to be the nadir and peak, it would be highly informative to examine the oscillatory respiration at other time points. It's also difficult to understand the rationale behind the 'clock gene scoring' in 3A as the expression of the clock genes are offset from one another and therefore wouldn't collectively accumulate? The only way I can think of this occurring is in an asynchronous state.

The reverse light cycle data in Figure 3 does quite nicely show the oscillation of IL-17A production but the conclusion that it is purely light entrained is inaccurate as the reverse light cycle after 3 weeks also involves a resetting of feeding and other behaviours which are known to synchronize peripheral clocks.

In Figure 4, why do the authors designate lipolysis/thermogenesis during the light phase when Mitch Lazar, Patrick Rensen, Paul Lee and others have shown that thermogenesis begins increasing just before the dark phase? The authors convincingly show the involvement of IL-17A in the regulation of DNL genes but the circadian rhythmicity is mechanistically questionable. Joseph Takahashi has shown that less than 50% of circadian transcriptional changes manifest into protein oscillation. This is particularly relevant for DNL in BAT where those enzymes are among the highest expressed in the entire proteome. Thus, it would be informative to determine the oscillation of some of the key DNL proteins. Also, is IL-17F circadian like IL-17A, given that cytokines can compensate for each other.

Author Rebuttals to Initial Comments:

Referee #1 (Remarks to the Author):

The manuscript by Douglas et al. presents a set of experiments designed to explore how circadian expression of gd T cell-derived IL-17 associates with adipose tissue lipogenesis and body temperature. Notably, the authors present data indicating that expression of IL-17 by gd T cells, as well as their intrinsic mitochondrial metabolism, follows oscillatory patterns. gdT cell-derived IL-17 associated with adipose lipogenesis, while germline Il17 mutants displayed altered lipogenesis.

The manuscript includes a significant set of descriptive data and provides incremental insight on the role of IL -17 in lipid metabolism. Despite addressing an interesting hypothesis, the conceptual framework of this work is not entirely novel, and the manuscript contains conclusions that are not supported by the experimental data and approaches. For example, there is a general lack of formal mechanistic genetic links to sustain the main manuscript claims. Finally, the main parts of the text are fragmented and loosely connected, which further contributes to a manuscript that reads as a compilation of incomplete independent studies.

We thank the reviewer for their positive points on the overview of our manuscript, and we agree all of their points and critique. The original submission was more descriptive, although we did identify a novel target of IL-17 action, *de novo lipogenesis*, which impacts the whole organism, which we believe is an important, new and unexpected finding. That said, the original manuscript lacked the genetic manipulation required to demonstrate circadian rhythm of IL-17 in gd using gold standard techniques, and the impact on adipose metabolism. The revised manuscript uses genetic manipulation through circadian reporter lines and immune specific knockout of circadian genes. The use of genetic manipulation has strengthened our findings and further highlights adipose IL-17 producing gd T cells as important circadian effectors in adipose homeostasis.

1. Fig.1 and 2 are mostly descriptive focusing on splenic and lymph node gd T cells. It is well established that tissue-resident lymphocyte signatures are significantly shaped by their local environment. Thus, descriptive analysis of diurnal expression patterns must be performed on gd T cell purified from adipose tissues.

The original figure focused on spleen and LN, but we have now made significant changes to the whole manuscript – the data on splenic and LN gds are provided in the supplementary figures. However, one important point from the original (and current Figure 1 A and B) is that usually tissue resident lymphocytes are shaped by their environment, and usually by scRNA seq, they cluster together with other cells from the same tissue, or with other cells of their lineage. In contrast, we have shown that all innate T cells that produce IL-17 cluster together, and not by tissue. This finding is novel, important and highlights the strong transcriptional imprint of innate-IL-17 producers regardless of tissue or cell type.

We thank the reviewer for their suggestion that circadian patterns should be analysed on purified gd T cells, and we agree. Isolation of adipose lymphocytes poses a challenges due to cell numbers. However, in the revised manuscript, we isolated adipose $\gamma\delta^{17}$ T cells at 4 time points over 24 hours for analysis of molecular clock genes throughout the 24 hr period (Figure 1G). Additionally, we used a Venus reporter mouse to quantify circadian oscillations of PER1 by adipose $\gamma\delta^{17}$ T cells at 4 timepoints from adipose tissue (Figure 1H-K). This analysis highlights how $\gamma\delta^{17}$ cells specifically, and not $\alpha\beta$ T cells, have a strong diurnal pattern in the circadian genes across three different adipose depots. This was not true of adipose $\alpha\beta$ T cells, or $\gamma\delta$ T cells that

produce IFN γ and agrees with our findings that adipose $\gamma\delta$ have the strongest circadian rhythm (Figure 1F).

2. In most tissues and immune cells, *Arntl* peaks at the transition between the active and rest phase; *Nr1d1* peaks around the beginning of the light phase; and *Per1* peaks at the transition between the rest and active phase. This is in large contrast to the data shown Figs. 1E, F and Suppl Figure 1C, which seemingly display a phase shift of these clock genes. This discrepancy likely results from the in vitro expansion and serum shock approach taken by the authors, which fails to represent the complex diurnal regulation of adipose-resident gd T cells. This approach is misleading.

We really appreciate the reviewers critique in pointing out the limitation in the serum shock approach. In complete agreement with the reviewer, we have instead isolated adipose $\gamma\delta^{17}$ T cells for circadian analysis of molecular clock genes, and by also using a Venus reporter for *Per1*, as mentioned above (Figure G-K).

3. By performing in vitro time course analysis from gd T cells purified at one single time point from the animal, the authors are not taking into account direct or indirect cues (neuronal, endocrine, feeding-related, etc) that may act on these cells beyond that specific timepoint in vivo. Thus, the expansion/serum shock strategy is not adequate to explore physiologically relevant oscillations in adipose-tissue resident gd T cells. The authors must define the diurnal expression of clock genes and gdT cell functions and/or genetic signatures from purified adipose tissue gd T cells, ie, mRNA analysis of FACS purified adipose gdT cell in 4-6 timepoints/24h. Cosinor or akin statistical analysis must be also provided in full.

In agreement with the reviewer, we have removed all data and figures from experiments that used serum shock. Instead we have FACS purified adipose $\gamma\delta^{17}$ T cells from 4 time points for circadian analysis of molecular clock genes, in addition to using a Venus reporter mouse for *PER1* and isolating adipose $\gamma\delta^{17}$ T cells at 4 different time points for flow cytometry analysis (Figure1 G-K). This data agrees with the original finding with serum shock method, but is a much better approach to isolate gd at different time points over 24hrs, as the reviewer pointed out. Cosinor analysis is performed on these new data and throughout the manuscript. We found adipose $\gamma\delta^{17}$ have a significant rhythm in circadian genes, while adipose $\alpha\beta$ T cells do not.

4. Fig.1 in vitro experiments. The presence of the grey area in the plots, which is assumed to represent the dark phase, should not be used since the zeitgeber in this case, is the time point of the serum shock. While these experiments can elucidate cell-intrinsic maintenance of diurnal oscillations in vitro, this claim must be validated in relevant in vivo settings.

We thank the reviewer for this advice. We have removed the data and figures from experiments that used serum shock due to the limitations pointed out by the reviewer. We sincerely believe that this reviewers advice has been invaluable in improving the circadian analysis of the manuscript.

5. Data analysis presents inconsistencies that are puzzling. For example, in Suppl Figure 1B-D, clock gene expression is normalized to *Ppib*, while *Actb* is used for normalization in Figure 1E-H. Why this inconsistency? This makes interpretation of the data uncertain.

We thank the reviewer for highlighting this discrepancy. This was due to simple human error by us, as *PPIB* is normally used as the housekeeping gene for adipose tissue qPCR analysis which is used throughout the manuscript, and was mistakenly switched for the cell specific

housekeeping gene β -actin. However, as previously mentioned all experiments with regards to the serum shock model have been removed from the manuscript due to the issues pointed out by the reviewer.

6. In Sup Fig 1F, ZT03, ZT15 and ZT18 do not display error bars. What is the sample size? This is not specified in the figure legend.

The data is from 4 individual mice, and it is representative of 2 independent experiments (n=8 mice total). The previous Sup Fig 1F (which is now Sup Fig 2I) did not display error bars at ZT03, ZT15 or ZT18 due to a very small spread of the data. The manuscript has been updated to reflect this in the figure legend (Supplementary figure 2).

7. Figure 1M, diurnal expression of IL-17A (MFIs) produced by ex vivo adipose-resident gd T cells must be shown. IL-17F data should also be provided (% and MFIs). Are the total numbers of gd T cells and IL-17 producing gd T cells in the adipose tissue oscillating in a diurnal manner?

Diurnal expression of ex vivo gd17 is shown in Figure 2A and B, as well as with reporter mice for IL-17A (Figure 5C). The primary focus of this manuscript is IL-17A expression by adipose $\gamma\delta^{17}$ T cells, as data shows that IL17A is the primary IL-17 family member to induce de novo lipogenesis. However often gd17 produce both IL17A and IL-17F at the same time, and in some cases we have also captured the IL-17F expression, which is also regulated by the molecular clock, and included it in the manuscript (example Figure 2H). In response to the reviewers second point, we find that adipose $\gamma\delta^{17}$ T cell numbers do not oscillate in adipose tissue. We have included the oscillations of adipose $\gamma\delta$ T cells (Supplementary Figure 3A, B).

8. How do putative adipose gd T cell rhythms compare to the daily pattern of IL-17A production by other immune cells and in other adipose depots (BAT, scWAT and epiWAT)?

This is an excellent point which we have now included. The main producers of IL-17 in adipose tissue are $\gamma\delta^{17}$ T cells (80% of IL-17 producers are $\gamma\delta^{17}$), more so than Th17, NKT17 or ILC3s. However, we suspect that NKT17 and MAIT17 may also have a circadian rhythm to their IL-17 production as these innate sources of IL-17 are also high in expression of molecular clock genes (Figure 1A-F). In particular Figure 1F shows that adipose NKT17 and MAIT17 are also enriched in molecular clock genes. Furthermore, Figure 5A-C shows that $\gamma\delta^{17}$ have diurnal expression of IL-17 in BAT, scWAT and epiWAT. We have now incorporated PER1 expression by adipose $\gamma\delta^{17}$ T cells in epiWAT, scWAT and mesWAT, in addition to using mice with a specific ablation of BMAL1 in the CD45 compartment in epiWAT, scWAT and IELs and the effects are most evident in $\gamma\delta^{17}$ T cells in adipose tissue (Figure 2 J-S).

9. The authors extensively correlate NFIL3 expression levels in gd T cells with IL-17 expression, indicating that, similarly to Th cells, NFIL3 may regulate gd IL-17 in a circadian manner, presumably in a cell autonomous manner via Rorc. This discussion is not supported by the data and the literature is controversial in this regard. While germline NFIL3 mutants display altered Th IL-17 (Yu et al 2013), this is unlikely due to the T cell intrinsic clock machinery, as demonstrated by the analysis of the master circadian Arntl mutants indicating that Th17 differentiation is independent of the cell-intrinsic clock activity in Th cells (Hemmers et al 2015). Such claims by the authors require additional experimental evidence.

We thank the reviewer for the insightful comments on NFIL3 interaction with the molecular clock. We have made changes in the manuscript to simply acknowledge we have analysed the circadian expression of NFIL3 by $\gamma\delta^{17}$ T cells, without any further correlations.

