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The thymoprotective function of leptin is indirectly mediated via suppression of obesity

Jayasree Sreenivasan,[1,2] Susan Schlenner,[1,2] Dean Franckaert,[1,2] James Dooley[1,2] and Adrian Liston[1,2]

[1]*VIB, Leuven, Belgium and* [2]*Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium*

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Correspondence: James Dooley, VIB and University of Leuven.
Email: james.dooley@vib-kuleuven.be
and
Adrian Liston, VIB and University of Leuven.
Email: adrian.liston@vib.be
Senior author: James Dooley, Adrian Liston

Summary

Leptin is an adipokine that regulates metabolism and plays an important role as a neuroendocrine hormone. Leptin mediates these functions via the leptin receptor, and deficiency in either leptin or its receptor leads to obesity in humans and mice. Leptin has far reaching effects on the immune system, as observed in obese mice, which display decreased thymic function and increased inflammatory responses. With expression of the leptin receptor on T cells and supporting thymic epithelium, aberrant signalling through the leptin receptor has been thought to be the direct cause of thymic involution in obese mice. Here, we demonstrate that the absence of leptin receptor on either thymic epithelial cells or T cells does not lead to the loss of thymic function, demonstrating that the thymoprotective effect of leptin is mediated by obesity suppression rather than direct signalling to the cellular components of the thymus.

Keywords: leptin; obesity; thymic involution; thymus.

Introduction

Leptin is an adipokine and neuroendocrine hormone inextricably linked to obesity. Genetic defects in either leptin or leptin receptor drive excessive food consumption and severe obesity in both humans[1,2] and mice,[3,4] suggesting a function for leptin as an appetite suppressant. Conversely, leptin levels are raised in obese individuals and deficient in anorexic individuals,[5] suggesting that leptin is a signal reporting on the quantity of adipose tissue. A reconciliation of these data suggests a complex role for leptin in regulating both satiety and energy expenditure, with obesity potentially being driven by shifts in leptin sensitivity in different tissues.[5]

The direct mediators of leptin function in obesity have come under intensive scrutiny in recent years, with the availability of mice bearing a floxed allele of the leptin receptor.[6] The function of leptin in suppression of appetite and adipose expansion has been demonstrated to be mediated through a subset of neurons in the lateral hypothalamic area expressing neuronal nitric oxide synthase, which in turn are capable of inhibiting orexin-producing neurons following leptin signalling.[7,8] In addition, leptin inhibits neurons of the parabrachial nucleus, suppressing the counter-regulatory response and inhibiting energy use,[9] while also activating neurons of the dorsomedial hypothalamic nucleus and promoting thermogenesis.[10]

Beyond regulating adipogenesis and energy balance, leptin has been proposed to have many additional roles. A variety of tissues beyond adipocytes are capable of secreting leptin, and likewise many cell types beyond hypothalamic neurons express the leptin receptor,[11–13] consistent with leptin functioning across multiple systems. Furthermore, the structural and sequence homology to interleukin-6 supports a function as a cytokine as well as a hormone. The extra-metabolic functions of leptin are suggested through the phenotype of leptin-deficient mice, which exhibit (in addition to obesity) phenotypes including excessive inflammation,[14] defects in reproduction,[15] altered bone metabolism,[16] altered angiogenesis[17] and reduced function of the thymus (the primary site of T-cell production).[18]

A role for leptin in maintaining the function of the thymus is supported by the observation of an involuted low cellularity thymus in leptin-deficient obese mice.[18–20] This role has been proposed to be a direct function of leptin because of the observed expression of the leptin receptor on the medullary thymic epithelium,[21] suggesting direct communication between leptin-producing adipocytes and thymus-supporting epithelium. As thymic function is critical for the continued production of T cells,

and becomes limiting in post-pubescent individuals, this leptin–thymus axis has the potential to alter the quality of the adaptive immune response, particularly in aged individuals where thymic function is reduced.[22] Despite the importance of this interaction, it has never been formally demonstrated that leptin directly acts on the thymus in a thymoprotective fashion. Here we used the Cre-Lox system to specifically excise the leptin receptor from both the epithelial and lymphocytic compartments of the thymus. We show that while global leptin receptor-deficiency results in thymic involution, thymic-specific loss of leptin receptor does not alter thymus function. These results demonstrate that the thymic involution described in leptin-deficient mice reflects indirect effects of obesity rather than loss of a direct thymoprotective function of leptin.

Materials and methods

Mice

LepR$^{db/db}$,[3] *LepR*^{flox},[6] *Foxn1*^{Cre}[23] and *CD127*^{Cre} mice[24] were all used on the C57BL/6 background. All experiments were carried out in agreement with the University of Leuven Ethics committee. Mice were housed in a specific pathogen-free environment.