10. A vast majority of body cells display circadian oscillations. Thus, it is not novel to see circadian oscillation of IL17, as demonstrated by previous studies. The authors correlate circadian gene and IL17 circadian oscillation, but fail to demonstrate that IL-17 production by adipose gd T cells is regulated by the molecular clock. Understanding whether rhythms in IL-17 expression and production are dependent on a functional cell-intrinsic molecular clock or on cell-extrinsic circadian signals is critical. In vivo conditional deletion of a master circadian regulator, such as *Arntl*, is required to claim that “Circadian expression of IL-17A by adipose gd T cells” impacts lipogenesis as referred in the title or in the text, eg, Lines 175-176: “While IL-17 production by lymph node $\gamma\delta$ T cells is regulated by the molecular clock...”. The authors do not present clear and unequivocal demonstration that IL17 production by adipose gd T cells is controlled by their circadian machinery.

We thank the reviewer for the helpful comment. We have now analysed IL-17A expression by adipose $\gamma\delta^{17}$ T cells which have been treated with the REV-ERB α agonist SR9009 (Figure 2 D-I; Supplementary Figure 3). Furthermore, we have quantified IL-17A expression by knocking out BMAL1 in immune cells (*Bmal1* ^{Δ Vav1}; Figure 2J-P), and additionally in cells expressing IL-7R (*Bmal1* ^{Δ Il7r}; Figure 2Q-S), of which adipose $\gamma\delta^{17}$ s are enriched. Additionally, we found that increased IL-17A expression in the BAT of *Bmal1* ^{Δ Il7r} correlated with increased *de novo* lipogenesis gene expression. We hope that this demonstrates that adipose $\gamma\delta^{17}$ T cells have a functional molecular clock which modulates IL-17A expression.

11. Data shown in Fig 2 (metabolic phenotype of gd T cells) does not connect to the main message of the manuscript and is fully dispensable to main story. Still, it would be important to have a full diurnal characterization (4-6 timepoints) of the basal, max and spare respiration.

The experiments on circadian cellular metabolism can be included in the supplement or removed. In the revised version they are removed. We agree they are not critical to the main message, but are just linked to the functional readout of $\gamma\delta^{17}$ which is linked to their intrinsic metabolism.

12. Figure 2B does not show that lipid uptake follows a diurnal oscillation, this is shown in Figure 2C. Please correct (line 151). What is the cause of gd T cells displaying increased mitochondrial metabolism during the light phase?

As above, experiments on circadian cellular metabolism have been removed, but could be included in the supplement.

13. Fig 3K. The authors must perform a circadian characterization, analyzing 4-6 timepoints, in order to determine complete inversion of the circadian profile. This should be demonstrated by circadian statistical analysis of the parameter from the Cosinor model or using another suitable method (eg.: CircaCompare).

We thank the reviewer for the suggestion, and we have carried out this experiment in two independent experiments. We isolated adipose $\gamma\delta^{17}$ T cells from normal light and light inverted mice across 4 time points for characterisation of circadian IL-17A expression (Figure 3E), in addition to parameters of metabolic rate (Figure B-D). All statistical analyses were performed using the Cosinor model which are now presented.

14. Inversion of light-dark cycles results in the inversion of whole-body behavior and physiology, including mouse feeding behavior. Since the adipose tissue can respond to many physiological signals, including feeding/fasting cycles, it is not clear whether IL-17 production by adipose tissue

gd T cells is entrained by light, by feeding or by other cue (microbial, temperature...). Together with cell-intrinsic clock disruptions (see point 10), definition of extrinsic circadian factors contributing to gd circadian regulation is critical to sustain the authors' claims.

We thank the reviewer for raising an important and interesting point, and we have performed multiple additional experiments to answer this question. To tease apart the regulation of light and feeding behaviour, we performed additional extensive experiments in metabolic cages. We have measured feeding in the light inverted experiments, in addition to performing inverted feeding experiments, where some mice only have access to food during the day (their inactive phase). We found this disrupted adipose IL-17 expression by $\gamma\delta^{17}$ T cells and whole-body circadian metabolic rhythm (Figure 3H-N). Thus disrupting feeding rhythm also disrupts IL-17 rhythm (Figure 3L). However, only having access to food during the light period caused reduced food intake. Therefore, we also carried out caloric restriction studies alongside the inverted feeding studies and found that caloric restriction caused reduced IL-17 but not a loss of IL-17 rhythm. Mice fed reduced calories retain their metabolic rhythm also – so in summary, disrupted feeding disrupts IL-17 rhythm. We believe that these additional experiments provide important insights for the manuscript.

15. In Fig.4K the authors must demonstrate full inversion of IL-17A rhythms expression rhythms and provide circadian analysis, including values and statistics for the acrophase, etc.

We agree with the reviewer's suggestion and this has been performed. We have isolated adipose $\gamma\delta^{17}$ T cells from regular light and light inverted mice across 4 time points for characterisation of circadian IL-17A expression (Figure 3G), in addition to parameters of metabolic rate (Figure 4B-C; Supplementary Figure 4A). All statistical analyses were performed using the cosinor model, amplitude and acrophase inclusive.

16. Data in Figure 4I are representative of one independent experiment, n=2. These data need to be replicated, the n must be increased and statistical analysis must be provided. This raises important uncertainty about data analysis.

These data have been replicated now and the findings agree with the original data. With the new revision experiments, these have been moved to the supplementary figures as they are not the main focus of the manuscript.

17. The novelty and excitement in the finding that gd T cell-derived IL-17 shapes lipid metabolism, is taken away by previous studies, from this group and others, showing a role for IL17a and IL17f in adipose tissue physiology. The use of double IL17a and IL17f mutants is incremental.

We respectfully disagree with the reviewer on this point. The previous manuscript by Kohlgruber et al focused on the identification of this clonal gd population in adipose for the first time. Using parabiotic experiments, we showed this adipose gd population was adipose tissue resident and clonal. We found that gd T cells and IL-17 was important for response to cold challenge. This current manuscript contains all novel findings. Firstly, we show that innate IL-17 producers are enriched for molecular clock genes compared to innate Th1-like cells, particularly in adipose tissue. We next describe a role for diurnal IL-17 production in adipose tissue, and then identify the mechanism, that IL-17 is produced nightly (in mice) in response to light and feeding, which targets adipose tissue de novo lipogenesis, which is critical for storing glucose as lipids. Without IL-17, there is whole-body disruption in adipose de novo lipogenesis. Although our previous study identified adipose $\gamma\delta^{17}$ T cells, their function was primarily examined in the context of cold challenge, and the role for $\gamma\delta^{17}$ T cells producing IL-17 daily for adipose tissue homeostasis was

not realised. This was also true for the Spiegelman paper who examined the role of gd17 in thermogenesis during cold, and the Spiegelman lab focused on IL-17F during cold, while we focused on IL-17A during cold. The addition of IL-17A and IL-17F double knockout is not intended to be an advance but rather a better model to study the role of IL-17 in adipose tissue as IL17A and IL-17F compensate for each other and both are produced by adipose $\gamma\delta^{17}$ T cells. The phenotype in the double knockout is stronger than either cytokine alone, and this mouse strain has never been examined in adipose tissue.

18. What is the outcome of Il17a/f^{-/-} mice upon cold exposure and/or high fat diet?

Previous work from our lab and the Spiegelman lab have demonstrated that Il17a^{-/-} and Il17f^{-/-} mice are susceptible to cold challenge. We were initially surprised that IL-17 peaked at night during lipogenesis, however, work from Dr Mitchel Lazar's lab has shown that defects in de novo lipogenesis have knock on effects for lipolysis. We also repeated the cold challenge on IL-17af^{-/-} mice and found they are also susceptible to cold challenge.

The effect of HFD on Il17a/f^{-/-} mice is currently under investigation in our lab, and data so far shows that they are resistant to weight gain. We hypothesise that this is due to defects in lipogenesis (this study could potentially be combined here, but it does not focus on circadian rhythm). In addition, we performed short-term HFD experiments (3-weeks) for this manuscript to examine the effects of blunted circadian rhythm of RER. We found that a 3-week HFD feeding regimen inhibited the circadian rhythm of RER and was sufficient to disrupt IL-17A signalling, with increased levels of IL-17 at all times over 24 hours (Figure 4D, E).

19. Do Tcrd^{-/-} mice display impaired de novo lipogenesis, altered respiratory exchange ratio (RER), blunted circadian RER rhythms, decreased body temperature / dampened circadian fluctuation of body temperature?

In response to the reviewer's comment, we performed these experiments on Tcrd^{-/-} mice and have included this data in the manuscript. Tcrd^{-/-} mice also have disrupted de novo lipogenesis (Figure 5F-H). This agrees with $\gamma\delta^{17}$ T cells being the main producers of IL-17 in adipose tissue.

20. The authors do not demonstrate which IL-17-producing immune(s) effector(s) are mediating de novo lipogenesis in the BAT. Whether this is mediated by gd T cells or by other(s) innate or adaptive IL-17-producing immune cells remains to be addressed. Cell-autonomous in vivo strategies altering gd IL-17 expression must be employed to support the author's claims.

We thank the reviewer for the thought provoking comment. We now show that $\gamma\delta^{17}$ T cells are the main producers of IL-17 in adipose tissue, of total lymphocytes (Figure 5A, B), and that Tcrd^{-/-} mice display a disrupted rhythm to de novo lipogenesis genes in BAT.

21. Do mice with conditional ablation of the molecular clock in gd T cells recapitulate the de novo lipogenesis and whole-body metabolic phenotype of Il17a/f^{-/-} mice?

We have not been able to generate $\gamma\delta$ -specific knock-out of clock genes, as tools to specifically knockout genes on $\gamma\delta$ T cells are limited compared to Tregs and other $\alpha\beta$ T cells. However, we have used molecular clock specific deletion in CD45⁺ cells (BMAL1 Δ^{Vav1}), in addition to IL-7R expressing cells. In adipose tissue, $\gamma\delta^{17}$ T cells express the most IL-7R.

Referee #2 (Remarks to the Author):

In the paper, Douglas and colleagues describe circadian expression of clock genes in innate T cells in the lymph node and white adipose tissue. Specifically, IL-17 is rhythmically expressed in these cells. Lack of both IL-17A and IL-17F reduces the amplitude in core body temperature in affected animals, yet the circadian rhythmicity remains.

While the link between IL-17 and circadian lipogenesis and body temperature is novel, it has previously been shown that IL-17 is important for thermogenesis by the authors themselves (PMID: 29670241). Since thermogenesis also is circadian (e.g. PMID: 19717669), it is thus not that surprising that there is a link between the two phenomena. Furthermore, the authors show a known circadian phenomenon - mitochondrial fission and fusion - in innate T cells. Thus, overall novelty is hampered by a combination of known circadian rhythms in a cell type where this has not been shown before.

The manuscript discusses circadian rhythms at length, yet there is no single functional circadian experiment performed, for example in the form of genetic ablation of circadian rhythms, ideally in a lineage-specific manner. In general, the manuscript is descriptive, with the first functional intervention in the form of loss of IL-17 shown in Figure 4. Other data are shown in the main figure that seem to replicate each other (lymph node data and WAT data for gamma delta T cells), which should be placed (for the LN) in the supplements. Thus, the manuscript suffers from an overall lack of mechanistic insights.