Flow cytometry

Thymus and spleen were analysed by flow cytometry. Samples were blocked in 2.4G2 (anti-CD16/32, hybridoma supernantent, clone 2.4G2, obtained from American Type Culture Collection (ATCC), Manassas, VA) before surface

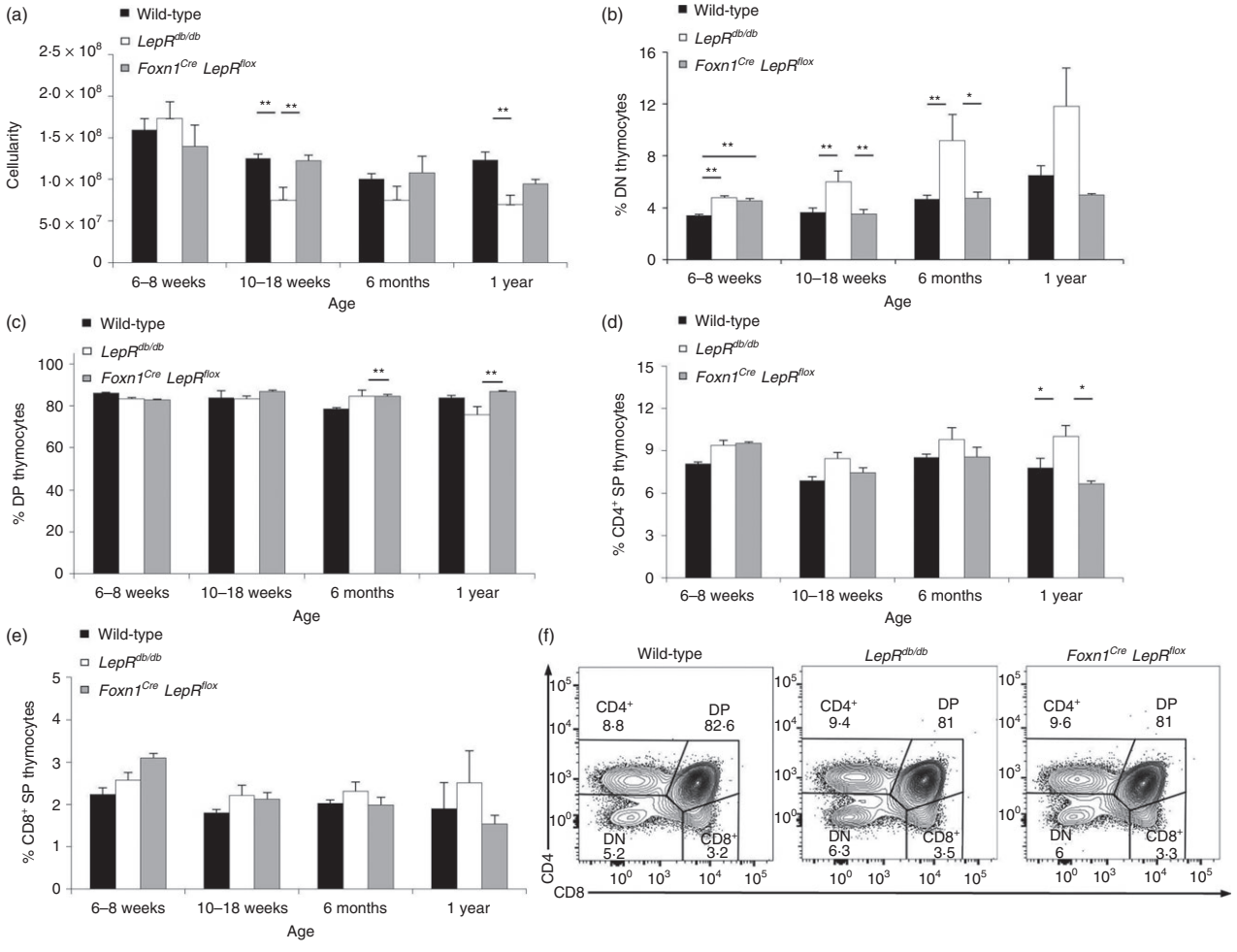

Figure 1. The thymoprotective effect of leptin is independent of leptin receptor expression on thymic epithelial cells. (a) Thymic cellularity of wild-type, *LepR*$^{db/db}$ mice and *Foxn1*^{Cre} *LepR*^{flox} mice at 6–8 weeks (n = 21, 8, 8), 10–18 weeks (n = 26, 6, 10), 6 months (n = 29, 5, 6) and 1 year of age (n = 20, 17, 7), respectively. (b–e) The percentage of thymocytes from wild-type, *LepR*$^{db/db}$ and *Foxn1*^{Cre} *LepR*^{flox} mice that are (b) double-negative (DN) T cells, (c) double-positive (DP) T cells, (d) CD4 single-positive (CD4 SP) T cells, and (e) CD8 single-positive (CD8 SP) T cells at 6–8 weeks (n = 8, 8, 3), 10–18 weeks (n = 26, 6, 10), 6 months (n = 29, 5, 6) and 1 year of age (n = 20, 17, 7), respectively. (f) Representative flow cytometry plots for wild-type, *LepR*$^{db/db}$ and *Foxn1*^{Cre} *LepR*^{flox} mice at 10–18 weeks of age. Mean ± SEM; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}005$.

staining with anti-CD4–allophycocyanin-Cy7 (GK1.5) and –efluor 450 (RM4-5), anti-CD8–phycoerythin (PE)-Cy7 and -allophycocyanin (53-6.7), anti-CD44-peridinin chlorophyll protein-Cy5.5 (IM7), anti-CD25-PE (IL-2Rα; p55), anti-CD62L-PE-Cy-7 (MEL-14), all from eBioscience (San Diego, CA). Cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience, San Diego, CA) before staining with anti-Foxp3-FITC (FJK-16s). The data were collected on a CantoII flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analysed using FLOWJO (Treestar, Ashland, OR).

Statistics

The statistics were calculated using an unpaired Student's *t*-test. Values with $P < 0{\cdot}05$ were considered significant.

Results

The thymoprotective effect of leptin is independent of leptin receptor expression on thymic epithelial cells

Leptin and its receptor have been studied extensively by using either the leptin-deficient (*ob/ob*) or leptin receptor-deficient (*db/db*) mice. These studies have established the presence of the leptin receptor in the thymus and have determined localization of expression to the medullary thymic epithelium.[21] To understand the role of leptin signalling in the thymic epithelial cells, we used a floxed version of the leptin receptor allele[6] and a thymic epithelial cell-specific Cre, driven by the *Foxn1* promoter,[23] to generate mice that were deficient in leptin receptor signalling only in the thymic epithelial compartment. Mice with thymic epithelial cell-specific deletion of the leptin receptor did not gain weight, unlike the control $LepR^{db/db}$ mice, which developed early onset obesity (data not shown), consistent with the anti-obesity function of leptin being restricted to the hypothalamic neurons. Compared with wild-type mice, $LepR^{db/db}$ mice, with global leptin receptor deficiency, developed premature thymic involution (Fig. 1). Thymic involution in $LepR^{db/db}$ mice was mild, with a ~ 30% reduction in thymic cellularity from 10 weeks of age onwards (Fig. 1a). This involution was accompanied by an increase in the double-negative thymocyte population (Fig. 1b). At 1 year of age $LepR^{db/db}$ mice also developed a decrease in the double-positive thymocyte population (Fig. 1c) and an increase in CD4 single-