We thank the reviewer for their comments, and have completely revised the manuscript in light of all of the reviewer comments. Firstly, we agree that there was some redundancy between data shown for both lymph node and adipose $\gamma\delta^{17}$ T cells, therefore all lymph node data has been moved to the supplemental figures, with a primary focus on adipose $\gamma\delta^{17}$ T cells. We have now also included functional manipulations of the molecular clock, including the use of a REV-ERB α agonist SR9009, in addition to mice lacking BMAL1 expression in immune cells expressing CD45 (BMAL1 Δ^{Vav1}) and all cells expressing IL-7R (BMAL1 Δ^{IL7R}), as adipose $\gamma\delta^{17}$ T cells are the main lymphocytes expressing IL-7R in adipose tissue. We also use a Per1-Venus reporter mouse, and IL-17 GFP reporter mouse to illustrate that adipose $\gamma\delta^{17}$ T cells are specifically and strongly regulated by the molecular clock, more so than $\alpha\beta$ T cells in adipose tissue, and more than $\gamma\delta$ T cells elsewhere.

Major points:

line 72: It is stated that the authors show that 'molecular clock components.... mediates circadian expression of ROR γ t, IL-17A, IL-17F, and their cellular metabolism'. There is no data in the paper showing that components of the clock mediate this circadian expression.

Line 77: '...demonstrate a crucial role for IL-17 which is regulated by the molecular clock'. Again, there is no data supporting this claim.

We have now analysed IL-17A expression by adipose $\gamma\delta^{17}$ T cells which have been treated with the REV-ERB α agonist SR9009 (Figure 2 D-I; Supplementary Figure 3). Furthermore, we have quantified IL-17A expression by knocking out BMAL1 in all cells expressing CD45 (Bmal1 Δ^{Vav1} ; Figure 2J-P), and in IL-7R expressing cells (Bmal1 Δ^{IL7R} ; Figure 2Q-S). Additionally, we found that increased IL-17A expression in the BAT of Bmal1 Δ^{IL7R} correlated with increased *de novo* lipogenesis gene expression. This demonstrates that adipose $\gamma\delta^{17}$ T cells have a functional molecular clock which modulates IL-17A expression.

Figure 1C: Clock genes exhibit strong time of day differences, with different clock genes peaking at different times. It is thus not clear how data can be merged when the time of analysis is not known and a molecular clock gene score on the expression is provided. E.g. example Bmal1 expression is high when Per1 expression is low. It is not clear how these can then be merged. Clearly, in both cases clock genes are expressed and rhythmic expression of clock genes is occurring. How were these individual genes weighted? Given that these molecules are rhythmically expressed, the amplitude in the rhythm in each subset would be much more functionally relevant than mere expression. The legend would also need to be adapted as not all clock genes were analyzed, e.g. the clock genes Nr1d2, Cry2, Per2/3 are missing.

We thank the reviewer for these important points which we have now addressed. Initially we did not split the innate T cell populations based on the paper that they were from, making the assumption that scSeq run was performed during the day time. However, our lab generated both the adipose and spleen scSeq datasets, and therefore know that these were run at the same time of day for the different subsets of gd T cells, and other innate t cells subsets, and different organs from the same mice. Therefore, we performed the analysis with the molecular clock score, broken down into the individual datasets (Figure 1E, Supplementary Figure 1), and find that type-17 innate T cells are enriched for molecular clock genes compared to type-1 innate lymphocytes harvested at the same time from the same mice. In agreement with the reviewer, we now include the additional genes, and the molecular clock score was adjusted to include Nr1d2, Cry2, Per2/3.

Figure 1E-H: light/dark conditions are indicated in grey shades; however, these experiments were performed in vitro without light input. Arntl should be high at the respective onset of the day not the night. These should be circadian time points and not Zeitgeber time points. What is ZT0 referring to? 0 h after serum shock? This is normally too early a time point after serum shock. The Y-axes should start at 0 or in case the Y-axis is cut, this should at least be indicated to not misrepresent relatively small oscillations (also for other Figures, e.g. 1K, 1M, 2K, 2L).

All experiments using the serum shock model have been removed from the manuscript due to the issues and limitations highlighted by the reviewers.

Figure 1I: These should be circadian time points as no Zeitgeber is present, i.e. time after serum shock. The general circadian terminologies should be applied to facilitate understanding. In the text, it should be stated how long cells were stimulated for. Line 124: How do the authors link the in vitro time points with the in vivo time points? The 'ZT18' should I presume be time after serum shock? Given then Arntl peaks in a phase-shifted manner in the previous data set, the authors need to verify which in vitro time point matches to which in vivo time point. In addition, the peak time point appears to be between 'ZT18' and 'ZT0'. The authors should include a 24h time point. In general, it should also always be stated whether samples were harvested from tissues and directly analyzed or whether they were kept in culture; this is not always clear (e.g. Figure 3B).

We thank the reviewer for pointing out the flaw in the serum shock approach. We have rectified this by isolating adipose $\gamma\delta 17$ T cells at 4 time points for circadian analysis of molecular clock genes (Figure 1G). Additionally, we used a Venus reporter mouse to quantify circadian oscillations of PER1 by adipose $\gamma\delta 17$ T cells (Figure 1H-K), thus removing any confusion with correlating *in vitro* models with *in vivo* models.

Figure 1L-N: The experimental setup is not clear - in general the paper would benefit from diagrams explaining the in vitro setups: Are T cells harvested and some are left for hours in vitro before being stimulated? Surely, this has an effect on cell phenotype and survival before the stimulation is performed?

We thank the reviewer for the suggestion, and have now incorporated experimental setups into the manuscript to make understanding of the protocols easier, for example in Figure 3.

Figure 2H: The images are not clear. There should be co-stainings and higher power analyses to be able to make any kind of claim.

The experiments with circadian cellular metabolism have been removed or can be added to the supplement as we felt they were not central to the main point of the paper.

Figure 3A: This is another representation of the same data shown in Figure 2D, which seems odd. Figure 2D should just be cited instead of showing Figure 3A. Figure 3A-F: This is very similar data (just white adipose tissue) to lymph node data already shown in previous figures. The data should only be shown once in the main Figure with the LN data going into supplements.

We have taken the reviewers comment into account and have moved all lymph node data to the supplementary figures to avoid redundancy and to highlight the main focus on adipose tissue $\gamma\delta$ T cells, which are most highly enriched for clock genes (Figure 1F and Figure 1I-K)), and regulated by the clock (Figure 2D-L) as evidenced by *Per1* reporter mice, and *Arntl-Vav-Cre* mice.

Figure 3G-K: This data is not surprising. Light is the main entrainment factor of the body and mice (and humans) simply adjust to new time zones over several days. The authors should have tried to tease light and food input apart, as the latter as the source of energy would be an important entrainment factor for adipose tissue, presumably more important than light.

We agree that light entrainment was not surprising, although still necessary to test. The reviewer also makes an excellent point that food and light are intricately linked, and we initially hypothesised the same as the reviewer - that food would be more important than light for adipose entrainment. We performed several different experimental approaches to tease apart the regulation of light and feeding behaviour. We performed experiments with inverted feeding, where one group of mice could only access food during the day and the other group during the night. and we found this disrupted adipose IL-17 expression by $\gamma\delta^{17}$ T cells and whole-body circadian metabolic rhythm (Figure 3H-N). We also found this caused significant reduction in food intake which could also be the cause for altered IL-17 production. Therefore, we performed calorie restriction experiments to match the reduced food intake from the reverse feeding, but to feed this reduced amount at night as usual. This calorie restriction caused a slight decrease in IL17 but did not cause a disruption in IL-17 rhythm, or in whole-body rhythm (although did cause differences in whole-body metabolism, but rhythm was maintained). Thus, we conclude from the experiments that food behaviour is an important factor in IL-17 diurnal production.

line 226: The authors state that they investigate whether 'absence of circadian IL-17 signalling impacted the circadian rhythm of de novo lipogenesis'. They use IL17a/f double KO mice to assess this but this is not absence of circadian IL-17 signaling, this is general absence of IL-17 signaling. These assays are not specific enough.

We have not been able to generate $\gamma\delta$ -specific KO mouse models, which are also lacking in the $\gamma\delta$ -field. Instead, we have used mice with a *Bmal* deletion specifically in all CD45 expressing cells (*BMAL1 Δ^{Vav1}*), and also in IL-7R expressing cells. In adipose tissue, $\gamma\delta^{17}$ T cells are the main cells expressing IL-7R and thus, this is the closest we could manage to get with a $\gamma\delta$ -specific molecular clock knock-out, as well as using $\gamma\delta$ T cell KO mice.

lines 271: 'Here, we show that tissue resident innate T cells express a molecular clock, which is more prominent in type 17 cells compared to type 1 cells, and this is important for maintaining normal physiological responses which change between night and day.' This claim cannot be made as there is no functional assessment of the importance the circadian clock plays in this process.

We agree, and we have now addressed this using in vivo genetic manipulation, and ex vivo with inhibitors of clock components. We analysed IL-17A expression by adipose $\gamma\delta 17$ T cells treated with the REV-ERB α agonist SR9009 (Figure 2 D-I; Supplementary Figure 3). We found that a REV-ERB α agonist almost completely inhibits IL-17A and IL-17F production, with several methods of stimulation, without impacting ROR γ t expression. We next used genetically modified mouse models (Bmal1 Δ^{Vav1} ; Figure 2J-P), and more specifically in IL-7R expressing cells (Bmal1 Δ^{IL7R} ; Figure 2Q-S). We found that loss of Bmal in immune cells or T cells specifically impacts gd17 cells, and does not impact gd T cells that produce IFN γ , and does not impact $\alpha\beta$ T cells in adipose tissue. Thus, the new data strongly agrees with the conclusion that adipose resident innate T cells are specifically controlled by the clock compared to $\gamma\delta$ T cells elsewhere and compared to other T cells in adipose tissue.

Minor points:

line 70: It is stated that 'Adipose $\gamma\delta 17$ T cells have the highest expression of molecular clock components across multiple organs'. Among which cells, all cells, leukocytes, innate lymphoid cells? This needs to be clarified as currently the statement is not correct.

We apologise for the confusion and have made it more clear in the manuscript that adipose $\gamma\delta^{17}$ T cells have the highest molecular clock expression among the innate lymphocytes, as shown in figure 1.

line 42: It should be stated which organism is being discussed here, likely mice. In humans, peak body temperature occurs in the evening, just before going to bed.

We have altered the manuscript to make it more clear that mice are the main organism being used throughout the manuscript.

It would be much easier to review when each figure is directly followed by the respective figure legend.

Line 130: "gdIFN γ T cells displayed a weak circadian rhythm for IFN γ production..." This figure (Fig S1F) shows a two star significance by the cosinor analysis. This is the same degree of significance that gd17 T cells showed for their diurnal IL-17 production in Figure 1M, however, the rhythm of IL-17 production was called "robust" (Line 128). The discrepancy in the adjectives does not reflect the similarity in the circadian nature of both IL-17 and IFN γ production by their respective cell types following PMAi stimulation.

We apologise for the confusion, we were referring to the difference in amplitude of the IL-17 and IFN γ waves. We have changed our wording in the manuscript to improve understanding.

Figure 3B-C: It is unclear if or how adipose gd17 T cells were stimulated. Please clarify in figure legend

PMA and ionomycin or cytokine stimulation are performed throughout the manuscript, and are now stated in the figure and figure legend and methods.

Referee #3 (Remarks to the Author):

Douglas et al. provide evidence for the circadian control of adipose tissue de novo lipogenesis through oscillating IL-17A levels from resident $\gamma\delta$ T cells. While the impact of IL-17A and IL-17F on adipose tissue DNL is quite convincing, the link to circadian regulation of de novo lipogenesis is less clear. The following points should be considered to strengthen the manuscript.

Major points

From a circadian clock standpoint, Figure 1 raises some major conceptual concerns/questions. One such consideration is whether the publicly available data used for the analysis, is from experiments/harvest performed at similar times of day, which would affect the interpretation.

We thank the reviewer for this important point. We have now split all innate T cell populations based on the paper that they were from, making the assumption that the scSeq run was performed at the same time of the day in the individual labs. Our lab generated both the adipose and spleen scSeq datasets, and therefore know that these were run at the same time of day (i.e. adipose and spleen subsets were isolated from the same mice at the same time). Here, we performed the analysis with the molecular clock score showing that from the same mice at the same time, type-17 innate T cells are enriched for molecular clock genes compared to type-1 innate lymphocytes. We have also done this breaking down the individual datasets. (Figure 1E, Supplementary Figure 1). Thus, separately and combined, type-17 cells are enriched in molecular clock genes, and the data with the genetic manipulation with Bmal in lymphocytes also agrees with this. We also adjusted the molecular clock score to include Nr1d2, Cry2, and Per2/3.

Most significantly, however, in Figure 1D, all the clock genes are higher in the $\gamma\delta$ 17 T cells, including the repressor Nr1d1 and the activator Arntl. This is perplexing given that Nr1d1 and Arntl physiologically oscillate in opposition to one another because Nr1d1 represses Arntl (see CircaDB database of circadian transcriptomes of organs throughout the body by John Hogenesch). This means that when Nr1d1 peaks, Arntl is near its nadir. This is further evidenced in Figure 1E and 1F, where Arntl and Nr1d1 exhibit overlapping oscillations, which need to be explained.

Figure 1D shows a heatmap of expression of the molecular clock genes which is relative to each other. In order to further define this, we have separated the T cell populations as stated above. Although the molecular clock genes do indeed fluctuate throughout the day, the expression of these is always higher in adipose $\gamma\delta$ ¹⁷ T cells. Indeed, using Venus reporter mice for PER1, we observe that PER1 expression by adipose $\gamma\delta$ T cells is always more highly expressed than in adipose $\alpha\beta$ T cells, even during the time of day when its expression is lowest. Thus, $\gamma\delta$ ¹⁷ T cells may have higher expression of molecular clock genes at all times of day compared to other adipose innate lymphocytes, although they do fluctuate during 24 hours as expected. We believe the data supports that this is because adipose $\gamma\delta$ ¹⁷ T cells are most strongly regulated by the clock compared to other cells.

As a general important comment, the graphs and text throughout the paper imply day/night in the oscillatory curves but to my understanding these are data almost exclusively from serum shocked cells which have been expanded 3 days in vitro? This gives a misleading impression and day/night or time-of-day should only be used when tissue or cell samples were isolated from animals at that particular time and analyzed. To that end, sorting and directly analyzing transcriptional rhythmicity

in $\gamma\delta$ T cells from mice at four or more times of day would be the best way to assess physiologically relevant rhythmicity.

We agree with the reviewer, and we have made extensive revisions, to remove serum shock as a model and replace it with analysis on immune cells isolated from adipose and other organs at 4 time points through the day/night. (In the previous version of the paper, most of the graphs had shown day/night oscillatory curves from tissues harvested at those exact time of day, with only figure one showing data from a serum shock model).

Given that post serum shock ZT5 and ZT17 time points don't appear to be the nadir and peak, it would be highly informative to examine the oscillatory respiration at other time points. It's also difficult to understand the rationale behind the 'clock gene scoring' in 3A as the expression of the clock genes are offset from one another and therefore wouldn't collectively accumulate? The only way I can think of this occurring is in an asynchronous state.

We have removed all serum shock data from the revised paper due to the limitations raised by all reviewers. Regarding the clock gene score, $\gamma\delta^{17}$ T cells have higher expression of molecular clock genes at all times of day compared to other adipose innate lymphocytes, as well as having the expected fluctuations over 24 hours. This is supported by the Per1-Venus reporter data in Figure 1.

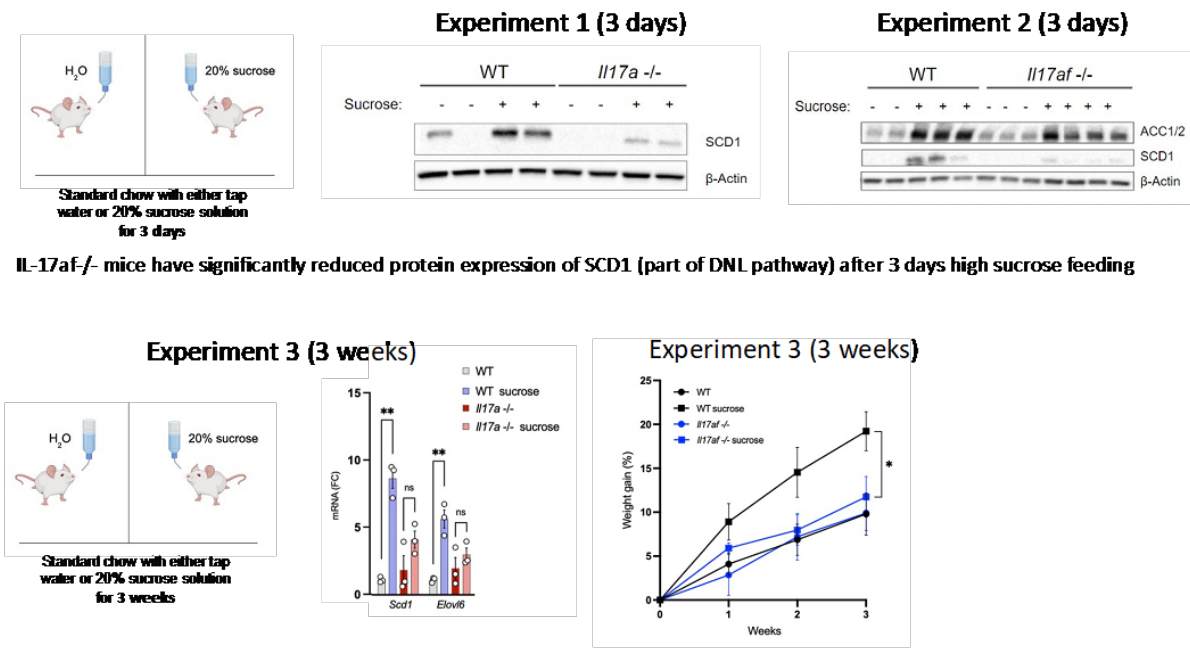
The reverse light cycle data in Figure 3 does quite nicely show the oscillation of IL-17A production but the conclusion that it is purely light entrained is inaccurate as the reverse light cycle after 3 weeks also involves a resetting of feeding and other behaviours which are known to synchronize peripheral clocks.

We thank the reviewer for raising an important and interesting point which promoted us to more extensively investigate entrainment of IL-17 and the role of feeding in this pathway. To tease apart the regulation of light and feeding behaviour, we performed experiments with inverted feeding, where one mice had access to food only during the light (inactive) phase, compared to mice that had access to food during the night phase as usual. We found this disrupted food intake and disrupted adipose IL-17 expression by $\gamma\delta^{17}$ T cells and whole-body circadian metabolic rhythm (Figure 3H-M). Because reverse feeding caused mice to eat less food, it was possible that reduced caloric intake caused the disrupted IL-17 rhythm (Figure 3L). Therefore, we designed another set of experiments to test this hypothesis alongside reverse feeding, where we fed mice reduced calories that matched what they ate during reverse feeding, but we fed them this reduced caloric diet at night (Figure 3O). We found that reduced calories caused reduced IL-17 production overall, but the IL-17 rhythm was intact (Figure 3P-U). Thus these data support the conclusion that reduced calories do not disrupt the rhythm and that disruption of feeding is the cause of disrupted IL-17 production, and that adipose IL-17 is entrained by light because of feeding behaviour.

In Figure 4, why do the authors designate lipolysis/thermogenesis during the light phase when Mitch Lazar, Patrick Rensen, Paul Lee and others have shown that thermogenesis begins increasing just before the dark phase? The authors convincingly show the involvement of IL-17A in the regulation of DNL genes but the circadian rhythmicity is mechanistically questionable. Joseph Takahashi has shown that less than 50% of circadian transcriptional changes manifest into protein oscillation. This is particularly relevant for DNL in BAT where those enzymes are among the highest expressed in the entire proteome. Thus, it would be informative to determine the oscillation of some of the key DNL proteins. Also, is IL-17F circadian like IL-17A, given that cytokines can compensate for each other.

The regulation of de novo lipogenesis gene expression at the protein level is also affected by IL-17, see reviewer Figure 1 below. In the revised manuscript, there are significant changes in de novo lipogenesis at the whole body level in *Il17a*^{f/f}, *Il17rc*^{-/-} and *Bmal1*^{Δil17r} mice which display different conditions of IL-17A absence, signalling absence and enhancement of IL-17 expression respectively. The western blot below was performed in a high sucrose challenge to enhance de novo lipogenesis, in order to detect a strong signal by western blot. We include it here rather than the main paper as it was performed during high sucrose (3 days of 20% sucrose in drinking water) which we felt could be confusing to introduce in the manuscript. However we could also include in the manuscript if the reviewer feels it is important to.

Reviewer Figure 1



Il-17a^{f/f} mice have significantly reduced protein expression of SCD1 (part of DNL pathway) after 3 days high sucrose feeding

Il-17a^{f/f} mice have significantly reduced de novo lipogenesis and resist weight gain with 3 week high sucrose feeding

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors should be lauded for their efforts to respond to the reviewers' comments. However, ultimately, the key criticism remains, namely that overall novelty is hampered by a combination of known circadian rhythms in a cell type where this has not been shown before.

The title now better reflects the data. There is a concern with the specificity of the promoters driving the induced lack of BMAL1. The Vav-cre drives expression in all leukocyte lineages and for example myeloid lack of BMAL1 has been associated with a pro-inflammatory phenotype and obesity. The IL7R driver mitigates some of these effects but here, in vitro synchronization data would have been very helpful, namely to assess lack of rhythmicity in isolated, synchronized $\gamma\delta 17$ T cells with or without expression of BMAL1.

For the enrichment of clock genes in the $\gamma\delta 17$ T cells versus the $\gamma\delta 1$ T cells subsets, these data are still difficult to interpret as the actual time points are not mentioned. Adipose $\gamma\delta 1$ T cells and NKT1 cells seem to have a higher expression in Per2 and Cry2 than their 17 counterparts, which in fact indicates that they have an inverse expression of their circadian clock. Indeed, the authors state that 'ex vivo stimulated $\gamma\delta 17$ T cells displayed a robust circadian rhythm for IL-17A production, with a nadir at ZT3 and a zenith at ZT15, and overall higher production at night (Supplementary Figure 2B-E). In contrast, $\gamma\delta$ IFN γ T cells displayed an opposing diurnal rhythm compared to IL-17A, with the percentage of IFN γ positive cells highest at ZT6, lowest at ZT18, with overall higher production during the day (Supplementary Figure 2F-I).' So, there is also a circadian rhythm in $\gamma\delta 1$ T cells, inverted to the $\gamma\delta 17$ T cells. That seems to fit the data from Figure 1, namely an inversion of the clock profile. Importantly, the fold change in IL17A or IFN γ production, respectively, appears stronger in the $\gamma\delta 1$ group, indicating that they do possess a circadian clock but that their phenotype is shifted. Simply assessing clock gene expression is not enough, as assessment of their oscillations would be much more relevant. In fact, it may be very well be the case that all these cells exhibit oscillations, even with overall lower expression. Finally, the whole first part of the Figure 1 (A-F) is not really relevant for the story (similar to some data in the first version of the manuscript), as the authors simply use it to reason why they focus on a specific subset of cells.