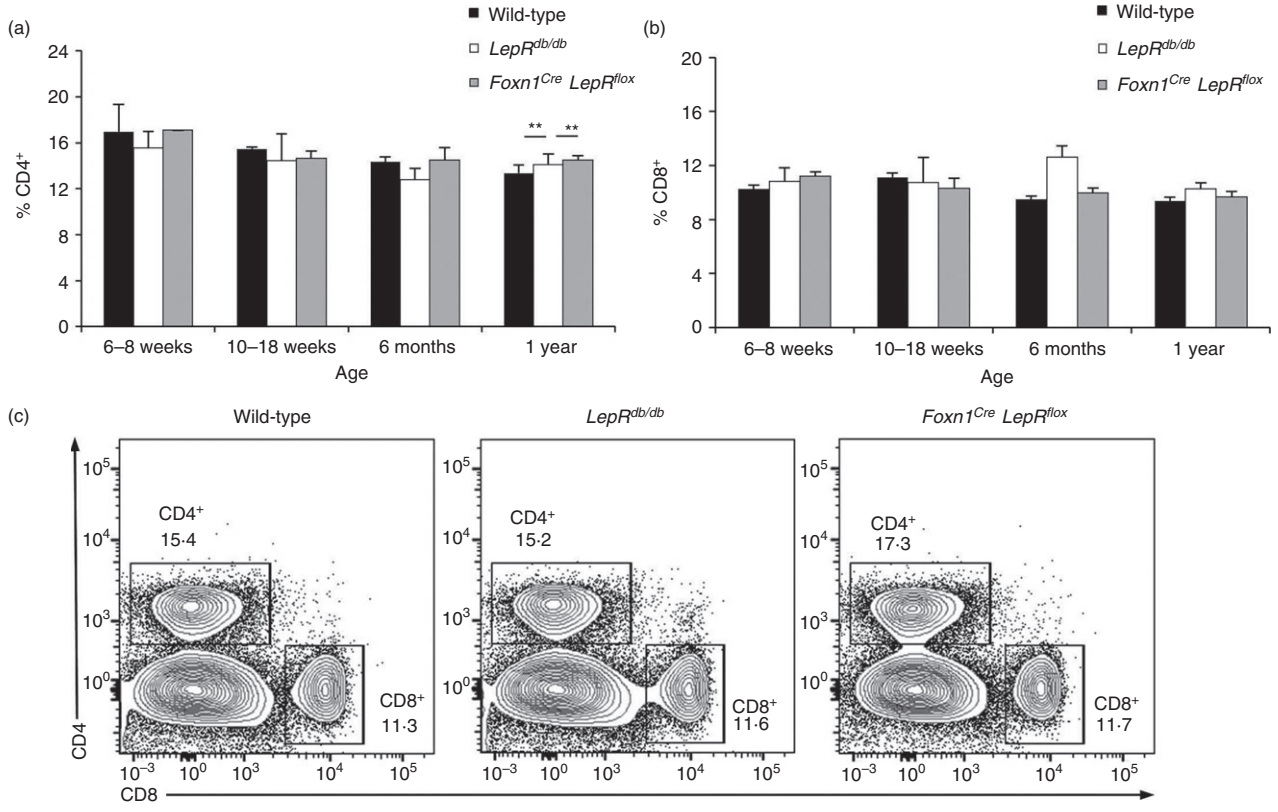

Figure 2. Thymic epithelial cell-specific deletion of leptin receptor does not affect peripheral lymphocyte populations. Splenic lymphocytes were evaluated by flow cytometry from wild-type, $LepR^{db/db}$ and $Foxn1^{Cre}$ $LepR^{flox}$ cohorts at 6–8 weeks (n = 8, 8, 3), 10–18 weeks (n = 26, 6, 10), 6 months (n = 29, 5, 6) and 1 year (n = 20, 17, 7), respectively, for (a) $CD4^+$ lymphocytes and (b) $CD8^+$ lymphocytes. (c) Representative flow cytometric plots for the wild-type, $LepR^{db/db}$ and $Foxn1^{Cre}$ $LepR^{flox}$ cohort at 10–18 weeks of age. Mean ± SEM; $*P < 0{\cdot}05$, $**P < 0{\cdot}005$.

positive (SP) thymocytes (Fig. 1d), $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells (see Supplementary material, Fig. S1) and as a trend towards increased CD8 SP thymocytes (Fig. 1e). Increased double negative, SP and Treg populations are routinely observed in involuted thymuses, which start to gain a secondary lymphoid organ-like profile after the reduction in thymopoiesis. These shifts between thymocyte subpopulations in the obese $LepR^{db/db}$ mice (Fig. 1f) are consistent with earlier studies of premature thymic involution in leptin-deficient mice, although notably the phenotype is milder than previously reported.[18–20] In stark contrast to $LepR^{db/db}$ mice, $Foxn1^{Cre}$ $LepR^{fl/fl}$ mice demonstrated normal thymic cellularity out to 1 year of age (Fig. 1a), and did not manifest the thymocyte differentiation defects observed in $LepR^{db/db}$ mice (Fig. 1b–f; see Supplementary material, Fig. S2). These results confirm the thymoprotective function of leptin, but exclude thymic epithelial cells as the mediators of this effect.

In addition to thymic involution, obese $LepR^{db/db}$ mice have been reported to have disturbed peripheral T-cell populations, including increased $Foxp3^+$ Treg cells.[25] In order to investigate this effect, we analysed the splenocytes from wild-type, $LepR^{db/db}$ mice and $Foxn1^{Cre}$ $LepR^{fl/fl}$ mice. Neither global deficiency nor thymic epithelial cell-specific deficiency in leptin receptor modified the splenic $CD4^+$ and $CD8^+$ T-cell populations (Fig. 2; see Supplementary material, Fig. S3). Likewise, the numbers of naive, effector and Treg subpopulations was unaffected (Fig. 3; see Supplementary material, Figs S4, and S5), and lymph node size was normal (see Supplementary material, Fig. S6). These results suggest that thymic epithelial expression of the leptin receptor is not critical for the differentiation or peripheral homeostasis of T cells.