Similarly, the EAE and HFD diet data are not relevant. HFD has been shown to decrease EAE survival and lack of rhythmicity in IL-17 cannot functional be linked to increased EAE severity.

Furthermore, the language used still overstates findings and is imprecise at times:

The authors state that 'While lymph node $\gamma\delta 17$ T cells express a higher molecular clock than $\gamma\delta$ IFN γ T cells, adipose tissue $\gamma\delta 17$ T cells have the highest expression of molecular clock genes.' A 'higher' molecular clock is difficult to grasp, as discussed above for $\gamma\delta$ IFN γ . These T cells still exhibit probably even more 'robust' circadian oscillations in their phenotype than $\gamma\delta 17$ T cells. Further, these cells may simply just be more synchronized and so the population shows a higher expression of clock genes.

The authors state that 'there was no change in total number of $\gamma\delta 17$ T cells over a 24-hour period (Supplementary Figure 3A, B). Although adipose $\gamma\delta 17$ T cells display a circadian rhythm to their proportions, no rhythm was observed in absolute adipose $\gamma\delta 17$ T cell number (Supplementary Figure 3A, B), suggesting that the fluctuations in proportions are due to changes in circulating $\gamma\delta$ IFN γ T cells, which are higher in the blood.' This is not understandable, is there blood contamination? Or is this due to tissue infiltration? This would need to be tested for such a statement to be made.

Referee #3 (Remarks to the Author):

The authors have done an impressive amount of work addressing the concerns I had and I highly commend the effort. The addition of the Bmal1 KO was particularly impacting in pointing the mechanistic underpinnings to Nr1d1.

My minor points are that in Figure 1G it would be nice to see Bmal1 expression overlaying Nr1d1 to demonstrate the inverse nature of their biorhythmicity and set a cleaner transition to the genetic studies with Bmal1 KO.

Referee #4 (Remarks to the Author):

In their resubmission, Douglas et al have largely reconfigured the manuscript while addressing several of the criticisms of the original submission. They have added a substantial amount of new data to further support conclusions that there is circadian control of IL-17 production by adipose tissue resident $\gamma\delta$ T cells and that the IL-17A and IL-17F cytokines regulate lipid balance in adipose tissue, promoting de novo lipogenesis (DNL). This group as well as Spiegelman previously showed that IL-17 cytokines produced by adipose $\gamma\delta$ T cells contribute to thermogenesis, through either direct or indirect effect on lipolysis. This paper argues that the production of IL-17 is dependent on cell-intrinsic circadian clock regulation by the $\gamma\delta$ T cells and that the cytokines stimulate de novo lipogenesis. Both of these processes, if experimentally substantiated, would be important and novel contributions (although Hooper has published that there is an intrinsic clock regulator for IL-17 production in gut Th17 cells – which is

controversial). However, the conclusions are largely based on correlative data. For example, there is very little evidence to support the link between DNL and IL-17 apart from qPCR data, which is unsatisfactory given that there are gold-standard techniques such as D2O labelling to measure DNL. The manuscript would be much stronger if it is shown that DNL can be directly attributed to IL-17 and that it is not just a consequence of reduced lipolysis. The role of circadian regulation in the expression of IL-17 also remains unclear, particularly as the authors now show that feeding behavior, normally regulated by the light-dark cycle, is a major contributor to IL-17 production. This finding, along with the disruption of IL-17 circadian expression in mice fed a high fat diet, raise additional questions regarding the cell intrinsic nature of the circadian clock in the $\gamma\delta$ T cells.

Major points

- 1) With respect to the data with the *Bmal1* knockout in CD45+ cells, the authors show a decrease in total $\gamma\delta$ T cells in the fat pad, but an increase in the proportion making IL-17A (based on in vitro re-stimulation). These results make it very difficult to assess whether there is indeed increased IL-17 signaling in the adipose tissue. Are there compensatory mechanisms independent of *Bmal1* in the CD45+ compartment that regulate the IL-17 content in the fat pads? Are both the decrease in $\gamma\delta$ T cells and the increase in IL-17A production by the cells that remain cell autonomous functions?
- 2) To make the case for cell autonomous function of the clock genes in $\gamma\delta$ T cells, use of Vav-Cre and IL-17R-Cre is not sufficient. The TCR- δ Cre-ER line is available at JAX (<https://www.jax.org/strain/031679>) and can be used for conditional inactivation of *Bmal*.
- 3) For Figure 3, to rule out if the reverse feeding effect is due to caloric restriction, it would help to compare reverse and control feeding with caloric restriction in the same experiment (rather than compare CR to ad-libitum during the night cycle).
- 4) The EAE experiment with HFD is interpreted as an increase in IL-17 production because of the disrupted circadian cycle, but HFD can also alter microbiota which influences Th17 induction and there are multiple cell types that would be affected by the switch to HFD, so it is very difficult to tease apart with the data shown. Moreover, the experiment does not seem relevant for the conclusions of the paper. Moreover, it has been well documented that mice start eating during the light cycle and that HFD disrupts the circadian rhythm in general (PMID: 17983587). Thus, there is little novelty and relevance for the entire Figure 4.
- 5) For Figures 5 and 6, the authors only use gene expression to conclude that there is a defect in the DNL. It would be better to show protein levels and, as mentioned above, perform ²H₂O labeling experiments, which has also been done with humans (PMID: 14600072).
- 6) To know if there is an actual consequence of the reduced DNL, it would be important to show that either the fat pads are smaller in the IL-17A/F DKO or Tcr δ KO or that there is resistance to weight gain in conditions such as increased caloric intake. Relating to this, was there any difference in the weight difference between IL-17 DKO and WT at baseline and after 3 weeks of HFD? There is reduced lipolysis in the IL-17 DKO as the authors and others reported previously. If there is no substantial difference in the fat pads at baseline, this could then easily explain the reduced DNL, since less fat would need to be made to maintain homeostasis. One potential way to counter this is to house mice at 30°C or thermoneutrality and thus reduce the need for lipolysis. In this scenario, if the IL-17 DKO mice do not become fat, it would imply that there is a genuine defect in DNL that is unrelated to lipolysis.
- 7) It would also be interesting to know if there is a circadian change in IL-17 production at various time

points in the *Bmal1 Δ Vav* mice, especially if there is a cell extrinsic mechanism that also regulates IL-17 production. Additionally, it would be interesting to put them in metabolic cages to see if there is an actual difference in RER due to potential effects on DNL and lipolysis as seen in the IL-17 DKO mice.

Minor points

- 1) In Figure S5, there is only N=2, which is not sufficient for a qPCR experiment.
- 2) In Figure 2D-H, the ability of SR9009 to inhibit ROR γ t function is expected, given that REV-ERBa and ROR γ t bind the same motifs (PMID: 30590045). This result need not be due to a cell-intrinsic circadian mechanism.
- 3) To address the comment of Referee 3 regarding *Nr1d1* and *Arntl* expression being correlated, it would be critical to show that the expression of *Arntl* also changes at the various time points as shown for *Nr1d1*.
- 4) The point raised by Referee 1 regarding different baseline genes is not fully addressed in the revision, as in Figure 6A, B, C there is use of *Rpl18*; in 6E, 6J, 5F-H it is *Ppib* and in 1G it is *beta-actin*.
- 5) Discussion is repetitive in several parts, can be abridged to summarize and provide context.

Author Rebuttals to First Revision:

Referee #2 (Remarks to the Author): Circadian/Lymphocytes

The authors should be lauded for their efforts to respond to the reviewers' comments. However, ultimately, the key criticism remains, namely that overall novelty is hampered by a combination of known circadian rhythms in a cell type where this has not been shown before.

We thank the reviewer for the recognition of the additional work, which was spurred by their previous comments. Although there are papers that describe the influence of the molecular clock on Th17 and ILC3 cells, all of these other studies investigate the gut, whereas our study focuses on tissue resident IL-17 producing cells in adipose tissue, in the absence of stimulus. These prior studies either do not demonstrate a full circadian rhythm of IL-17A (PMID: 24202171 & PMID: 31534216), or their primary focus is predominantly on IL-22 expression not IL-17A (PMID: 31586011 & PMID: 31586012). Further, in adipose, IL-17 does not have the same physiological function of the previously described roles of circulation IL-22 or IL-17 in the gut. These studies do not correlate the circadian expression of cytokines with the physiological function and maintenance of the tissue itself, except in one instance with regard to gut barrier integrity (PMID: 31534216), but again they correlate this finding with IL-22, and not IL-17A. We find our data is of particular value because we not only report the regulation of IL-17A by the molecular clock, for the first time in adipose tissue, but also how this impacts tissue physiology and whole-body metabolism.

The title now better reflects the data. There is a concern with the specificity of the promoters driving the induced lack of BMAL1. The Vav-cre drives expression in all leukocyte lineages and for example myeloid lack of BMAL1 has been associated with a pro-inflammatory phenotype and obesity. The IL7R driver mitigates some of these effects but here, in vitro synchronization data would have been very helpful, namely to assess lack of rhythmicity in isolated, synchronized $\gamma\delta 17$ T cells with or without expression of BMAL1.

We thank the reviewer for raising these concerns. We have data that demonstrate there is no difference in body mass or fat pad weight between the *Arntl^{fl}* and *Arntl^{ΔVav1}* which can be included in the manuscript. We do find however that there is a difference in fat pad weight between the *Arntl^{fl}* and *Arntl^{ΔIL7r}*, - *Arntl^{ΔIL7r}* mice have decreased fat pad mass. Furthermore, studies involving adipocyte specific KO or myeloid specific Ko of *Arntl* only seem to be susceptible to obesity when fed a high-fat diet, and not on a standard fat diet (PMID: 23970558; PMID: 23142819, PMID: 35238641). We appreciate the suggestion on the in vitro synchronization data, which we have but we have removed, based on the previous round of revisions. In place of the synchronization data that we removed (although it was positive in support of the overall findings), we instead isolated $\gamma\delta 17$ T cells directly from adipose at 4 different time points for PCR analysis, as suggested by reviewer 1 who stated 'Thus, the expansion/serum shock strategy is not adequate to explore physiologically relevant oscillations in adipose-tissue resident gd T cells', in addition to reviewer 3 who stated 'sorting and directly analyzing transcriptional rhythmicity in $\gamma\delta$ T cells from mice at four or more times of day would be the best way to assess physiologically relevant rhythmicity'.