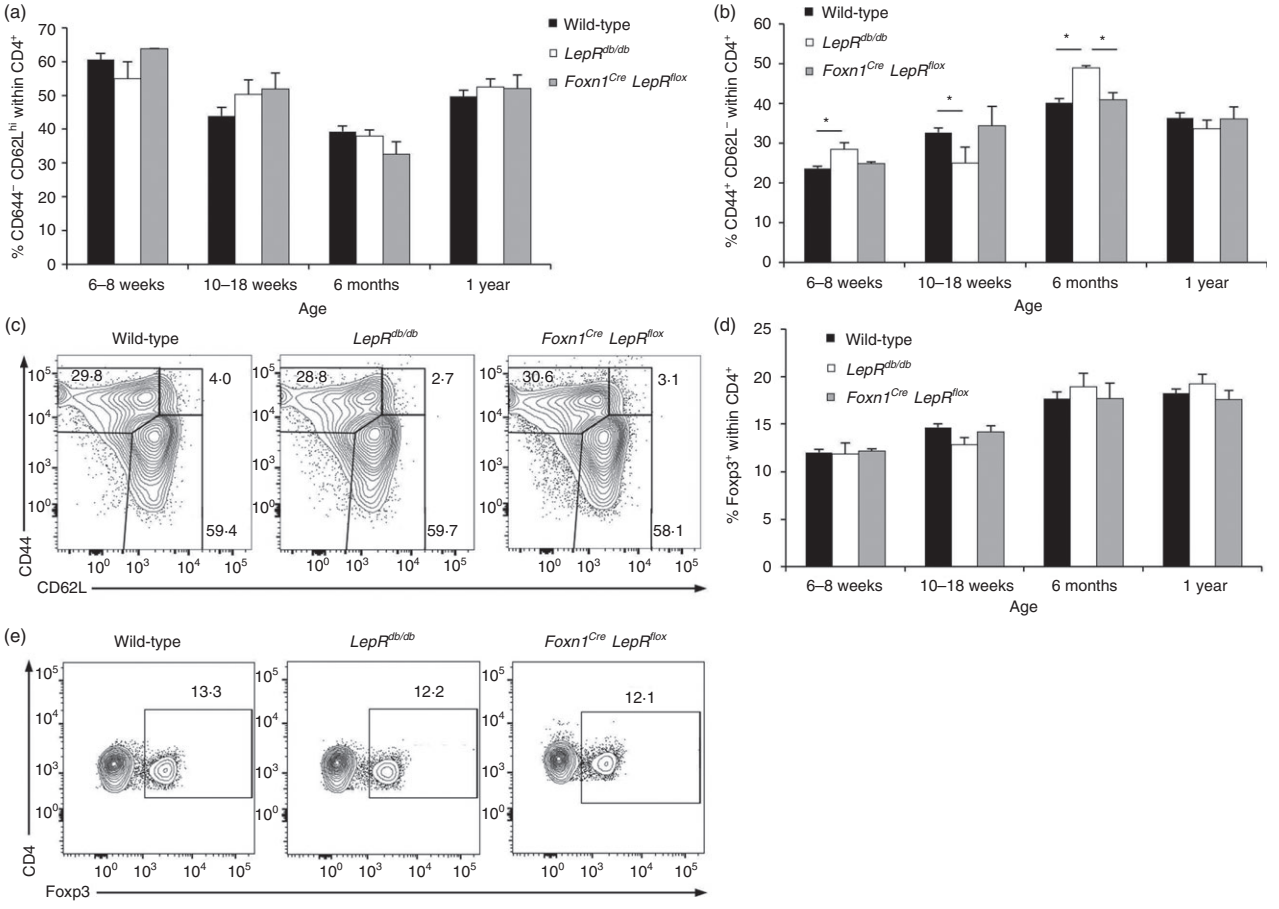

Figure 3. Thymic epithelial cell-specific deletion of leptin receptor does not affect naive, effector or regulatory T cells. Splenic lymphocytes were analysed by flow cytometry for the wild-type, $LepR^{db/db}$ and $Foxn1^{Cre}$ $LepR^{flox}$ cohorts at 6–8 weeks (n = 8, 8, 3), 10–18 weeks (n = 26, 6, 10), 6 months (n = 29, 5, 6) and 1 year (n = 20, 17, 7), respectively. (a) The percentage of $CD4^+$ naive T cells ($CD44^-$ $CD62L^+$) and (b) $CD4^+$ activated T cells ($CD44^+$ $CD62L^-$). (c) Representative flow cytometric plots of naive and effector populations for the wild-type, $LepR^{db/db}$ and $Foxn1^{Cre}$ $LepR^{flox}$ cohort at 10–18 weeks of age. (d) $CD4^+$ $Foxp3^+$ (regulatory T) lymphocytes within the $CD4^+$ population. (e) Representative flow cytometric plots of regulatory T cells for the wild-type, $LepR^{db/db}$ and $Foxn1^{Cre}$ $LepR^{flox}$ cohort at 10–18 weeks of age. Mean ± SEM; $^*P < 0{\cdot}05$.

T-cell deletion of leptin receptor does not alter thymic differentiation or peripheral homeostasis

As leptin receptor in the thymic epithelium was not essential for the immune phenotype observed in the obese $LepR^{db/db}$ mice, we looked to block leptin signalling in T cells and T-cell progenitors. For these sets of experiments, we used the *Cd127* (IL7R) Cre mice[24] in conjunction with the floxed allele of leptin receptor. As $Cd127^{Cre}$ is active from the bone-marrow T-cell precursor stage, $Cd127^{Cre}$ $LepR^{fl/fl}$ mice allow the determination of whether leptin receptor is important in the lymphocytic compartment of the thymus. On analysis of $Cd127^{Cre}$ $LepR^{fl/fl}$ mice and wild-type siblings at 6–8 weeks and 6 months of age, $Cd127^{Cre}$ $LepR^{fl/fl}$ mice did not show any differences in body weight. Thymic cellularity remained unaltered at 6–8 weeks and decreased normally with age (Fig. 4a). Flow cytometry was performed on the thymic populations isolated from these mice. We found no significant changes in the double-negative (Fig. 4b), double-positive (Fig. 4c), CD4 SP (Fig. 4d), CD8 SP (Fig. 4e) and thymic Treg (see Supplementary material, Fig. S7) populations across the two-time points analysed, in percentage or absolute number (Fig. S7). Likewise, in the periphery $Cd127^{Cre}$ $LepR^{fl/fl}$ mice had normal $CD4^+$ T-cell (Fig. 5a) and the $CD8^+$ T-cell (Fig. 5b) populations, indicating that these cells were not affected by loss of leptin signalling, in percentage or absolute number (see Supplementary material, Fig. S8). The assessment of naive, effector and regulatory compartments also reflected no changes at either age (Fig. 6; see Supplementary material, Fig. S9) and lymph node size was normal (see Supplementary material, Fig. S10). Together, these data indicate that leptin signalling in either the thymic epithelial or T-cell compartment is not required for normal T-cell differentiation or homeostasis, and suggests that the immune phenotype observed in obese $LepR^{db/db}$ mice are secondary to the anti-obesogenic function of leptin.

Discussion

Leptin and leptin receptor signalling play an important role in regulating both metabolism and extra-metabolic phenotypes, ranging from inflammation[14] to thymopoiesis.[18] Although the cellular control over the metabolic functions has been dissected in meticulous detail (e.g. the appetite suppressive function[7,8]), the control over extra-metabolic phenotypes has lagged behind, and, indeed, it is not even clear that all of the functions documented for leptin are direct effects. In this study we have followed up previous reports of a direct thymoprotective function for leptin, based on the expression of leptin receptor on the medullary thymic epithelium[21] and the premature thymic involution in leptin-deficient mice.[18–20] Our work on *db/db* mice recapitulates the original findings of premature thymic involution, although surprisingly the effect we

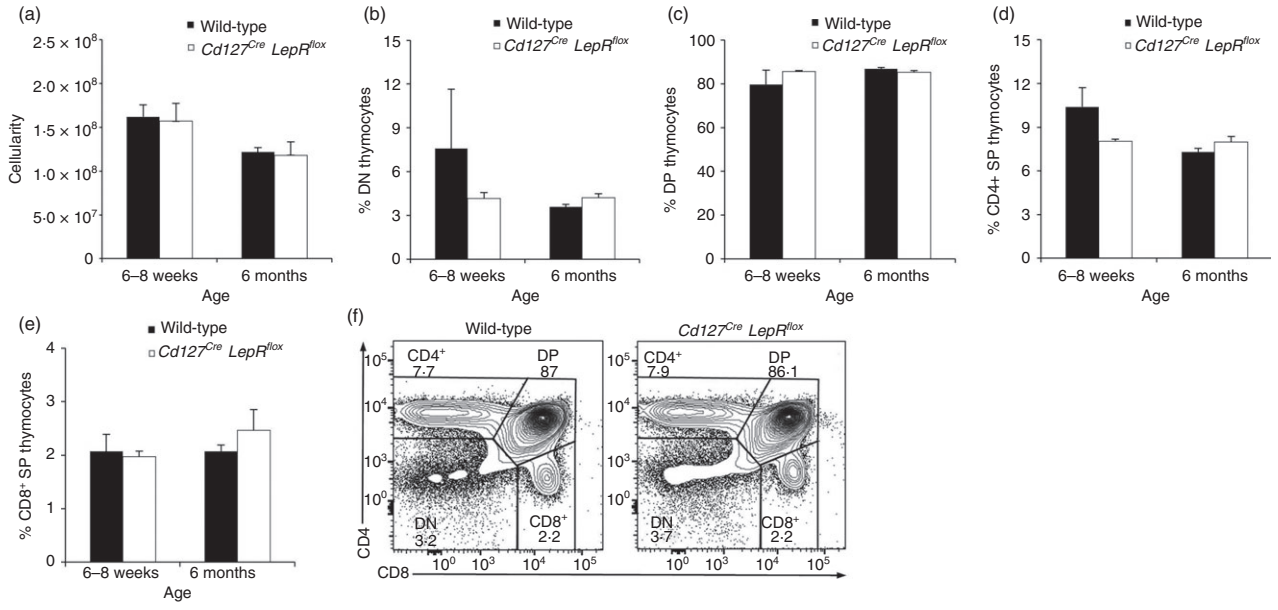

Figure 4. The thymoprotective effect of leptin is independent of leptin receptor expression on thymocytes. (a) Thymic cellularity of wild-type and $Cd127^{Cre}$ $LepR^{flox}$ mice at 6–8 weeks (n = 7, 6) and 6 months (n = 20, 10) of age, respectively. (b–e) The percentage of thymocytes from wild-type and $Cd127^{Cre}$ $LepR^{flox}$ mice that are (b) double-negative (DN) T cells, (c) double-positive (DP) T cells, (d) CD4 single-positive (CD4 SP) T cells, and (e) CD8 SP T cells at 6–8 weeks (n = 7, 6) and 6 months (n = 20, 10) of age. (f) Representative flow cytometry plots for wild-type and $Cd127^{Cre}$ $LepR^{flox}$ mice at 6–8 weeks of age. Mean ± SEM.