For the enrichment of clock genes in the $\gamma\delta 17$ T cells versus the $\gamma\delta 1$ T cells subsets, these data are still difficult to interpret as the actual time points are not mentioned. Adipose $\gamma\delta 1$ T cells and NKT1 cells seem to have a higher expression in *Per2* and *Cry2* than their 17 counterparts, which in fact indicates that they have an inverse expression of their circadian clock. Indeed, the authors state that 'ex vivo stimulated $\gamma\delta 17$ T cells displayed a robust circadian rhythm for IL-17A production, with a nadir at ZT3 and a zenith at ZT15, and overall higher production at night (Supplementary Figure 2B-E). In contrast, $\gamma\delta$ IFN γ T cells displayed an opposing diurnal rhythm compared to IL-17A, with the percentage of IFN γ positive cells highest at ZT6, lowest at ZT18, with overall higher production during the day (Supplementary Figure 2F-I).' So, there is also a circadian rhythm in $\gamma\delta 1$ T cells, inverted to the $\gamma\delta 17$ T cells. That seems to fit the data from Figure 1, namely an inversion of the clock profile. Importantly, the fold change in IL17A or IFN γ production, respectively, appears stronger in the $\gamma\delta 1$ group, indicating that they do possess a circadian clock but that their phenotype is shifted. Simply assessing clock gene expression is not enough, as assessment of their oscillations would be much more relevant. In fact, it may be very well be the case that all these cells exhibit oscillations, even with overall lower expression. Finally, the whole first part of the Figure 1 (A-F) is not really relevant for the story (similar to some data in the first version of the manuscript), as the authors simply use it to reason why they focus on a specific subset of cells.

Our intention was not to suggest that IFN γ producing cells do not have a molecular clock, as certainly almost every mammalian cell expresses a functional molecular clock. Our intention was to demonstrate increased expression of molecular clock genes in IL-17 producing innate T cells compared to IFN γ producing cells. This data is reinforced by Fig 2P where the loss of *Arntl* does not appear to have an effect on IFN γ production.

Indeed, we do not have the exact time of day for every dataset, however if we split each dataset down to their individual papers, this result still stands as we can assume each lab performed their sequencing at the same time of day. We know from the spleen and adipose datasets, which we generated, that they were run at the same time of day. This is also observed in ILC3s, where the time of day that the cells were isolated is also known (PMID: 31534216, PMID: 31586011 & PMID: 31586012).

Similarly, the EAE and HFD diet data are not relevant. HFD has been shown to decrease EAE survival and lack of rhythmicity in IL-17 cannot functional be linked to increased EAE severity.

Seminal studies by the Mills lab and others have demonstrated a crucial role for IL-17 expression in the induction and progression of EAE, and particularly a role for $\gamma\delta$ T cells in the induction (PMID: 32023490). However, we completely agree that this is a correlation, which may or may not be the determining factor, but nevertheless we feel the novel finding that IL-17 is elevated at all times of the day in obesity is valuable.

Furthermore, the language used still overstates findings and is imprecise at times: The authors state that 'While lymph node $\gamma\delta 17$ T cells express a higher molecular clock than $\gamma\delta$ IFN γ T cells, adipose tissue $\gamma\delta 17$ T cells have the highest expression of molecular clock genes.' A 'higher' molecular clock is difficult to grasp, as discussed above for $\gamma\delta$ IFN γ . These T cells still exhibit probably even more 'robust' circadian oscillations in their phenotype than $\gamma\delta 17$ T cells. Further, these cells may simply just be more synchronized and so the population shows a higher expression of clock genes.

Thank you for your feedback on our phrasing, which is very helpful. With regards to the term 'higher molecular clock' the data does show higher expression of molecular clock genes in IL-17A producing cells compared to IFN γ producing cells. This is also the case for $\gamma\delta$ T cells when compared with $\alpha\beta$ T cells for Per1 expression at the protein level across the entire 24-hours. Considering that the expression of all the molecular clock components are higher in IL-17A producing cells, this could be indicative of higher expression in general, as shown at the RNA and protein level [here](#).

The authors state that 'there was no change in total number of $\gamma\delta 17$ T cells over a 24-hour period (Supplementary Figure 3A, B). Although adipose $\gamma\delta 17$ T cells display a circadian rhythm to their proportions, no rhythm was observed in absolute adipose $\gamma\delta 17$ T cell number (Supplementary Figure 3A, B), suggesting that the fluctuations in proportions are due to changes in circulating $\gamma\delta$ IFN γ T cells, which are higher in the blood.' This is not understandable, is there blood contamination? Or is this due to tissue infiltration? This would need to be tested for such a statement to be made.

We demonstrate that there is no fluctuation in the cell numbers of IL-17 producing $\gamma\delta 17$ T cells in the tissue, and that circadian IL-17A expression cannot be explained by differences in cell numbers across 24-hours. However, there were differences in proportions, but we show this is based on differences in the IFN γ producing subset. These analyses were included in response to a point by reviewer 1 who asked 'Are the total numbers of gd T cells and IL-17 producing gd T cells in the adipose tissue oscillating in a diurnal manner?'. We found that the answer was no, but we still needed to explain the difference in proportions (which is percentage based) and absolute number of cells.

Referee #3 (Remarks to the Author): BAT/Circadian

The authors have done an impressive amount of work addressing the concerns I had and I highly commend the effort. The addition of the Bmal1 KO was particularly impacting in pointing the mechanistic underpinnings to Nr1d1.

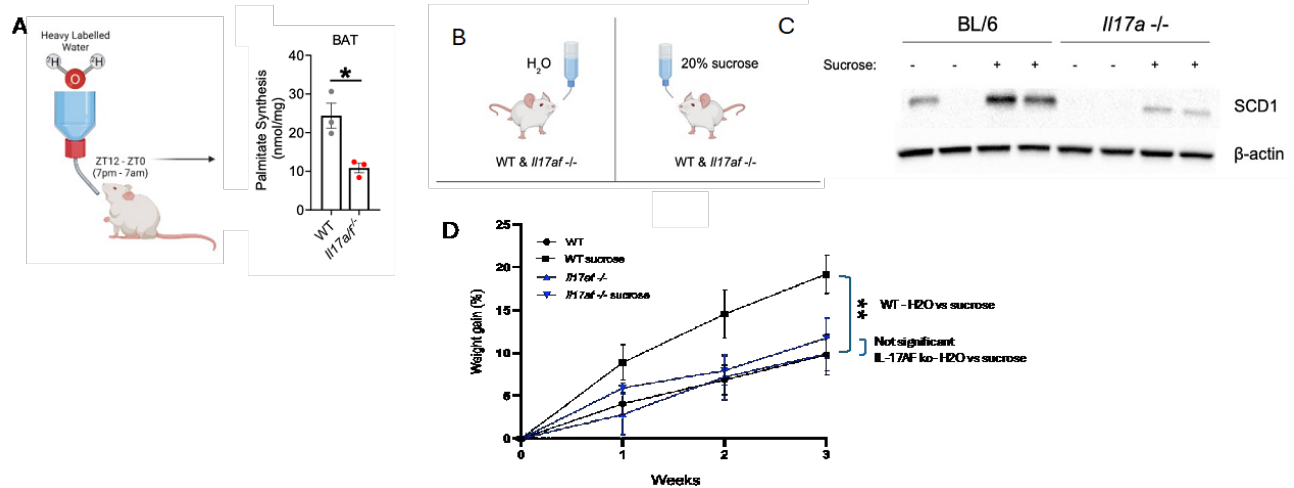
My minor points are that in Figure 1G it would be nice to see Bmal1 expression overlaying Nr1d1 to demonstrate the inverse nature of their biorhythmicity and set a cleaner transition to the genetic studies with Bmal1 KO.

We thank the reviewer for taking the time to review our manuscript and for the feedback provided in the initial submission, which we are sure has strengthened our manuscript significantly.

Referee #4 (Remarks to the Author): Innate Lymphocytes

In their resubmission, Douglas et al have largely reconfigured the manuscript while addressing several of the criticisms of the original submission. They have added a substantial amount of new data to further support conclusions that there is circadian control of IL-17 production by adipose tissue resident $\gamma\delta$ T cells and that the IL-17A and IL-17F cytokines regulate lipid balance in adipose tissue, promoting de novo lipogenesis (DNL). This group as well as Spiegelman previously showed that IL-17 cytokines produced by adipose $\gamma\delta$ T cells contribute to thermogenesis, through either direct or indirect effect on lipolysis. This paper argues that the production of IL-17 is dependent on cell-intrinsic circadian clock regulation by the $\gamma\delta$ T cells and that the cytokines stimulate de novo lipogenesis. Both of these processes, if experimentally substantiated, would be important and novel contributions (although Hooper has published that there is an intrinsic clock regulator for IL-17 production in gut Th17 cells – which is controversial). However, the conclusions are largely based on correlative data. For example, there is very little evidence to support the link between DNL and IL-17 apart from qPCR data, which is unsatisfactory given that there are gold-standard techniques such as D2O labelling to measure DNL. The manuscript would be much stronger if it is shown that DNL can be directly attributed to IL-17 and that it is not just a consequence of reduced lipolysis. The role of circadian regulation in the expression of IL-17 also remains unclear, particularly as the authors now show that feeding behavior, normally regulated by the light-dark cycle, is a major contributor to IL-17 production. This finding, along with the disruption of IL-17 circadian expression in mice fed a high fat diet, raise additional questions regarding the cell intrinsic nature of the circadian clock in the $\gamma\delta$ T cells.

We thank the reviewer for taking the time to not only review the revised manuscript, but for also taking the time to read the previous version along with the reviewer comments. We completely agree that providing additional evidence will strengthen the manuscript, in particular using gold-standard techniques to demonstrate the role of IL-17 in de novo lipogenesis. We have performed heavy labelled D2O labelling experiments in WT and IL-17AF ko mice at steady state and found that IL-17AF KO mice have significantly decreased DNL, which is the gold standard to demonstrate this *in vivo* (A, Figure below). Furthermore, when WT and IL-17 DKO mice are placed on a high sucrose diet for 3 weeks, IL-17 DKO mice do not gain weight compared to WT littermate controls (B&D Figure below), and have significantly less SCD1 protein upregulated by sucrose, compared to WT mice (C Figure below), demonstrating a defect in DNL in IL-17AF mice at steady state and in response to high sucrose.



On the reviewers second point – effect of IL-17 on lipolysis vs lipogenesis, we also completely agree, We started this project with the hypothesise that the primary impact of IL-17 would be on lipolysis. This could be the case in cold challenge, but we found this was not the primary pathway affected at steady state. We demonstrate that IL-17AF KO mice have decreased expression of DNL genes and proteins (Fig 6J) but not of lipolysis genes (Supp Fig 5 C, D). Previous studies by other labs including Dr Mitchell Lazar found that defects in DNL could impact lipolysis, and hence we think this is the primary mechanism for lipolysis defects in IL-17AF KO during cold challenge. Finally, with regards to the feeding modulation of IL-17, we find that light and feeding are zeitgeber of IL-17 production by adipose $\gamma\delta 17$ T cells, light strongly affected IL-17 production rythm, which we think a large part was due to feeding, as disrupting feeding disrupted IL-17. Further, mice fed a high fat diet also have disrupted feeding, even in normal light schedule, because it had been widely reported that mice fed HFD eat more during the day than they would on a SFD (a frequent grazing phenotype) – and we also see that HFD causes increases IL-17 at night and day.

Major points

1) With respect to the data with the Bmal1 knockout in CD45+ cells, the authors show a decrease in total $\gamma\delta$ T cells in the fat pad, but an increase in the proportion making IL-17A (based on in vitro re-stimulation). These results make it very difficult to assess whether there is indeed increased IL-17 signalling in the adipose tissue. Are there compensatory mechanisms independent of Bmal1 in the CD45+ compartment that regulate the IL-17 content in the fat pads? Are both the decrease in $\gamma\delta$ T cells and the increase in IL-17A production by the cells that remain cell autonomous functions?