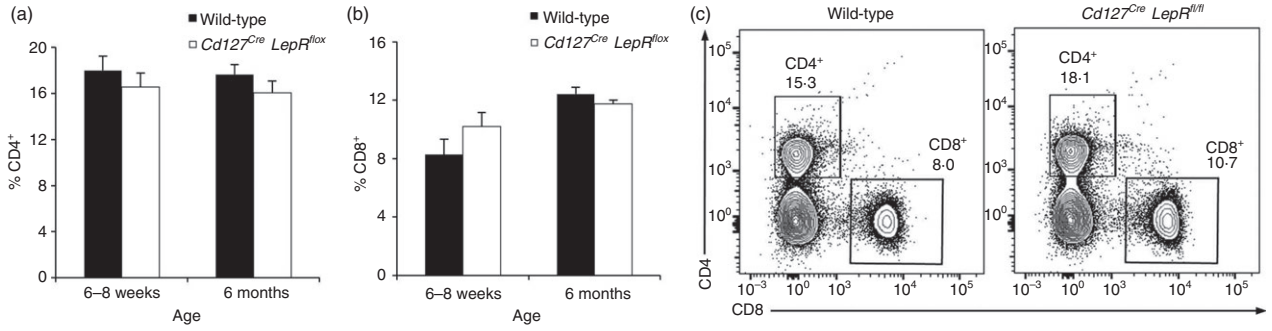

Figure 5. Thymocyte-specific deletion of leptin receptor does not affect peripheral lymphocyte populations. Splenic lymphocytes were evaluated by flow cytometry from wild-type and *Cd127*^{Cre} *LepR*^{flox} cohorts at 6–8 weeks (n = 7, 6) and 6 months (n = 20, 10) of age, respectively, for (a) CD4$^+$ lymphocytes and (b) CD8$^+$ lymphocytes. (c) Representative flow cytometric plots for the wild-type and *Cd127*^{Cre} *LepR*^{flox} cohort at 6–8 weeks of age. Mean ± SEM; *P < 0·05.

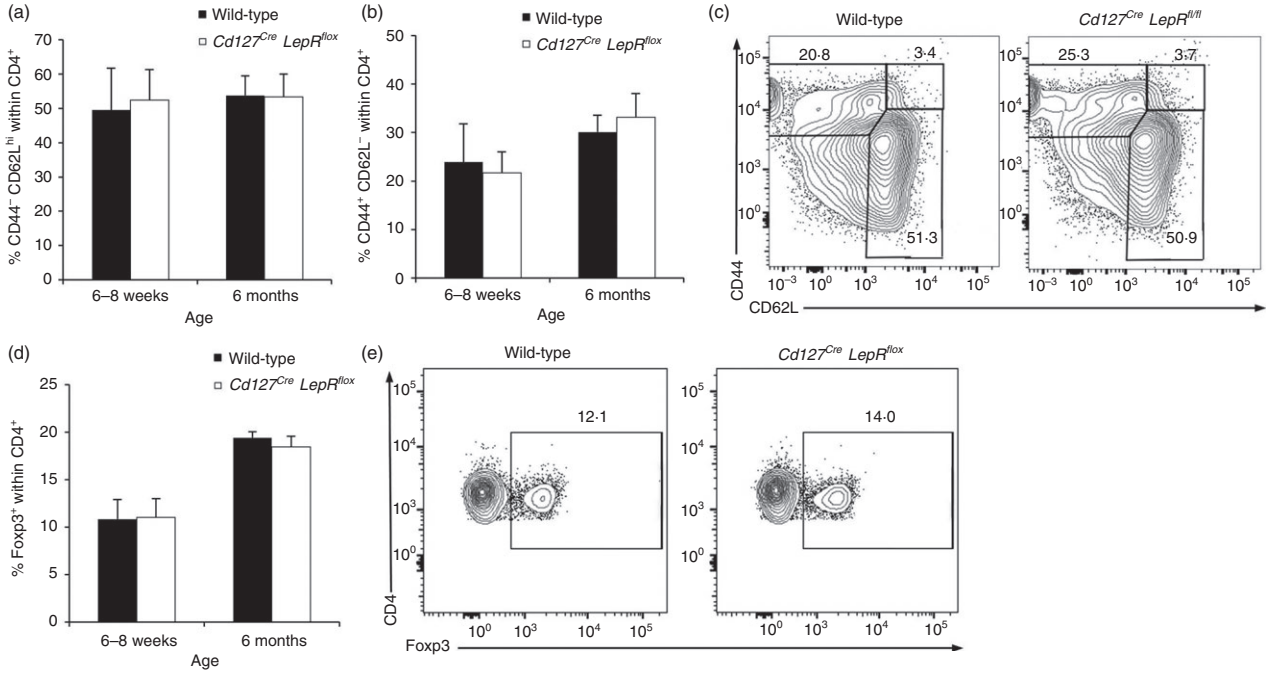

Figure 6. Thymocyte-specific deletion of leptin receptor does not influence regulatory T cells. Splenic lymphocytes were analysed by flow cytometry for the wild-type and *Cd127*^{Cre} *LepR*^{flox} cohorts at 6–8 weeks (n = 7, 6) and 6 months (n = 20, 10) of age, respectively. (a) The percentage of CD4$^+$ naive T cells (CD44$^-$ CD62L$^+$) and (b) CD4$^+$ activated T cells (CD44$^+$ CD62L$^-$). (c) Representative flow cytometric plot of naive and effector populations for the wild-type and *Cd127*^{Cre} *LepR*^{flox} cohort at 6–8 weeks of age. (d) CD4$^+$ Foxp3$^+$ (regulatory T) lymphocytes within the CD4$^+$ population. (e) Representative flow cytometric plot of regulatory T cells for the wild-type, *Cd127*^{Cre} *LepR*^{flox} cohort at 6–8 weeks of age. Mean ± SEM.

observed was relatively mild and constant, unlike the severe progressive loss of thymic cellularity previously reported, a difference that may be due to differing microflora across colonies.