We thank the reviewer for their comment, and agree that it is difficult to know the reason for increased IL-17 levels in Vav-Cre-Bmal ko mice. It could be that Bmal restrains IL-17, and loss of Bmal increases IL-17, or it could be that Bmal is important for gd survival and their loss is associated with increased IL-17 to fill the niche. The evidence is in favor of the former because this was true in vitro also when ‘quorum sensing’ in the tissue was removed. Further, this result can be explained by the lack of RORyt competition by REV-ERBa, as loss of BMAL1 results in loss of REV-ERBa, but not complete loss of RORyt, which now has no competition of binding

sites, leading to increased IL-17A expression. Increase IL-17A due to loss of REV-ERBa activity has been demonstrated previously. (PMID:24202171, PMID: 31534216, PMID: 31586012). Although oscillations beyond BMAL1 have been described to exist, it is beyond the scope of the paper currently, although it is certainly very interesting to investigate further.

2) To make the case for cell autonomous function of the clock genes in $\gamma\delta$ T cells, use of Vav-Cre and IL-17R-Cre is not sufficient. The TCR- δ Cre-ER line is available at JAX (<https://www.jax.org/strain/031679>) and can be used for conditional inactivation of Bmal.

We agree, that using a TCR- δ Cre-ER would be very interesting. We contemplated the pros and cons of doing this, as because the mice don't yet exist, it would be a long undertaking (an additional 1 year for the PhD student and postdocs career), and the being unsure of the penetrance of the Cre ko. We do not know whether the TCR- δ Cre-ER would provide complete KO of BMAL (similar to some reported issues of penetrance in the ab TCR ko-Cre mice), while Vav-Cre works very well to KO the gene of interest. Furthermore, the key point that we would like to make is that innate sources of IL-17 production in adipose tissue nightly is important for tissue and systemic metabolism, regardless of the immune cell of interest.. In this case, adipose gd T cells are the primary source of IL-17, but we want to highlight it is IL-17 that is the effector we want to highlight. Further, when gd-17 cells are knocked out (Vg4/6 ko mice), other IL-17 producing cells can compensate, and MAIT17, iNKT17 and Th17 are increased in adipose tissue of gd ko. That said, future planned studies that we are very interested in pursuing will be to understand what are the circadian molecular/antigenic stimulus of adipose gd to produce IL-17, but we think this will take several years (3+) to work out). Therefore we felt that Vav-Cre and IL17R-cre mice nicely demonstrated that gd17 are most affected by loss of Bmal compared to other T cells and lymphocytes, which is a novel finding, and allows us to investigate the impact of this in adipose tissue, appreciating it is not specific for gd.

3) For Figure 3, to rule out if the reverse feeding effect is due to caloric restriction, it would help to compare reverse and control feeding with caloric restriction in the same experiment (rather than compare CR to ad-libitum during the night cycle).

We appreciate the reviewers comments. I may be misinterpreting the comment, but as I understand it, I believe we have performed this experimental comparison and the data is in Figure 3 S-U. We have done many additional versions of inverse light and feeding, including ruling out that reverse feeding effects is not due to caloric restriction, we have done side by side. The data included in the manuscript are the ones that regulate adipose IL-17.

4) The EAE experiment with HFD is interpreted as an increase in IL-17 production because of the disrupted circadian cycle, but HFD can also alter microbiota which influences Th17 induction and there are multiple cell types that would be affected by the switch to HFD, so it is very difficult to tease apart with the data shown. Moreover, the experiment does not seem relevant for the conclusions of the paper. Moreover, it has been well documented that mice start eating during the light cycle and that HFD disrupts the circadian rhythm in general (PMID: 17983587). Thus, there is little novelty and relevance for the entire Figure 4.

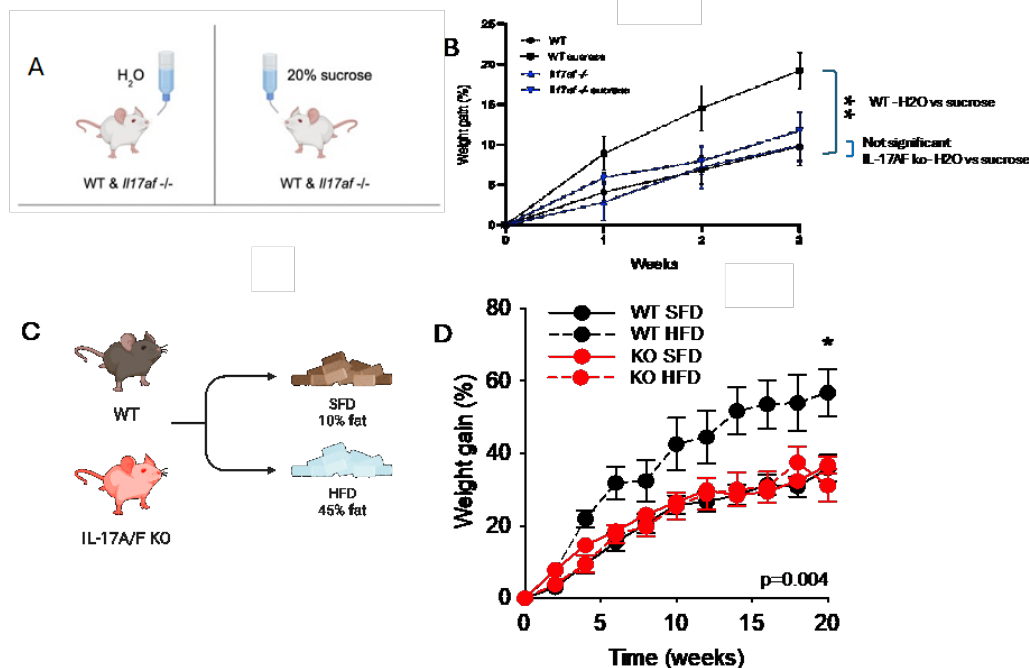
We completely agree with the reviewer, we can exclude or add to supplement, as this is not the key message of the paper. Rather a likely side effect of chronically elevated IL-17 in HFD, either due to disrupted circadian regulation, or microbial differences, or likely a combination of both, or other reasons!

5) For Figures 5 and 6, the authors only use gene expression to conclude that there is a defect in the DNL. It would be better to show protein levels and, as mentioned above, perform ²H₂O labeling experiments, which has also been done with humans (PMID: 14600072).

We have now performed both labelled D₂O experiments and have protein expression, using the gold standard to show that IL-17ko mice have significant defects in adipose DNL.

6) To know if there is an actual consequence of the reduced DNL, it would be important to show that either the fat pads are smaller in the IL-17A/F DKO or Tcrgd KO or that there is resistance to weight gain in conditions such as increased caloric intake. Relating to this, was there any difference in the weight difference between IL-17 DKO and WT at baseline and after 3 weeks of HFD? There is reduced lipolysis in the IL-17 DKO as the authors and others reported previously. If there is no substantial difference in the fat pads at baseline, this could then easily explain the reduced DNL, since less fat would need to be made to maintain homeostasis. One potential way to counter this is to house mice at 30°C or thermoneutrality and thus reduce the need for lipolysis. In this scenario, if the IL-17 DKO mice do not become fat, it would imply that there is a genuine defect in DNL that is unrelated to lipolysis.

This is a very important and interesting point raised by the reviewer, and we have performed the experiments to answer these questions. Firstly on a high sucrose diet, IL-17AF ko mice have defects in upregulation of DNL genes (as early as days 1-3 of high sucrose drinking water intake). If you leave mice on high sucrose water for 3 weeks they show significant weight gain. IL-17AF ko do not gain weight on a high sucrose diet (Figure A-B below). We also placed WT and IL17AF KO mice on HFD for 20 weeks, and found that while WT mice gain weight as expected, IL-17AF ko mice do not (Figure C-D below).



The lack of weight gain on high sucrose data completely supports our overall findings. Initially we wondered about the HFD data because de novo lipogenesis is not a major pathway at play for weight gain in HFD. Indeed, it has been reported that HFD can inhibit de novo lipogenesis overall. However, we believe we have data to explain our findings- it has been reported that adipose-specific inhibition of DNL leads to reduced weight gain on HFD through increased thermogenesis and energy expenditure in WAT <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3467338/>. Thus, our findings that IL-17AF KO mice don't gain weight on high sucrose or high fat diet supports the role of IL-17 in lipogenesis.

Minor points

- 1) In Figure S5, there is only N=2, which is not sufficient for a qPCR experiment.

We agree and this experiment was repeated independently.

- 2) In Figure 2D-H, the ability of SR9009 to inhibit ROR γ t function is expected, given that REV-ERBa and ROR γ t bind the same motifs (PMID: 30590045). This result need not be due to a cell-intrinsic circadian mechanism.

Yes, we agree and this was the purpose of the experiment, to agonise REV-ERBa activity in order to decrease IL-17A. This adds to the cell intrinsic function of the molecular clock.

- 3) The point raised by Referee 1 regarding different baseline genes is not fully addressed in the revision, as in Figure 6A, B, C there is use of Rpl18; in 6E, 6J, 5F-H it is Ppib and in 1G it is beta-actin.

The previous difference in baseline genes was due to human error in labelling. However, this time it is done purposely, as in Figure 1 B-actin is used as these are purified cells isolated from adipose

tissue, and B-actin is one of the most commonly used baseline genes for cells. In the figures PPIB and Rpl18 are used for brown adipose tissue, and the differences are purely due to collaboration between labs.

5) Discussion is repetitive in several parts, can be abridged to summarize and provide context.

This can be easily shortened to be more concise.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #4 (Remarks to the Author):

In their newly revised manuscript, Douglas et al. have added new data that strengthens their conclusions. Most notably, heavy water labelling and weight gain experiments with HFD and sucrose provide additional evidence supporting a role for IL-17 at steady state in de novo lipogenesis, which is the major novelty in the paper. There remain questions as to the relative effect of IL-17 on lipid balance and metabolic health as well as the cellular sources of the cytokine.

1. The role of IL-17 from gammadelta T cells as opposed to other lymphocytes remains unsubstantiated. The authors argue that in the absence of gdT cells there is a compensatory increase in NKT17, MAIT17 and conventional Th17 cells in the adipose tissue, but do not show the relative roles of those cells in the increased IL-17 observed on loss of Bmal. The observation of reduction in gdT cells in adipose tissue in the lymphoid lineage Bmal ko along with increase in proportion that produce IL-17 during the cycle, is still not clearly explained in the context of elevated IL-17 resulting in DNL. Ultimately, total amount of IL-17 produced in the tissue in a circadian manner is what is important to show. Can the levels of IL-17a/f in adipose tissue be measured in WT and mutant mice?
2. For the heavy water labelling, the authors have now shown data for BAT. Given the data with the HFD IL17A/F KO not gaining weight, reduced DNL would also be expected in WAT, and it would be informative to have data for WAT (and liver, which is only shown as schematic in Fig. 6H – as potential negative control). Finally, showing a connection of DNL/heavy water labeling with circadian regulation (Bmal conditional KO) would further strengthen the paper.
3. In the Discussion, the argument is put forth that accumulation of fat tissue mass in the conditional Bmal mutant mice may involve effect of increased IL-17 on intestinal epithelial cells. There is no evidence for this, whereas there is evidence that IL-22 reduction in such mice results in increased lipid uptake. This part of the Discussion can be omitted.
4. The HFD EAE data, if included, is better suited for the supplement rather than as a main figure, especially since the authors also agree that there are multiple explanations for the data.
5. The reviewer response figures with 20% sucrose water and HFD could be included in the paper, as they strengthen the DNL argument. Measurement of fat pad mass and adipocyte area could further substantiate the claims.
6. Line 324-6: The brodalumab data is shown in suppl fig 6I, not in main Fig 6H; and the ixekizumab data is in main Fig. 6L. (in text it is 6I). Figures 6K and 6L are not mentioned in the text. It appears that the numbering was not updated in the revised manuscript.

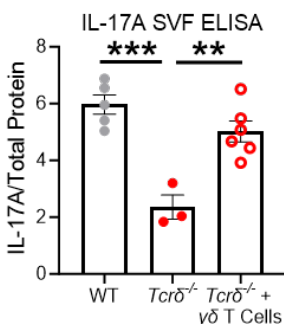
Author Rebuttals to Second Revision:

In their newly revised manuscript, Douglas et al. have added new data that strengthens their conclusions. Most notably, heavy water labelling and weight gain experiments with HFD and sucrose provide additional evidence supporting a role for IL-17 at steady state in de novo lipogenesis, which is the major novelty in the paper. There remain questions as to the relative effect of IL-17 on lipid balance and metabolic health as well as the cellular sources of the cytokine.

We thank the reviewer for taking the time to review our updated manuscript, with additional experiments added spurred on by their previous comments, including the addition of protein data from OPABAT, circadian protein levels of FASN from WT and IL-17^{-/-} mice, and heavy water labelling from WT and IL-17^{-/-} which have further substantiated our findings.

1. The role of IL-17 from gamma delta T cells as opposed to other lymphocytes remains unsubstantiated. The authors argue that in the absence of gdT cells there is a compensatory increase in NKT17, MAIT17 and conventional Th17 cells in the adipose tissue, but do not show the relative roles of those cells in the increased IL-17 observed on loss of Bmal.

In all adipose depots, >70% of IL-17 is made by $\gamma\delta$ T cells (Figure 5B). In $\gamma\delta$ ko mice, there is significantly less IL-17, but it is not absent, due to increased IL-17 from other T cells, which increase but do not 'fill' the niche. We measured IL-17 protein levels in adipose tissue by ELISA showing that IL-17 levels are significantly lower without $\gamma\delta$ in adipose tissue. We adoptively transferred $\gamma\delta$ and then IL-17 levels are restored. (See Reviewer Figure 1 below, now added to manuscript as Figure 5C). To answer the reviewer specifically, on how Bmal loss in CD45 impacts IL-17 by other cells, we found that in BMAL1 ^{Δ Vav1} mice, adipose $\gamma\delta$ T cells have increased expression of IL-17 (Figure 2O), while loss of BMAL1 in adipose CD4⁺ T cells had no effect on IL-17 production or CD4⁺ T cell number (Figure 2 K, M). NKT17 and MAIT17 are negligible levels in adipose tissue. Thus, our data supports the conclusion that $\gamma\delta$ are the main source of IL-17 in adipose, are the cells most significantly expressing clock genes, and are the only significant source of IL-17 that is affected by loss of BMAL.



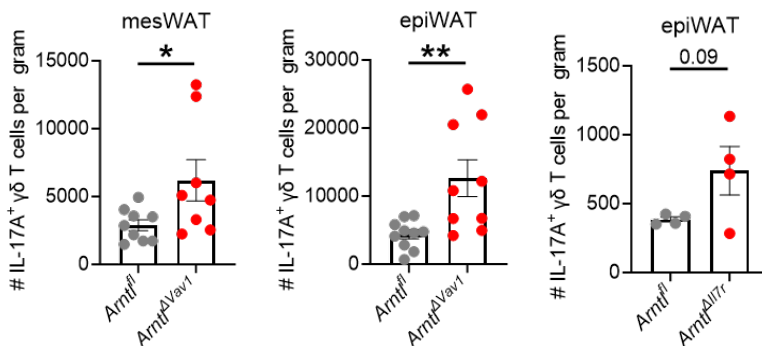
Reviewer Figure 1, and new Figure 5C

The observation of reduction in gdT cells in adipose tissue in the lymphoid lineage Bmal ko along with increase in proportion that produce IL-17 during the cycle, is still not clearly explained in the

context of elevated IL-17 resulting in DNL. Ultimately, total amount of IL-17 produced in the tissue in a circadian manner is what is important to show. Can the levels of IL-17a/f in adipose tissue be measured in WT and mutant mice?

Yes we agree, and we appreciate this important point by the reviewer, and have addressed it. We measured the total $\gamma\delta$ proportion and number that make IL-17, compared to other adipose lymphocytes also. Bmal deletion caused reduced $\gamma\delta$ T cells in total but an increase in the proportion producing IL-17. Here we include additional calculations showing increased numbers of IL-17 producing $\gamma\delta$ T cells in the adipose tissue of *Arntl* ^{Δ Vav1} and *Arntl* ^{Δ Il7r} mice (below, and now added as **Figure 2O**), which demonstrates there are more IL-17 producing cells, rather than just a skewed proportion of gd.

As background to why we think $\gamma\delta$ make more IL-17 when Bmal is deleted: Previous studies have demonstrated that loss of BMAL1 leads to loss of REV-ERBa, which normally competes with ROR γ t for binding sites. Due to a loss of REV-ERBa in BMAL1 deficient mice, ROR γ t activity is free to bind and transcribe its target genes leading to an upregulation of IL-17 and IL-22 (PMID: [31534216](#), PMID: [31586011](#), PMID: [31586012](#)). Based on these findings we hypothesised a similar mechanism for adipose $\gamma\delta$ T cells, having found a REV-ERBa antagonist inhibited IL-17 (Figure 2D-I, Supplementary Figure 3E-L). As IL-17 is increased in adipose $\gamma\delta$ T cells from *Arntl* ^{Δ Il7r} mice, we hypothesised these mice would have increased expression of DNL genes, which we found in BAT (Figure 6D, E). Thus demonstrating that loss of BMAL1 upregulates IL-17 in adipose tissue leading to increased DNL gene expression.

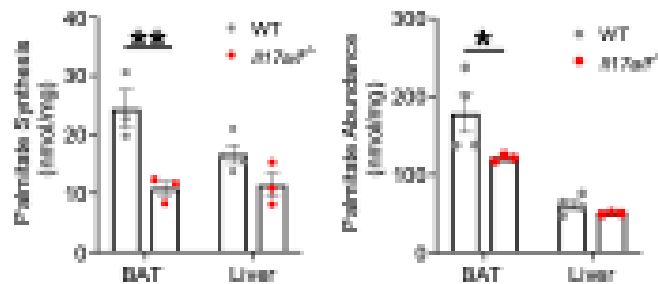


Reviewer Figure 2

2. For the heavy water labelling, the authors have now shown data for BAT. Given the data with the HFD IL17A/F KO not gaining weight, reduced DNL would also be expected in WAT, and it would be informative to have data for WAT (and liver, which is only shown as schematic in Fig. 6H – as potential negative control). Finally, showing a connection of DNL/heavy water labeling with circadian regulation (Bmal conditional KO) would further strengthen the paper.

We did not focus on DNL in WAT because DNL is not a major mechanism for fat accumulation in WAT - it is more uptake and storage than DNL, while in BAT, DNL is a major pathway. Prof Christian Metallo (Salk Institute, who we collaborated with for the heavy labelled water studies) had suggested we focus on BAT and liver, as it is harder to detect DNL by heavy labelled water

tracing in WAT because it is a minor pathway and there are so many lipids present. However, we did perform heavy water labelling for liver also, and found that there is a trend for decreased DNL in liver, but BAT is more striking, and is significant. (Reviewer Figure 3 below). In addition, we have transcript levels for WAT under different steady state, high sucrose and HFD conditions, and found DNL is inhibited.



Reviewer Figure 3

3. In the Discussion, the argument is put forth that accumulation of fat tissue mass in the conditional Bmal mutant mice may involve effect of increased IL-17 on intestinal epithelial cells. There is no evidence for this, whereas there is evidence that IL-22 reduction in such mice results in increased lipid uptake. This part of the Discussion can be omitted.

We agree – we can omit this part of the discussion

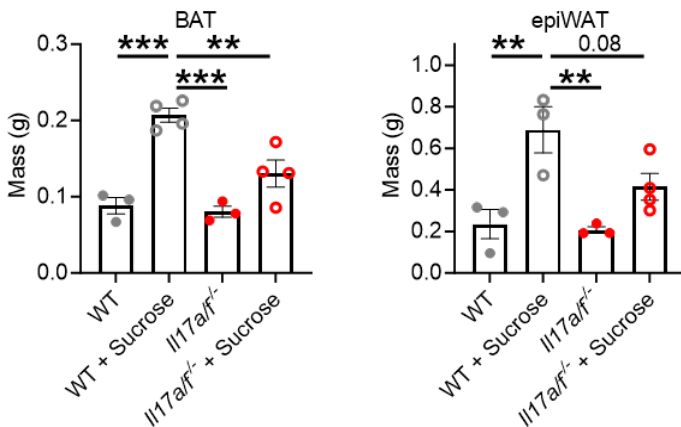
Our rationale for including it was - although previous studies have demonstrated increased adipose mass in mice where BMAL1 is deleted from all ROR γ t expressing cells (*Arntl*^{ΔRorc}), they did not look at production of IL-17 or IL-22, only cell numbers (PMID: [31534216](#)). Other studies published at the same time show that IL-22 is increased in *Arntl*^{ΔRorc} rather than decreased due to the same mechanism we have described above, which would also lead to enhanced IL-17 which these studies demonstrate (PMID: [31586011](#), PMID: [31586012](#)). Therefore, we thought that it is up for discussion whether the increased fat mass in *Arntl*^{ΔRorc} is due to IL-17 or IL-22, which remains to be seen, but can be omitted here until further studies are done.

4. The HFD EAE data, if included, is better suited for the supplement rather than as a main figure, especially since the authors also agree that there are multiple explanations for the data.

We agree, and have moved the HFD EAE data to the supplemental section of the paper.

5. The reviewer response figures with 20% sucrose water and HFD could be included in the paper, as they strengthen the DNL argument. Measurement of fat pad mass and adipocyte area could further substantiate the claims.

We thank the reviewer and agree that the 20% sucrose water and HFD weight data supports or argument for IL-17 regulating adipose tissue DNL and we have now included it in the paper (Figure 4I,K,J,L). The fat pad weight is shown in Reviewer Figure below and can also be added to the paper (omitted for space reasons). We have also included the lack on weight gain on HFD feeding in IL17afko to the supplemental section of the paper (Supplementary Figure 5).



6. Line 324-6: The brodalumab data is shown in suppl fig 6I, not in main Fig 6H; and the ixekizumab data is in main Fig. 6L. (in text it is 6I). Figures 6K and 6L are not mentioned in the text. It appears that the numbering was not updated in the revised manuscript.

We thank the reviewer for catching this error, which is now updated.

Reviewer Reports on the Third Revision:

Referees' comments:

Referee #4 (Remarks to the Author):

Douglas et al have revised their manuscript and significantly improved it. There are no other major concerns.

Minor points:

- 1) The authors did not do the heavy water tracing experiment on any Bmal cKO mice to show a potential increase in DNL. However, the data showing increased numbers of IL17 positive cells in total and the gene signature is acceptable, especially since they showed the decrease in DNL in the IL17A/F KO and the reverse change in gene signature.
- 2) The fat mass data shown in reviewer figure 4 could be included, perhaps added to Supplementary figure 5.