Most strikingly, the proposed function of leptin as a thymoprotective adipokine is not mediated by the expression of leptin receptor on either the epithelial or lymphocytic compartment. Both *Foxn1*^{Cre} *LepR*$^{fl/fl}$ mice and *Cd127*^{Cre} *LepR*$^{fl/fl}$ mice, with excision of the leptin receptor gene in thymic epithelial cells and thymocytes, respectively, demonstrated normal thymic cellularity and function, with no signs of the premature thymic involution that were documented in *ob/ob* or *db/db* mice. Indeed, even the peripheral T-cell compartment was comparable between the wild-type, *Foxn1*^{Cre} *LepR*$^{fl/fl}$ mice and *Cd127*^{Cre} *LepR*$^{fl/fl}$ mice. This is despite the documented expression of leptin receptor on T cells,[26,27] expression that has been used as the grounds for proposed leptin functions in T-cell

hyporesponsiveness and expanded regulatory T-cell numbers.[28,29] Importantly, our study here is directed towards assessing the thymoprotective function of leptin, and does not negate earlier work on additional functions of leptin in the T-cell compartment.

The exclusion of a direct function for leptin as a thymoprotective factor demonstrates that the thymic involution observed in *ob/ob* and *db/db* mice is a secondary effect. The most likely cause of this secondary effect is the expansion of adipose tissue that is the hallmark of *ob/ob* and *db/db* mice. Normal age-related thymic involution is associated with an increase in adipocytes in the thymus.[30] This association may in part drive thymic involution, as adipocytes seem to have a direct toxicity effect on T-cell differentiation, either *in vitro* or following adipocyte transplantation *in vivo*,[30,31] while calorie restriction (which reduces both adiposity and circulating leptin) increases thymic function.[30,32] The reasons for this indirect effect of leptin are likely to be complex and multifactorial,[22] however, a leading factor is likely to be the inflammatory cytokines produced by obese adipose tissue.[33,34] In this context, the thymoprotective effect observed when mice are injected with exogenous leptin[18,35] is likely to reflect the impact of non-obese adipocyte tissue on regulating inflammation,[36,37] rather than any direct effect on thymic epithelial cells or T cells. One caveat to this interpretation is the presence of non-epithelial non-thymocyte cell types in the thymus (dendritic cells, fibroblasts), which could be acting as an alternative secondary signal provider. As the role of leptin receptor in these cell types was not tested, it cannot be excluded that leptin provides a secondary thymoprotective function via these cell types, rather than through the suppression of obesity.

Finally, beyond dissecting the mode of activity of leptin as a thymoprotective adipokine, this study serves as a note of caution on interpreting the phenotype of *ob/ob* and *db/db* mice. Although the multi-faceted nature of leptin and the wide expression of the leptin receptor encourage extrapolation from the *ob/ob* and *db/db* phenotypes to putative direct functions of leptin, it should be perhaps considered the default explanation that any phenotype observed in these mice is a secondary effect of obesity, until that hypothesis has been formally disproven. In this regard, the availability of new molecular tools for dissecting the leptin pathway may result in a contraction of the proposed direct functions and an expansion of the indirect effects of obesity, as we have observed here.

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Disclosures

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Thymic regulatory Tcells are not influenced by epithelial cell-specific deletion of leptin receptor.

Figure S2. The thymoprotective effect of leptin is independent of thymic epithelial leptin receptor expression.

Figure S3. Thymic epithelial cell-specific deletion of leptin receptor does not affect T-cell homeostasis in the periphery.

Figure S4. Thymic epithelial cell-specific deletion of leptin receptor does not affect naive or effector CD4 T cells in periphery.

Figure S5. Thymic epithelial cell-specific deletion of leptin receptor does not influence peripheral regulatory T-cell numbers.

Figure S6. Thymic epithelial cell-specific deletion of leptin receptor does not affect lymph node cellularity.

Figure S7. The thymoprotective effect of leptin is independent of leptin receptor expression on thymocytes.

Figure S8. Thymocyte-specific deletion of leptin receptor does not affect T cells in periphery nor the naive or effector T-cell compartments.

Figure S9. Thymocyte-specific deletion of leptin receptor does not influence regulatory T-cell numbers.

Figure S10. Thymocyte-specific deletion of leptin receptor does not affect lymph node cellularity.